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RATE OF RELEASE OF SPRUCE BUDWORM[1] PHEROMONE FROM VIRGIN FEMALES AND SYNTHETIC LURES[2]

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Abstract—The rate of pheromone [(*E*)-and (*Z*)-11-tetradecenal] release from calling virgin *Choristoneura fumiferana* females and synthetic lures was determined in both static and aerated atmospheres. In a static system ca. 2 ng/hr was recovered per female. Owing to the >75% adsorption onto the females' bodies in static atmospheres, the actual release rate has to be corrected to roughly 9–27 ng/hr, depending on the percentage adsorbed. In the air-flow system, females were found to release between 4 and 20 ng/hr. On a 16:8 light–dark cycle, calling began 1–2 hr before lights-off and continued nonstop until lights-on. Pheromone was emitted throughout calling, while no pheromone was detected during the noncalling periods.

Key Words—*Choristoneura fumiferana*, spruce budworm, sex pheromone, pheromone release rates, (*E*)-11-tetradecenal, (*Z*)-11-tetradecenal, airborne collection.

INTRODUCTION

Pheromone trapping systems are being used for over 50 species of Lepidoptera as a survey tool, and yet pheromone release rates from females and synthetic lures are known only for a few species. In the eastern spruce bud-

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worm, *Choristoneura fumiferana*, the pheromone glands contain primarily tetradecenyl acetates, with the corresponding alcohols and aldehydes being present in very small quantities (Weatherston and Maclean, 1974; Wiesner et al., 1979). The female, however, releases primarily aldehydes (Silk et al., 1980) into the atmosphere. In fact, the identity of the *C. fumiferana* pheromone was determined by means of effluent collections (Weatherston et al., 1971).

To date the various methods used to determine release rates have included: weight loss measurement, extraction of residual semiochemials, trapping of effluent vapors, and direct observation of volume changes in capillary reservoirs (see Weatherston et al., 1981, and references therein). Of these methods the vapor collection method is the most reliable and is the only method aplicable to live females.

A vapor collecting method using glass beads to adsorb entrained pheromone was used to determine pheromone release rates of *Lymantria dispar* (Charlton and Cardé, 1982). This paper describes the use of a similar all-glass apparatus to determine pheromone release rates of *C. fumiferana*.

METHODS AND MATERIALS

Insects. C. fumiferana larvae were reared on a semisynthetic diet (Grisdale, 1970). Pupae were sexed, and emerging adults were removed daily before 0900 hr and held in cages with access to a 5% sucrose solution.

Chemicals and Formulated Lures. (*E*)- and (*Z*)-11-tetradecenal (11-14:Ald) were purified by $AgNO_3$ reverse-phase HPLC (Phelan and Miller, 1981). Solutions of the pheromone were diluted serially and dispensed in 100 μl hexane onto rubber septa (A.H. Thomas Company) to yield the required dosages.

Formulated *E*- and *Z*-11-14:Ald were obtained from Albany International and Hercon Group of Herculite Products Incorporated. The Conrel formulations were of two types, black Celcon and UV-stabilized Celcon, formulated in 0.1 or 0.2 mm ID fibers (see Table 2), containing 96% *E* and 4% *Z*11-15:Ald with 2% Bonax 20BA antioxidant. The two Hercon formulations, L-217-11-1 and L-217-11-2, were plastic sandwich laminates containing ca. 15.7 mg/cm^2 active ingredient.

Static Pheromone Collection Method. The procedure developed by Baker et al. (1980) was used to determine pheromone release rates in a static atmosphere. This involved placing the pheromone source, either rubber septa or five 3-day-old virgin females, in a 250-ml glass flask with a 24/40 ground glass stopper. After 3 hr, the pheromone source was removed and the flask rinsed with 8 ml redistilled hexane in three volumes. Following the addition of the internal standard, (*Z*)-11-hexadecenal (*Z*11-16:Ald), the rinse was filtered, evaporated to about 6 μl under a gentle steam of dry N^2, and

analyzed by gas–liquid chromatography (GLC). Percent adsorption of pheromone onto the female's body was determined by placing five dead females (3 days old and with minimal handling to prevent loss of scales) in the flask with rubber septa containing synthetic pheromone and determining pheromone release as before and comparing with release rates from synthetic sources alone.

Air-Flow Pheromone Collection Method. The apparatus used for this method has been used to determine release of rate of *L. dispar* pheromone (Charlton and Cardé, 1982) and is a modification of the type used by Weatherston et al. (1981). The pheromone source, either four moths or a synthetic dispenser, was placed in a cage in the holding chamber (see Charlton and Cardé, 1982, for details on apparatus) and air pulled through the apparatus with a vacuum pump at ca. 60 cm^3/sec for 3 hr, whence the entrained pheromone was trapped by adsorption onto 1-mm glass beads in a 1 × 3-cm trap. The temporal pattern of pheromone release was determined by obtaining sequential 3-hr collections from females for 48 hr after emergence. Every 3 hr the lower holding chamber (LHC) and pheromone trap (PT) were replaced with clean pieces. Six sets of four moths each were used for pheromone collection. Moths in the apparatus were checked for calling behavior every hour. The holding chamber and trap were rinsed with 10 ml redistilled hexane in three volumes. Following the addition of internal standard, the rinse was filtered, evaporated to 6 μl, and injected on a GLC.

When two traps were used in series, pheromone did not break through to the second trap in 3 hr, and hence the apparatus was used for 3 hr for all further release-rate determinations. Efficiency of the system was determined to be ca. 85% when entrainment of 100 ng *E*11–14:Ald from a coverslip resulted in ca. 84.9% ± 7.9 SD of the pheromone being trapped in the PT and LHC. The methodology is given in detail in Charlton and Cardé (1982).

GLC Analyses. The concentrated rinses from the two methods of pheromone collection were injected onto a 2-mm × 1.8-m glass column with 3% OV-1 (on 100/120 mesh Gas Chrom Q) at 190°C. The pheromone and internal standard eluted between 2 and 3.5 min and were collected in 30-cm glass capillaries cooled with dry ice and rinsed out with 10 μl CS_2. This rinse was concentrated under N_2 and 2 μl was injected on a 2-mm × 1.8-m glass column with OV-275 (on chromosorb RZ) at 160°C. Under these conditions, *E*11–14:Ald and *Z*11–16:Ald eluted at 9 and 14 min, respectively. Pheromone was quantified by using relative peak heights and retention times.

RESULTS AND DISCUSSION

Using 250-ml glass flasks, 2.1 ng/hr/calling female were recovered from 3-day-old virgin *C. fumiferana* females (Table 1). There was a large difference in the percentage adsorbed onto females' bodies when two different dos-

TABLE 1. RELEASE RATES OF *E*11–14:ALDEHYDE FROM VARIOUS SOURCES DETERMINED IN A STATIC SYSTEM

Source	Release		Adsorption (%)	Corrected release (ng)
	Mean ± SD (ng/hr)	5 moths, mean ± SD (ng/hr)		
3-day-old virgin female	2.1 ± 0.2			26.9 or 8.9
10-μg septum	3.4 ± 0.2	0.8 ± 0.4	76.5	
100-μg septum	36.0 ± 9.2			
1000-μg septum	106.7 ± 14.1	8.3 ± 5.8	92.2	

ages of synthetic *E*11–14:Ald were used. With five dead females in the flasks, 10-μg and 1000-μg dispensers emitted ca. 0.8 and 8.3 ng/hr, respectively, an adsorption rate of ca. 76 and 92%. All dead females used were 3 days old, of similar size, and experienced minimal handling. Accounting for adsorption onto females' bodies, the actual pheromone release rates from 3-day-old virgin females would be 8.9 or 26.9 ng/hr/calling female (Table 1). As suggested by Baker et al. (1980), glass adsorption is a simple and convenient technique, but a substantial correction factor has to be used to account for the pheromone lost due to adsorption onto the females' bodies. Sower and Fish (1975) reported that *Plodia interpunctella* releases ca. 2 ng and 6 ng/hr/female of (*Z*,*E*)-9,12-tetradecadienyl acetate and (*Z*,*E*)-9,12-tetradecadien-1-ol, respectively, in a static atmosphere, but these rates do not account for adsorption onto females' bodies. The present study on *C. fumiferana* and that of Baker et al. (1980) on *Grapholitha molesta* show a >75% adsorption onto females' bodies.

The adsorptive properties of glass led Weatherston et al. (1981) to develop an air-flow system to determine release rates of synthetic pink bollworm (*Pectinophora gossypiella*) pheromone from formulated lures. Using this system, they determined that Celcon fibers containing 230 μg of the synthetic pheromone emitted at the rate of ca. 125 ng/hr when the system was aerated at ca. 17 ml/sec. Charlton and Cardé (1982) used a modified version of this apparatus and reported peak emission rates for 2-day-old laboratory-reared and wild moths of ca. 28 ng and 25 ng/2 hr, respectively. The apparatus was used to determine release rates of *C. fumiferana* pheromone. Three-day-old virgin females emit ca. 10.6 ng/hr of *E*11–14:Ald (Table 2). This compares with corrected release rates of 8.9 or 26.9 ng/hr determined in a static system (Table 1). Determination of pheromone release rates from virgin females indicated similar rates of release using the two systems (Tables 1 and 2). But the rate in the static system had to be corrected substantially for adsorption onto females' bodies. The static system underestimates re-

TABLE 2. RELEASE RATES OF *E*11–14:ALDEHYDE FROM VARIOUS SOURCES DETERMINED ON AN AIR-FLOW SYSTEM

Source	Release (Mean ± SD, ng/hr)
3-day-old virgin female	10.6 ± 3.5
1000-μg septum	287.5 ± 48.3
100-μg septum	115.0 ± 10.0
10-μg septum	40.5 ± 7.8
Black Celcon (1980 formulation)	
1 fiber (0.20 mm diam)	229.7 ± 65.5
3 fiber (0.20 mm diam)	578.3 ± 76.7
UV-stabilized Celcon (1980 formulation)	
1 fiber (0.20 mm diam)	378.5 ± 173.2
3 fiber (0.20 mm diam)	640.0 ± 268.7
Black Celcon (1981 formulation)	
3 fiber (0.10 mm diam)	17.5 ± 6.0
Hercon (1979 formulation)	
1 × 1 cm (slow release) L-217-11-1	4730.7 ± 1672.7
1 × 1 cm (fast release) L-217-11-2	8617.3 ± 2323.9

lease rates not only due to adsorption (Baker et al., 1980; this paper) but probably also due to reduced vaporization of the pheromone from the females' pheromone glands.

Using a Porapak-Q-filled cartridge to trap entrained pheromone, Silk et al. (1980) determined *C. fumiferana* release rates to be a maximum of ca. 40 ng/females for the entire calling period or, because females call for 8–10 hr/night, about 0.4–4 ng/hr/female. Silk et al. (1980) used 100 virgin females in a ca. 2-liter chamber. We have noticed that increasing the number of females in an enclosed chamber leads to agitation among the moths, and this may decrease the proportion calling. Additionally, the air flow rate was less than half that used in our tests. Comparing the release rates from rubber septa containing synthetic pheromone (Tables 1 and 2), the air flow system estimated higher release rates than the static system, presumably due to the increased vaporization of pheromone in wind.

The temporal patterns of pheromone release and calling by female *C. fumiferana* were closely correlated (Figure 1). Moths assumed the characteristic calling position (Sanders and Lucuik, 1972) 1–2 hr before lights-off (2000 hr) and remained in this position until lights-on (0400 hr). They did not resume calling until the next evening just before lights-off. Sequential fractions of pheromone obtained from these females every 3 hr for 48 hr indicated that there was no detectable pheromone (< 1 ng/hr/female) during the noncalling hours (Figure 1). Once calling began, females emitted pheromone continuously until the end of the calling period. The highest mean re-

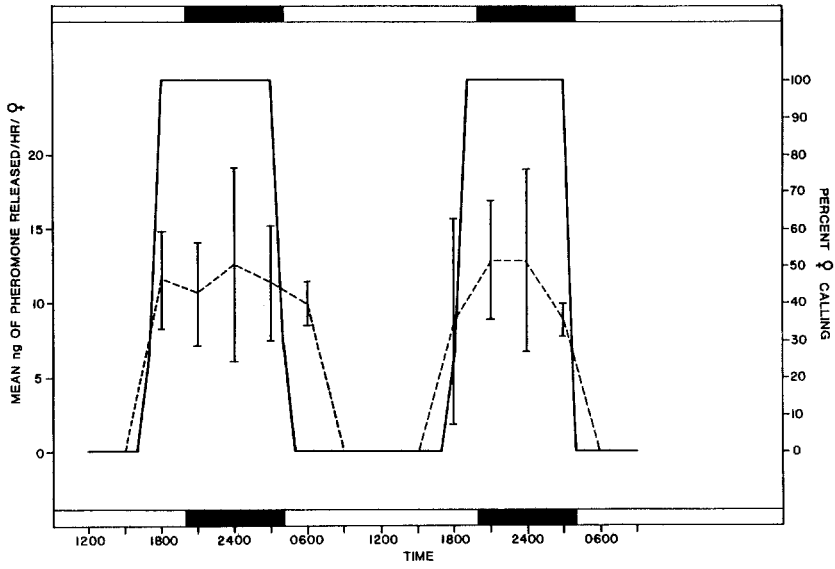

FIG. 1. Temporal pattern of pheromone emission and calling by virgin *C. fumiferana* females. Moths were placed in holding chamber at 0900 hr on day of emergence and pheromone collected at 3-hr intervals for 48 hr (dashed line). Calling was recorded every hour (solid line). White and black bars on *x* axis are photo- and scotophase. Vertical lines on dashed line = SD.

lease of pheromone was determined to be ca. 12 ng/hr/female. Variation from one collection to another during the same period was considerable (range 4–20 ng/hr). No difference in calling and pheromone release were noticed between the first and second day (Figure 1).

The Conrel and Hercon lures formulated in 1979 and 1980 emitted pheromone at very high rates (Table 2), more so than the rubber septa containing 10,000 μg *E*11-14:Ald. These high rates in part contributed to saturation in trap catches of *C. fumiferana* in the field (Ramaswamy and Cardé, 1982; unpublished observations). The 1981 Conrel formulation was tailored to emit pheromone at approximately the same rate as virgin females (Table 2). These lures were useful as monitoring lures in field traps.

The air-flow apparatus used here overcomes many of the problems (Weatherston et al., 1981; Charlton and Cardé, 1982) associated with polymeric adsorbents and cold traps to trap entrained pheromone. This apparatus was also instrumental in allowing us to have tailor-made lures that release pheromone at rates comparable to that of females.

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ROLE OF (*Z*)- AND (*E*)-11-TETRADECENYL ACETATE PHEROMONE COMPONENTS IN THE SEXUAL BEHAVIOR OF THE (*Z*) STRAIN OF THE EUROPEAN CORN BORER, *Ostrinia nubilalis* (LEPIDOPTERA: PYRALIDAE)[1]

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Abstract—A glass tube olfactometer bioassay was used to examine pheromone response of males of the (*Z*)-pheromone strain of *Ostrinia nubilalis* (Hubner). The presence of (*E*)-11-tetradecenyl acetate at the natural ratio to (*Z*)-11-tetradecenyl acetate (97:3; Z:E) did not consistently elevate wing-fanning, upwind walking, or clasper extrusion over (*Z*)-11-tetradecenyl acetate alone. This bioassay did not reveal the behavioral role of (*E*)-11-tetradecenyl acetate.

Key Words—*Ostrinia nubilalis*, Lepidoptera, Pyralidae, European corn borer, pheromone, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate.

INTRODUCTION

The (*Z*)-pheromone strain of the European corn borer, *Ostrinia nubilalis* (Hübner), produces a 97:3 *Z*:*E* blend of 11-tetradecenyl acetates (Kochansky et al., 1975) and greatest trap capture occurs with this ratio of components (Klun et al., 1973, 1975, 1979). However, trap catch also occurs with

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(*Z*)-11-tetradecenyl acetate (*Z*11-14:Ac) alone, suggesting that (*E*)-11-tetradecenyl acetate (*E*11-14:Ac) is not required for attraction. The role of the pheromone components of *O. nubilalis* in eliciting attraction behaviors remains inadequately defined. In studies where pheromone-mediated behavior in *O. nubilalis* was examined, only male precopulatory response (a composite of wing vibration, extension of genitalia, and clasper response) to one concentration of pheromone was reported (Chapman et al., 1978a,b; Klun et al., 1979). Behavioral responses that appear to be relevant to attraction and copulation, such as upwind orientation, wing-fanning, and clasper extrusion, were either not observed or not distinguished. Using an orientation tube olfactometer (Sower et al., 1973), we investigated the effect of the addition of 3% *E*11-14:Ac to *Z*11-14:Ac upon distance of upwind walking, wing-fanning, and clasper extrusion at several concentrations in the (*Z*) strain of *O. nubilalis*.

METHODS AND MATERIALS

The culture of *O. nubilalis* was established from field-collected insects from Ames, Iowa, and was maintained on a 16-hr photoperiod at 24°C, on an artificial wheat germ diet (Miles, 1970). *Z*11-14:Ac was purified on reverse-phase $AgNO_3$ HPLC (Phelan and Miller, 1981). Compound purity was determined by GLC on 20% OV-275 on Gas Chrom RZ. The *Z*11-14:Ac contained $<0.025\%$ *E*11-14:Ac and tetradecenyl acetate. A 97:3 *Z* to *E* mixture was prepared volumetrically and the ratio verified by GLC. Samples for bioassay were prepared by serial dilutions in petroleum ether. Treatments were dispensed in 100 μl onto silicone rubber septa (Arthur H. Thomas Co.). Pure *Z*11-14:Ac and *Z*11-14:Ac (3%E) were tested in dosages ranging in decade steps from 10 ng to 100 μg.

Behavioral bioassays of male response to synthetic pheromone were conducted in glass orientation tubes (Sower et al., 1973) 90 cm long and 2.5 cm diam. In-house air was filtered with activated charcoal, passed through distilled water to increase relative humidity, and passed into the orientation tubes where the air velocity was 1.2 m/sec. Individual males were placed in the tubes 1 hr before the initiation of scotophase and tested 7 hr into scotophase. Air flow and exhaust were initiated 1 hr before testing. Each moth was observed for 200 sec prior to and following introduction of the test stimulus. Moth activity was described and recorded on audiotape and later transcribed. All moths bioassayed were 2 days old and used only once. Bioassays were conducted at 24° and during scotophase light intensity was 0.5 lux. The following behaviors were recorded for each male: (1) duration of wing-fanning (duration of wing-fanning following introduction of test stimulus minus wing-fanning during the 200 sec prior to stimulus introduction); (2) mean duration of wing-fanning bouts (periods of wing-fanning >1 sec);

(3) duration of clasper extrusion (when wings were held vertically and the abdomen was waved slowly from side to side); and (4) distance of upwind walking (while wing-fanning), defined as the distance from the initial position 200 sec after stimulus introduction.

RESULTS AND DISCUSSION

In the (*Z*) strain of *O. nubilalis*, the percentage of males wing-fanning, the duration of wing-fanning, duration of longest wing-fanning bout, mean duration of wing-fanning bouts, and upwind orientation were similar for the *Z*11–14:Ac and the *Z*11–14:Ac with 3% *E* treatments at all the dosages tested (Table 1). The addition of 3% *E*11–14:Ac resulted in a small but statistically significant increase in the duration of clasper extrusion at the 100 μg dosage. Significant dose effects were observed for all behaviors, with 10 μg and 100 μg dosages eliciting the greatest behavioral responses.

In this assay, the presence of *E*11–14:Ac at the natural ratio to *Z*11–14:Ac did not appear to elicit behaviors distinguishable from those evoked by *Z*11–14:Ac alone. Indeed, pure *Z*11–14:Ac evoked pheromone trap catch 1/4 to 1/3 that of the natural blend (Klun et al., 1973, 1975b, 1979; Cardé et al., 1975). This suggests that *Z*11–14:Ac alone can elicit behaviors culminating in mate-finding and trap catch. These results support the conclusion of Chapman et al. (1975a,b) that precopulatory behavior in the (*Z*) strain of *O. nubilalis* is relatively insensitive to the presence or absence of *E*11–14:Ac at a natural ratio to *Z*11–14:Ac.

Among other Lepidoptera employing multicomponent pheromone blends, the behavioral effects of modifying the natural ratio are often substantial. The natural pheromone blend of the redbanded leafroller moth, *Argyrotaenia velutinana* (Walker), is a (3)-component mixture: *Z*- and *E*11–14:Ac in 92:8 ratio, and dodecyl acetate (12:Ac) (Klun et al., 1973; Roelofs et al., 1975). More wing-fanning while walking was evoked by *Z*11–14:Ac with 8% *E* than by pure *Z*11–14:Ac, *Z*11–14:Ac (30% *E*), or pure *E*11–14:Ac, over a range of four decade steps in a bioassay similar to ours. The *Z*:*E* ratio rather than absolute amounts of either isomer was a key factor in eliciting optimal response in *A. velutinana*. Dodecyl acetate combined with *Z*11–14:Ac (8% *E*) prolonged wing-fanning and in the field increased the frequency of landing and close approach to the pheromone source over *Z*11–14:Ac (8% *E*) alone. In *A. velutinana* *Z*11–14:Ac alone did not evoke the full sequence of behaviors that would result in trap catch or copulation (Baker et al., 1976).

However, *E*11–14:Ac did elevate trap capture in the (*Z*) strain of *O. nubilalis* (Klun et al., 1973, 1975, 1979; Cardé et al., 1975). *E*11–14:Ac must have a function in mate-finding, even though in our bioassay the combination did not appear to elicit any behavior distinguishable from that evoked

TABLE 1. WING-FANNING RESPONSES, ORIENTATION, AND CLASPER EXTRUSION RESPONSE BY *Ostrinia nubilalis* MALES TO PURE *Z*11–14:Ac AND *Z*11–14:Ac + 3% *E*11–14:Ac ($N = 37$)

	Treatments									
	*Z*11–14:Ac + 3% *E*11–14:Ac					*Z*11–14:Ac				
Dosage observation[a,b]	−2 10μg	1 10μg	0 10μg	1 10μg	2 10μg	−2 10μg	1 10μg	0 10μg	1 10μg	2 10μg
% moths responding with wing fanning	43c	59c	86ab	97a	100a	38c	51c	76b	97a	97a
$\bar{x}$ duration of wing-fanning (sec)	13.9a	19.4a	67.8b	111.0c	121.2c	12.6a	28.5a	51.0b	120.9c	122.5c
$\bar{x}$ duration of longest wing-fanning bout (sec)	5.3a	4.8a	31.3b	49.8c	66.2c	7.1ab	10.4ab	19.8ab	59.9c	59.7c
$\bar{x}$ duration of all wing-fanning bouts (sec)	2.2a	2.4a	14.3abc	26.5cd	38.3d	2.4a	3.5ab	7.0ab	36.7cd	29.1cd
% moths responding with clasper extrusion	0b	0b	8b	14b	35a	0b	0b	3b	8ab	22a
$\bar{x}$ duration of clasper extrusion	0a	0a	4.5a	0.3a	7.1b	0a	0a	0.8a	1.4a	2.8a
$\bar{x}$ distance of upwind orientation (cm)	3.2a	3.7a	17.1ab	30.1bc	27.7bc	1.5a	7.7a	11.7a	32.7c	30.7b

[a]Percentages in same row followed by same letter are not significantly different at the 5% level using a χ^2 2 × 2 test of independence with Yates' correction.

[b]Means in the same row followed by the same letter are not significantly different at the 5% level as determined by Student-Newman-Keul's multiple-range test.

by *Z*11–14:Ac alone. It is apparent that our assay did not define the behavioral role of *E*11–14:Ac and its influence on trap catch. Only in a flight tunnel bioassay, where behaviors such as landing can be observed, will it be possible to determine the precise behavioral responses elicited by the natural pheromone components.

Chapman et al. (1978a,b) argued that the conformation of the antennal chemoreceptors of *A. velutinana* and *O. nubilalis* (*Z* strain) could be characterized with a bioassay of precopulatory behaviors (rapid wind vibration and extension of genitalia) using chiral analogs [(+)-(*R*)- and (-)-(*S*)-9-(2-cyclopenten-1-yl) nonyl acetates] of the achiral *Z*11–14:Ac. Attraction and precopulatory behavior were viewed as distinct pheromone systems, with the latter being relatively insensitive to the presence or absence of *E*11–14:Ac. They proposed multiple chiral chemoreceptors that sense different conformations of a single achiral compound (*Z*11–14:Ac) in *A. velutinana* and a single stereoselective chemoreceptor for *Z*11–14:Ac in the (*Z*) strain of *O. nubilalis*, both of which could be characterized by the precopulatory response.

Based upon electroantennogram (EAG) studies (Baker and Roelofs, 1976) and single-cell recordings (O'Connell, 1975), at least two functionally different chemoreceptor cell types exist on male *A. velutinana* antennae. O'Connell (1975) found in the sensillum trichodea two cell types characterized by different response spectra to the pheromone components. In one cell *Z*11–14:Ac elicited the greatest responses, 12:Ac synergized the responses to *Z*11–14:Ac, and *E*11–14:Ac acted as an inhibitor. The other cell was relatively insensitive to 12:Ac, and *E*11–14:Ac elicited a greater response than *Z*11–14:Ac. O'Connell (1975) suggested that in *A. velutinana* perception involves across-fiber patterning in which the firing pattern across an ensemble of cells determines the behavior evoked.

In *A. velutinana* the chiral analogs of *Z*11–14:Ac can be expected to interact, as does *Z*11–14:Ac, with the receptor cell type most sensitive to *E*11–14:Ac and thereby alter the response threshold. If one of the two chiral analogs stimulated the cell most sensitive to the *E* receptor, then the presence of both chiral analogs could increase the proportion responding. Other pheromone analogs, also presumably interacting with the *E* and *Z* receptors, evoke attraction. (*Z*)- and (*E*)-11-tridecenyl acetates in a 85:15 blend produced trap catch of *A. velutinana* equivalent to that elicited by the three-component natural blend (Cardé and Roelofs, 1977). Thus, a bioassay of precopulatory responses to chiral analogs of *Z*11–14:Ac may not distinguish between the stimulation of two kinds of chemoreceptors in the receptor cell types for *Z*11–14:Ac alone and the stimulation of two receptor cell types maximally responsive to either *Z*11–14:Ac or *E*11–14:Ac. Single-cell studies and an understanding of the neural integration of the peripheral responses elicited by the natural pheromone components and their chiral analogs would be necessary to confirm in *A. velutinana* the presence of two (or more) chiral chemoreceptors for the achiral *Z*11–14:Ac.

The behavioral responses to the chiral analogs to Z11-14:Ac suggest only a single stereoselective chemoreceptor for Z11-14:Ac in the (Z) strain of *O. nubilalis* (Chapman et al., 1978a,b). Indeed, EAG responses to Z- and E11-14:Ac indicate that the receptors for the two isomers were highly interactive (Nagai et al., 1977). However, single-cell recordings and wind-tunnel bioassays of the natural pheromone components and their chiral analogs are necessary for definition of the precise behavioral effects of these compounds.

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EFFECT OF PHEROMONE CONCENTRATION ON ORGANIZATION OF PREFLIGHT BEHAVIORS OF THE MALE GYPSY MOTH, *Lymantria dispar* (L.)[1,2]

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Abstract—Male gypsy moths (*Lymantria dispar* L.) in a wind tunnel at 24° respond to decreasing dosages (1 μg to 0.1 pg) of (+)-*cis*-7,8-epoxy-2-methyloctadecane with a decrease in probability of wing-fanning, an increase in wing-fanning latency, and an increase in the number of behaviors (body jerks, antennal twitches, steps, and wing tremors) preceding fanning. Males initiating any behavior prior to wing-fanning had a 70% probability of wing-fanning and 97% of the males that wing-fanned eventually flew. The sequence of behaviors from quiescence to flight is not organized in a hierarchy, as this concept is used in ethology, nor is it dependent upon the concentration of pheromone. The time-average threshold concentration of pheromone for response of ca. 90% or more quiescent males is ca. 1.9×10^{-17} g/cm^3 over < 0.3 min.

Key Words—Gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, preflight male behavior, (+)-*cis*-7,8-epoxy-2-methyloctadecane, (+)-disparlure, wing-fanning.

INTRODUCTION

The first behavioral reaction of a quiescent male gypsy moth, *Lymantria dispar* (L.), to relatively high concentrations of female pheromone is wing-fanning. As the concentration of pheromone is decreased, the proportion re-

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sponding is lowered, time until the reaction occurs (latency) is increased (Cardé and Hagaman, 1979), and behaviors, such as antennal elevation, may occur between quiescence and wing-fanning. The wing-fanning response of the gypsy moth was the behavioral reaction used in the field to delimit active spaces and to test models of pheromone dispersion in a deciduous forest (Elkinton et al., 1984). The experiments reported here establish the time-average threshold concentrations of pheromone eliciting wing-fanning and define the temporal organization of wing-fanning and other behaviors preceding flight.

METHODS AND MATERIALS

Male gypsy moth pupae from the Gypsy Moth Methods Development Laboratory were held until adult emergence in 30 × 30 × 36-cm screen cages in a 16:8 light–dark cycle at 24 ± 1°C and 70–80% relative humidity. Adults were maintained, 30–60 per cage, under the same conditions. Behavioral tests were conducted in a wind tunnel (Cardé and Hagaman, 1979) under a similar environmental regime with a wind velocity of 0.7 m/sec. All tests were conducted between the 8th and 11th hour of photophase on the second day after adult emergence, when sensitivity to pheromone is optimal (Cardé, unpublished).

Pheromone stimuli consisted of eight dosages of synthetic (+)-disparlure [(+)-*cis*-7,8-epoxy-2-methyloctadecane, Farnum et al., 1977] ranging in decade steps from 10^{-6} to 10^{-14} g. Each dose was dispensed in 2 μl of petroleum ether on filter paper. A control group was tested with solvent alone.

We estimated the rate of pheromone release from 1000- and 100-ng dispensers by residue analysis of the dispensers on GLC using *cis*-9,10-epoxyeicosane as an internal standard (Charlton and Cardé, 1982). The $\bar{X}$ rates of release over 24 hr at 24° were 18 and 0.9 ng/hr, respectively. The plume dimensions were visualized by emitting cold "smoke" from titanium tetrachloride on a filter paper dispenser identical in size to those used for the pheromone (Cardé and Hagaman, 1979).

Thirty minutes prior to data collection, moths were selected randomly from the holding cages, placed individually on 5 × 7.5-cm glass slides, and covered with 4-cm-diam. clear plastic cups. Collection of data was initiated when the cup was removed from a randomly selected slide, and the slide and moth were placed 25 cm upwind of the exhaust vent of the wind tunnel. Each moth was observed for 3 min, and then the test stimulus (or control) was placed 2 m upwind of the moth. Observation continued until 7 min after the stimulus introduction or the male's flight off the platform.

Behaviors were videotaped from the side of the wind tunnel. Subsequent frame-by-frame playback allowed us to time behaviors to 1/60 sec. Our

analysis focused upon the occurrence, latency, and duration of the following five behaviors: (1) an antennal twitch, defined as movement of one or both antennae along the body axis; (2) a wing tremor, defined as movement of the wing not exceeding 15% of full amplitude fanning; (3) a step, lifting a tarsus off the substrate; (4) a body jerk, which entailed elevating the head and thorax and which was accompanied by a twitch of both antennae; and (5) wing-fanning, a continuous, full-amplitude wing movement, usually accompanied by walking. The first four behaviors had durations of less than 1 sec.

We have followed the techniques of Baerends et al. (1955), Halliday (1975), and others for the analyses of sequences of behaviors. The frequencies of first-order transitions between behaviors were transferred to a probability matrix. The resulting flow diagrams (Figure 1) show both conditional probabilities (i.e., the probability that a different behavior will occur, given that a change in behavior will take place) and the probabilities of a self-transition (i.e., the probability that a particular behavior followed by quiescence will be followed by the same behavior). "Rare" transitions ("random" as defined by a χ^2 test at $P = 0.05$) were excluded from Figure 1.

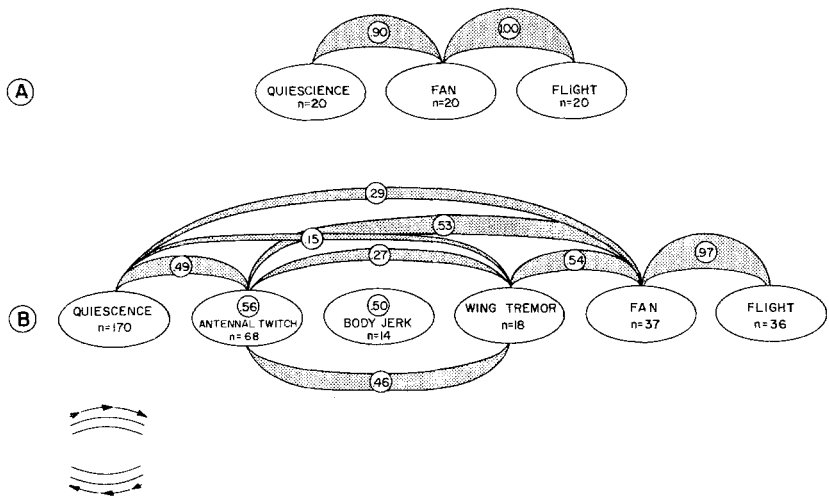

FIG. 1. Conditional transition probabilities of behaviors elicited by (+)-disparlure that are significantly greater by χ^2 than no transition at the 0.05 level. A circled proportion within an oval is a self-transition or the probability of repeating a behavior following quiescence. The number of transitions following that behavior is given by n. Figure 1A is the group of males exposed to the 1000-ng dose. Figure 1B is the group of males exposed to the 10- to 0.0001-ng dosages (see Table 1), excluding males that remained quiescent.

RESULTS AND DISCUSSION

As the amount of the (+)-disparlure on the dispenser was decreased (Table 1), the proportions of moths initiating wing fanning were lowered, while the wing-fanning latencies (both the number of behaviors preceding wing-fanning and the time until full wing-fanning) increased. The two lowest dosages were excluded from the analyses of differences among treatments because of the few moths responding in those groups.

Across all dosages 135 "early" components were observed: 60% antennal twitches, 15% wing tremors, 13% steps, and 12% body jerks. The probabilities of these behaviors being the first response were 0.60, 0.13, 0.07, and 0.20, respectively.

Analyses of the conditional probabilities of transitions between behaviors (Figure 1A) shows that at the comparatively high dosage of 1000 ng, wing-fanning followed quiescence in most males (0.90) and that wing-fanning was followed by flight. At all the lower dosages (pooled observations from 10 ng to 0.0001 ng, Figure 1B), several behaviors could precede wing-fanning, and self-transitions were common for antennal twitches and body jerks. However, even at these relatively low stimulus intensities, wing-fanning, once initiated, was very likely (0.97) to be followed by the relatively

TABLE 1. EARLY COMPONENTS OF SEXUAL BEHAVIOR IN MALE *Lymantria dispar* ELICITED BY VARIOUS DOSAGES OF (+)-DISPARLURE

Dose (ng)	N	Responses preceding fanning[a,b] ($\bar{X} \pm$ SD)	Percent fanning[c]	Fanning latency (min)[a,d] ($\bar{X} \pm$ SD)	Time to full fanning (sec)[a,e] ($\bar{X} \pm$ SD)
1000	20	0.35b ± 0.81	100a	0.30c ± 0.54	0.08b ± 0.12
100	20	0.84ab ± 1.30	95ab	0.26c ± 0.18	0.50b ± 1.53
10	20	1.07ab ± 0.83	70b	1.14b ± 1.10	0.25b ± 0.53
1	30	1.63ab ± 1.60	23cd	1.42b ± 1.70	0.63ab ± 1.22
0.1	30	5.60a ± 7.93	30c	4.41a ± 2.52	0.35b ± 0.65
0.01	30	2.80a ± 3.11	13de	2.08a ± 4.73	2.95a ± 5.25
0.001	30	2.00 ± 1.41	0e		
0.0001	30	3.00 ± 2.65	10e	2.40 ± 0.28	0.45 ± 0.27
Control	30		0e		

[a]Means in the same column followed by the same letter do not differ at the 5% level according to one-way ANOVA and the Student-Newman-Keuls' test after a log $(X + 1)$ transformation.
[b]Total number of antennal and body movements preceding wing-fanning.
[c]Percentages followed by the same letter do not differ at the 5% level by χ^2 with Yates' correction.
[d]Latency is the time between the introduction of the stimulus and the initiation of wing-fanning.
[e]Time to full fanning is the time between the first wing movement and full-amplitude wing-fanning.

"late" behavior of flight. The probability of a responding moth eventually wing-fanning was 70% and flight was always preceded by wing-fanning. The mean interval between behaviors was 16 sec (SD = 28). There were no significant differences in times between self-transitions and transitions to other behaviors.

The concept that in moths male behavioral responses to female-emitted sex pheromone is organized in a "hierarchy" originated with Schwink (1958). Increases in pheromone concentration were considered to elicit successive steps in a sequence of behavioral responses. Ideally, the sequence of behaviors would be relatively stereotyped and the categories of behavior mutually exclusive (Slater, 1973; Fagen and Goldman, 1977). The tests of Bartell and Shorey (1969) on *Epiphysas postvittana* (Walker) males appear to support Schwink's model in that, at most doses tested, relatively early behaviors (e.g., antennal elevation) occurred more frequently than late behaviors (e.g., wing-fanning). The sequence of reactions was not given and only the proportion of males exhibiting a behavior was presented. In a similar analysis, Rust (1976) found that "successive" behavioral reactions in *Periplaneta americana* (L.) tended to be released by increasing concentrations of pheromone. Thus, observations on these two species are compatible with a casual relationship between pheromone concentration and increased probability of eliciting comparatively "late" behaviors in a sequence. Our analysis of the organization of pheromone-mediated preflight reactions of the male gypsy moth does not demonstrate a sequence elicited by changes in concentration.

The use of the term "hierarchy" to describe the organization of a sequence of pheromone reactions (e.g., Shorey, 1973; Bartell, 1977) is at variance with the widespread usage of this term in ethology, as originally elaborated by Tinbergen (1950) and others (Baerends, 1976; Dawkins and Dawkins, 1976). In a traditional view of a hierarchical organization, behavioral elements are clustered according to function or occurrence in time; a sequence of behaviors is not implied. Demonstration of hierarchical organization requires empirical generation of a transition matrix in which there exist close associations within clusters and weak associations between clusters (Colgan and Slater, 1980).

Sufficient pheromone was emitted from the 1000- and 100-ng dispensers to evoke similar patterns of behavioral reactions, latencies, and probabilities of response (Table 1). The 1000-ng stimulus, emitting at ca. 18 ng/hr, is similar to the maximal rate of emission of disparlure from a virgin female (28.9 ng/hr) and the $\bar{X}$ maximal rate (9.3 ng/hr) Charlton and Cardé, 1982). The time-average airborne concentration (and the threshold over <0.3 min) of pheromone generated by the 100-ng stimulus at the location where the male was assayed was ca. 1.9×10^{-17} g/cm^3 or 4.1×10^4 molecules/cm^3. Lower concentrations of pheromone on a dispenser (e.g., 10 ng) obviously elicit

preflight wing-fanning and upwind flight, but the proportion of individuals responding is lower than for males exposed to sources ≥ 100 ng (Cardé and Hagaman, 1979).

The differences between the behavioral reactions to comparatively high or low concentrations of pheromone stimulus are remarkably similar to the responses to a comparatively high concentration of pheromone in either high (24–28°) or low (16°) ambient temperatures (Cardé and Hagaman, 1983). The low assay temperature caused an increase in the wing-fanning latency and the occurrence of the same behaviors prior to wing-fanning as found at the lowest dosages in the current tests at 24°. The functional significance of the behaviors preceding wing-fanning could involve either optimal positioning of the male to detect additional chemical stimulus or to sample a different section of the airstream.

In moderate to dense wild populations of adult gypsy moths, the preponderance of males are in flight from late morning to late afternoon, when mating takes place (Cardé et al., 1974). Low ambient pheromone concentrations in these densities (typically > 100 ♀ / hectare) could initiate flight. However, the proportion of males quiescent in sites where there are few or no calling females and where ambient pheromone concentrations would be reduced is not yet known.

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MATE LOCATION STRATEGIES OF GYPSY MOTHS IN DENSE POPULATIONS[1]

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Abstract—In high-density populations, the male gypsy moth (*Lymantria dispar*) "appetitive" flight is primarily vertical and within 1–50 cm of tree boles. Preceding location of a female, males land on trees or occasionally tree foliage and walk while wing-fanning. In high-density populations females may mate before calling, often prior to wing expansion or hardening. Additional matings may occur before any or full deposition of egg masses. Virgin females are not coy, whether their wings are unexpanded, or hardened and held rooflike, or whether they are calling or not. They generally mate with the first willing male and do not exercise sexual selection by an acceptance or rejection reaction. The mating structure in dense populations would seem to reduce selective pressure for female emission of and direct male anemotactic flight to attractant pheromone. A low proportion of males "search" appropriate objects, especially tree trunks, in the walking–wing-fanning state. Such males often are successful in locating virgin females before calling commences. This male strategy presumably would not be successful in low population densities.

Key Words—*Lymantria dispar*, Lepidoptera, Lymantriidae, gypsy moth, attractant, pheromone, population densities, sexual selection, location strategy.

INTRODUCTION

In low- and medium-density populations, male gypsy moths, *Lymantria dispar* (L.), locate virgin females by upwind anemotactic flight that is elicited by female-emitted pheromone. The chemical mediating this behavior was

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identified as *cis*-7,8-epoxy-2-methyloctadecane by Bierl et al. (1970) and named disparlure. The (+) enantiomer of disparlure elicits up to a 10-fold increase in trap catch in the field (Cardé et al., 1977; Miller et al., 1977; Plimmer et al., 1978). The enantiomeric composition of the natural attractant remains unresolved, although likely it is predominantly or all (+) enantiomer.

The behavioral reactions and sensory inputs involved in mate location in the gypsy moth have been investigated in a number of field studies (reviewed by Cardé, 1981). Richerson et al. (1976a,b) and Richerson (1977) focused attention on possible behavioral differences in the mate location process among populations of differing densities. Our study examines mate location strategies and behavior in a dense adult population.

METHODS AND MATERIALS

Field studies were conducted on Bald Eagle Mountain, Centre County, Pennsylvania in July of 1978 and 1979 in forests predominantly of oak (*Quercus* spp.). Oaks in these areas were 30–90% defoliated. A census of female densities in 100-m^2 sections by visual inspection of tree trunks indicated between 1000 and 6000 females/hectare. Male densities were difficult to determine precisely during most of the day because many males were flying simultaneously. In the sites where our observations and tests were conducted, visual inspection from 1000 to 1600 hr indicated that the ratio of flying males to females varied from about 2:1 to 1:2.

Male Behavior. Male behavior was observed in detail between 1100 and 1300 hr on 2 days when the temperature ranged between 22 and 24°C. A census was taken by visual examination of male moths, except for males in copula. Tree height varied from about 10 to 15 m, and males (and females) were distributed up into the canopy. Males in some behavioral states (i.e., quiescence) were more difficult to discern at the highest levels of the canopy, and consequently those categories could be underestimated. Male behavior at first sighting was categorized as: (1) vertical (up and down) flight within 1–50 cm of a tree trunk, including occasional in flight contact with the tree but not landing; (2) walking while wing-fanning on the tree trunk; (3) quiescent on the tree trunk (wings in a rooflike position); (4) flying horizontally within 2 m of the ground; and (5) mating.

Female Mating. The mating status of females on tree trunks within 0–2.5 m of the ground was determined on randomly selected trees. Females were categorized as: (1) laying eggs; (2) mating (in copula) with no eggs laid; (3) mating after partial or complete egg mass deposition; (4) laying an egg mass with a quiescent male apparently mating but (upon examination of the genitalia) not actually in copula; (5) "multiple" mating, with one male

in copula and 1–2 other males in "false" copula; and (6) laying an egg mass with a male actively attempting to mate.

Additional observations on the frequency of female mating were accomplished between 1000 and 1100 hr by selecting 25 naturally mated pairs and marking the wings of each sex with ink. This procedure did not appear to disturb either sex. These females and males were checked hourly until 1500. New males mating with these females were marked, so that the number of second and third matings of females could be determined.

Mating Behavior. Sequences of mating were recorded in the field on videotape. This system allowed discrimination of time intervals of 1/60 sec on frame-by-frame playback. Natural matings were recorded by locating females as they eclosed from pupae and videotaping their behavior until copulation. Additional sequences were obtained by placing 1-day-old virgin females with hardened wings (collected as pupae and held in cages in the field under natural photoperiod conditions) on tree trunks and recording their behavior on videotape until mating. Verbal descriptions of behavior recorded on the videotape supplemented the visual record.

RESULTS

Male Behavior. During the late morning to midafternoon census, a total of 382 males (78%) were in vertical flight close to tree trunks, 38 (8%) were walking while wing-fanning on the tree trunks, 30 (6%) were mating, and 20 (2%) were quiescent on the tree trunks. Only 30 males (6%) were noted flying horizontally 0–2 m above ground level. These proportions reflect behavior at first sighting. Flight near the tree trunks was predominantly vertical, that is up and down along the tree trunk, and males often contacted the trunks briefly. Undoubtedly the proportion of males exhibiting these behaviors changes with time of day and wind field, but these observations show that vertical flight near trees (where, of course, most females would be situated) is the predominant male flight behavior in a high density population.

Female Mating. Two censuses of female mating status revealed that mating occurred from within several minutes of female eclosion from the pupa, throughout the process of egg mass deposition, and even after depletion of the egg complement. Occasionally we found males mating with dead females whose bodies were partially consumed by a predator. Richerson et al. (1976b) also found some multiple mating in females confined with males in 140 m^3 field cage. Of the 609 females examined, 539 (88%) evidently had mated and were depositing a normal egg mass; 17 (3%) were depositing eggs and had males which were walking while wing-fanning on them and were probing with their abdomen in an attempt to mate; 24 (4%) were in copula and had not oviposited; 24 (4%) had deposited between 1/4

to the full egg mass and were in copula with a male; 1% similarly had deposited some or all of their eggs but had a male in "false" copulation. Not surprisingly, these latter males were dislodged easily during our inspection.

Of the 24 females (4%) that were mating prior to any egg deposition, the wing condition varied from unexpanded to expanded and fully hardened. One pair that appeared to be mating was not actually in copula, 16 females were in normal copula, 6 females in copula also had a false pairing with another male, and 1 female in copula had two false pairings. Censuses taken at other times had shown a higher proportion of females mating prior to egg deposition with one to two additional false pairings (e.g., 17 of 25 at 1200 on July 24, 1979).

Of the 25 naturally mating pairs that were marked and observed from 1100 to 1500 hr, 3 (12%) females mated with a second male, and one of these females (4%) mated with a third male. At 1500 hr 21 (84%) females were in copula with the same male as at 1100.

Female Behavior. Females emerged and walked a short distance (<5 cm) (usually upward) as their wings expanded and hardened. Calling behavior (a rhythmic extrusion of the pheromone gland, see Charlton and Cardé, 1982) did not begin before the wings were sclerotized.

Mating Behavior. We observed 34 pairings: 18 entirely natural, undisturbed matings of freshly emerged females and 16 matings of 1-day-old females which emerged from collected pupae and were placed on tree trunks. Complete videotape records were obtained for 26 pairs with partial video and audio records for the rest. There were no evident differences between the mating behavior of the undisturbed and placed 1-day-old virgin females. Most (1-day-old) females placed on tree trunks initiated calling several minutes after placement.

Eighty-eight percent of the naturally emerging and 38% of the placed 1-day-old females were located by a male and were mated before they called. All but two females (6%) mated with the first male to arrive and contact the female. Approach to the female over the last 10 cm was via wing-fanning while walking, with the male almost always in a head-up orientation. Successful final approach was rapid (mean walking speed 6.5 cm/sec, SD = 3.7) and fairly direct, although the successful males made an average of 1.5 (SD = 3.7) unsuccessful passes within 5 cm of the female before locating her.

When a fanning male contacted a female with his tarsi, he immediately oriented his body parallel along side of her, curved the distal third of the abdomen up to 90° toward her, and began probing with his claspers. As probing continued, fanning gradually decreased until the male's genital claspers were attached to the female's abdomen. Wing-fanning while probing lasted 11 ± 9 sec. Four males (12%) showed pauses in wing-fanning of 1.3 ± 0.5 sec prior to cessation of wing-fanning. Females remained quiescent throughout the courtship and initiation of mating.

DISCUSSION

Observations by Richerson et al. (1976b) of adult gypsy moths in dense populations similar to those in our study area indicated that during midday ca. one third of the males were flying vertically near trees. They suggested males in dense populations orient to vertical silhouettes (trees) and "not to specific pheromone sources over long distances (>25–30 cm)." Our observations are similar in that a large proportion of males was engaged in vertical flight near trees and that some males attempted to copulate with females that were laying eggs. However, we have observed that males in dense populations fly upwind to synthetic (+)-disparlure sources over several or more meters (e.g., Cardé and Webster, 1979), indicating that males in dense populations are capable of positive anemotactic flight to (+)-disparlure.

In dense populations we found freshly emerged females often were mated for the first time before they initiated calling and often before wing expansion. In these adult densities males land and walk while wing-fanning, perhaps, locating noncalling females by a "programmed" walking behavior rather than by a directed upwind anemotaxis. In high-density populations "surface walking" could be induced by low levels of naturally emitted pheromone, or possibly it could be influenced by male-to-male interactions. Visual cues do not appear effective in the male's recognition of the female even at close range, although cues presented by the calling site, such as a tree, are important to inflight orientation and landing (Cardé, 1981).

Major selective pressures in communication systems involving a sex attractant pheromone include: (1) temporal synchrony between the sexes in the time of sexual activity, often timed to energetically favorable environmental conditions (Cardé et al., 1975a), optimal dispersion of pheromone in the wind, or lessened predation; (2) an unambiguous pheromone message; (3) a low pheromone threshold and optimal appetitive "searching" strategy in the responding sex (Cardé, 1981); and (4) a high pheromone emission rate in the sending sex. Of course, in some species the orientation and mating behaviors of the responders are elicited by a comparatively narrow range of pheromone concentrations, as in *Grapholitha molesta* (Busck), the Oriental fruit moth (Baker and Cardé, 1979), so that there is an upper boundary for response and emission. And emission of pheromone at low rates may act to select responders that have low thresholds of sensitivity (Cardé and Baker, 1984).

In low population densities, gypsy moths appear to locate females mainly by pheromone-modulated upwind anemotaxis (Doane, 1968; Cardé, 1981; Richerson et al., 1976b), in which a low male threshold and high female emission rate (Q/K) ratio could be favored. Our observations suggest a different set of selective pressures in dense populations. Many females are mated before they commence calling and hence before they emit sufficient

pheromone to elicit upwind anemotaxis. Thus, selective pressure to release attractant is relaxed.

Low concentrations of pheromone could elicit vertical flight near trees in dense populations (Richerson et al., 1976a,b; Cardé et al., 1975b). Males possessing a searching behavior solely of appetitive flight would not be as likely to mate as males that land frequently on tree trunks, and walk over the trunk surface, thereby encountering females prior to calling. Once a male contacts a female (usually the forewing) with a tarsus, he usually attempts copulation, a response that in a high-density population can frequently result in males attempting to copulate with mated, egg-laying females. Thus, one successful male strategy in dense adult populations involves landing and walking on substrates likely to harbor females.

The reproductive success of males that mate with previously fertilized females is not clear. Females require only 5–7 min of copulation for transfer of sufficient sperm to fertilize their entire egg complement, although natural matings typically last for 1–2 hr or more (Doane, 1968). Males remaining in copula beyond the relatively short time necessary for fertilization may succeed in converting the female to mated behavior (noncalling) and also prevent additional matings that would occur in high-density populations whether the female calls again or not. The degree of sperm precedence in the gypsy moth is unknown, so that an increase in her fitness by a second-mating (through added genetic diversity of her offspring) remains possible. The spermatophore left by the male breaks down in 1–2 hr (Taylor, 1967), and it seems unlikely that it imparts a nutritional advantage to the eggs, as is known in some long-lived Lepidoptera (Boggs and Gilbert, 1979). After a successful mating, females commence laying eggs within several hours, do not call again (Doane, 1968), and are not attractive (Cardé et al., 1973). Males attempting to copulate with females in the process of egg-laying often spent over 1 min in abdominal probing. In several cases where we observed mating of a female laying eggs, the male succeeded in copulating by lifting the female's forewing up sufficiently to gain access to the female's abdomen. The degree of sperm precedence, if any, would determine the selective advantage in a male mating a previously fertilized female.

Validating these hypotheses on the mate location strategies of the gypsy moth will require additional field studies, particularly of male gypsy moth behavior in populations of medium and sparse densities. Of particular importance will be the prevalence in males of vertical flight near silhouettes and walking while wing-fanning on trees and the definition of the sensory inputs that govern these behaviors.

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EFFECT OF ALLYL ISOTHIOCYANATE ON FIELD BEHAVIOR OF CRUCIFER-FEEDING FLEA BEETLES (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—When water traps baited with allyl isothiocyanate (AIC)diffusing through polyvinyl chloride (PVC) and rubber membranes were used to monitor four species of crucifer-feeding flea beetle adults in a rutabaga field at L'Assomption, Que. in 1980–1981, differential responses to AIC were observed. *Phyllotreta cruciferae* was more attracted to AIC than *P. striolata*, whereas the behavior of *Psylliodes punctulata* was not affected by the presence of AIC. The traps with the PVC membrane caught significantly more flea beetles than the traps with the rubber membrane in 1980, but caught a similar number in 1981. Sticky traps covered with AIC mixed with Tangletrap® caught significantly more flea beetles than control sticky traps.

Key Words—Mustard oil, allyl isothiocyanate, Coleoptera, Chrysomelidae, flea beetle, beetle, *Phyllotreta, Psylliodes*, crucifer, rutabaga, behavior, isothiocyanate.

INTRODUCTION

Görnitz (1953) observed that extracts of rapeseed were attractive to several crucifer-feeding flea beetle species including *Phyllotreta cruciferae* (Goeze) and *P. striolata* (F.). Later Feeny et al. (1970) demonstrated that both species were attracted by an aqueous solution of 1% allyl isothiocyanate (AIC), one of the isothiocyanates naturally found in crucifers (Kjaer 1960). Mustard oil glucosides, secondary plant chemicals, also function as selective barriers to non-crucifer-feeding insects (Feeny 1977).

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Several methods of dispensing AIC have been used. After laboratory trials, Burgess and Wiens (1980) proposed a dispenser model with a rubber membrane allowing slow release of AIC which enhanced capture of flea beetles in boll weevil traps while lowering the risks of chemical burn associated with the manipulation of AIC in the field. Vincent and Stewart (1981) used hollow polyethylene caps as AIC dispensers to monitor crucifer-feeding flea beetle adults. However, these caps released AIC too quickly and had to be refilled monthly. In preliminary tests in our laboratory, a polyvinyl chloride membrane allowed slow diffusion of pure AIC from a vial. Although AIC appears to have an effect on estimates of sex ratio of *P. cruciferae* and *P. striolata* (Wylie 1981), it is not known if it exerts a differential effect on the different species here considered.

We here report the results of a two-year field study. The objectives were (1) to compare a PVC membrane to a rubber membrane as a suitable barrier for slow release of AIC, (2) to measure the effect of AIC on relative abundance and sex ratio of flea beetle adults as estimated using AIC-baited and unbaited water traps, and (3) to measure the effect of AIC mixed with Tangletrap® on the number of captures of flea beetles.

METHODS AND MATERIALS

The studies were conducted at the "Station de la Défense des Cultures," Ministry of Agriculture of Quebec at L'Assomption, Quebec, in 1980 and 1981.

Testing PVC Membrane in the Field. Ten 4-ml vials (No. 3338 A 1 Dr., Fischer Scientific Co.) containing 2 ml of freshly distilled AIC (No. 0322, 50 g, Anachemia Chemicals Ltd., P.O. Box 147, Lachine, Quebec, Canada H8S 4A7) diffusing through a 0.50-mm-thick PVC (Miller Plastics Inc., 10229 Cote de Liesse, Dorval, Quebec, Canada, H9P 1A3) membrane were put into a standard meteorological box (Stephenson screen) 1 m above ground level in the field on June 23, 1981. Release of AIC was measured by weighing the dispensers individually and weekly until September 23, 1981. Temperature and relative humidity fluctuations inside the Stephenson screen were recorded with a thermohygrograph.

Water Traps. Field plots measuring 30 x 30 m (rows 1 m apart, 66,000 plants/hectare) of rutabaga (var. Laurentian) were established on May 20, 1980, and June 1, 1981. Commercial fertilizer was applied, but no pesticides were used, weeding being carried out manually. Eighteen water traps, 15 cm diameter by 10 cm high, were randomly positioned between the rows of rutabaga with the top of the traps at 25 cm from ground level. They were half filled with tap water, and a few drops of detergent were added to reduce surface tension. Three groups (treatments) of six traps (replicates) were positioned in a complete randomized design. The groups were assigned as follows:

Group 1 consisted of 2 ml of freshly distilled AIC (boiling point 151–152°C) in a 5-ml vial with a rubber membrane (No. 06-406-10, Serum Bottle Sleeve type, Fischer Scientific Co.) as described by Burgess and Wiens (1980). The vial, painted black to screen the AIC from the sun and increase AIC evaporation, was suspended by a wire over the center of the trap a few centimeters above the water.

Group 2 consisted of 2 ml of freshly distilled AIC in a 4-ml vial painted black with PVC membrane stretched over a 5-mm hole drilled in the plastic cap of the vial, arranged as previously described.

Group 3 was the control, no AIC and no vial.

The traps were emptied every 3–4 days from establishment of the crop until any movements of flea beetles had stopped in late October. Specimens were sexed, identified as to the species, and counted on the same day when possible.

Sticky Traps. In an adjacent field with the agronomic characteristics described previously, 20 sticky traps were randomly positioned between the rows. The traps consisted of 6 x 6-cm^2 plastic gutter painted white (White gloss No. 514, Valspar Corp.). The four sides were covered with Tangletrap® (The Tanglefoot Co., Grand Rapids, Michigan, 49504) from 5 to 20 cm from ground level. Each trap therefore presented a trapping surface area of 15 x 6 cm on each of the four sides giving a total of 360 cm^2/trap. Two trap types were used: type 1 (control), vertical surface coated with Tangletrap only, and type 2, 10 ml of freshly distilled AIC was thoroughly mixed with 500 ml of Tangletrap before application. This apparently did not change the physical properties of the Tangletrap. Two trapping runs were carried out. The first run of 20 traps (10 AIC baited and 10 with Tangletrap only) was begun on August 18, 1981, and the captures recorded on August 28, 1981. The second run, with the same trap arrangement, was begun on September 4 and the captures recorded on September 14, 1981.

Voucher specimens were deposited at the Biosystematics Research Institute (Ottawa) (lot No. 81-1158) and at the Lyman Entomological Museum and Research Laboratory, Macdonald Campus of McGill University.

RESULTS AND DISCUSSION

The dispensers in the Stephenson screen released AIC constantly from June 23 to September 23, 1981 (Figure 1). The cumulative release was linearly related to time, as indicated by the value of the coefficient of correlation (r = 0.97**), and did not appear to be affected by temperature fluctuations. However, this picture might have been different if the PVC had been exposed to wind, sunshine, and rainfall. Four species, *P. cruciferae*, *P. striolata*, *P. bipustulata* (F.), and *Psylliodes punctulata* Melsh. were recorded (Figure 2).

In both years, the AIC-baited water traps caught more specimens than

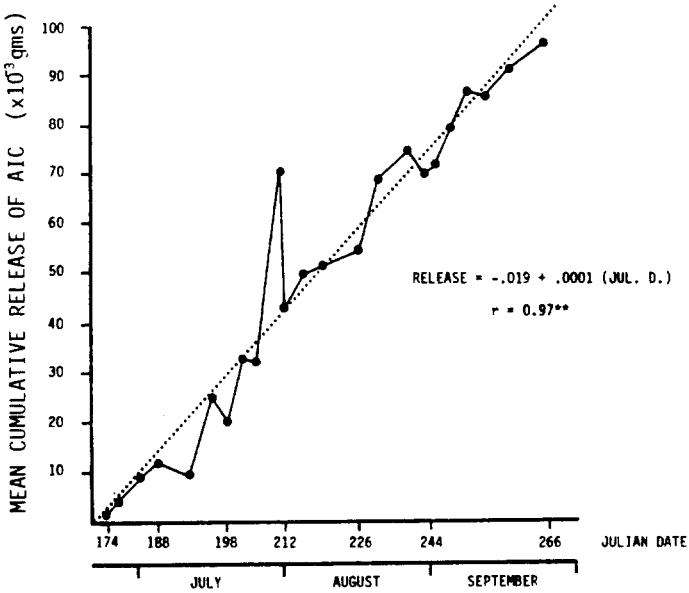

FIG. 1. Mean cumulative release of allyl isothiocyanate through a polyvinyl chloride membrane. Each dot represents an average for 10 dispensers.

the unbaited water traps, 1656 and 1158 against 251 in 1980, and 389 and 386 against 39 in 1981. The dispenser with PVC membrane caught significantly more flea beetles over the two years (χ^2 test, $\alpha = 0.01$), its performance being similar to the rubber membrane in 1981 (χ^2 test, $\alpha = 0.01$). The use of AIC as an attractant affected the estimates of relative abundance of flea beetle species. The control traps indicated that *P. striolata* was more prevalent (61.3% of the total catches in 1980 and 71.8% in 1981), and this agreed with absolute population estimates obtained by D-Vac® in the same fields, where *P. striolata* represented 74 and 79% of flea beetles sampled in 1980 and 1981, respectively (Vincent and Stewart, unpublished data). However, this species comprised only 32% (PVC) and 26% (rubber) of seasonal captures in AIC-baited traps in 1980 and 52% (PVC and rubber) in 1981. *P. cruciferae* was relatively more abundant in AIC-baited than in the control traps. Therefore both species are attracted to AIC, *P. cruciferae* more so than *P. striolata.* This is consistent with the finding that *P. cruciferae* is a more specialized herbivore than *P. striolata* (Hicks and Tahvanainen 1974). The behavior of *Ps. punctulata* was not affected by AIC, the relative abundance estimate being constant in the control and AIC-baited traps. Again, this is consistent with D-Vac estimates taken in this field (Vincent and Stewart, unpublished data).

For both *P. cruciferae* and *P. striolata*, wherever AIC was used, the number of females trapped was significantly higher than the number of males

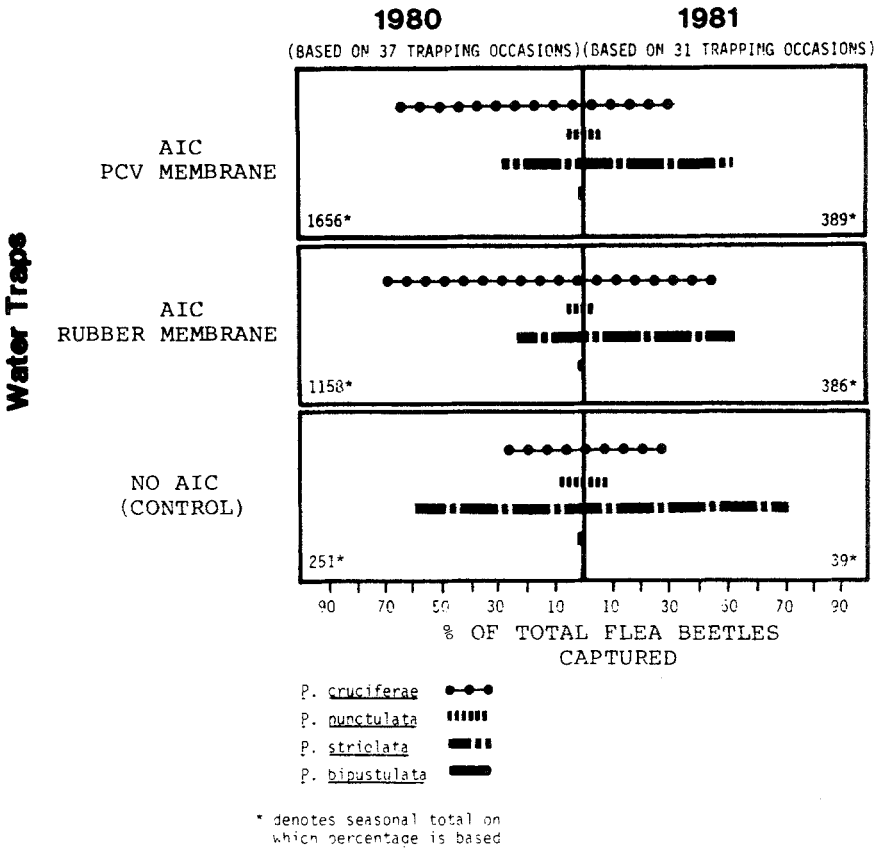

FIG. 2. Proportions of flea beetle species as determined by water traps in rutabaga fields, L'Assomption, 1980–1981.

(Table 1). The observed sex ratio was 1 : 1 when no AIC was used except for *P. striolata* in 1980. This greater attraction for females suggests that AIC might not only be a feeding stimulant as glucosinolates are (Hicks 1974), but also an oviposition stimulant, as Traynier (1965) demonstrated for the cabbage maggot. The observed sex ratio for *Ps. punctulata* was unaffected by the use of AIC. Wylie (1981) observed a higher percentage of female *P. striolata* and *P. cruciferae* in AIC-baited traps.

Sticky traps with AIC captured significantly more (t test, $\alpha = 0.01$) flea beetles than those without AIC. Mean captures were 43.1 per trap (control) and 104.1 per trap (AIC) and 20.2 (control) and 55 (AIC) in the first and second trials, respectively. We suggest that intrafield movement is influenced by AIC and the movements of flea beetles to new host plants may also be affected by mustard oils.

TABLE 1. FLEA BEETLE SEASONAL CAPTURES AND SEX RATIO IN A RUTABAGA FIELD, L'ASSOMPTION, QUÉBEC, 1980–1981.

	Phyllotreta cruciferae			*Psylliodes punctulata*			*Phyllotreta striolata*			*Phyllotreta bipustulata*		
	Male	Female	χ^2	Male	Female	χ^2	Male	Female	χ^2	Male	Female	χ^2
1980												
AIC PVC membrane	329	761	171.2**[a]	32	43	1.6NS	169	317	45.0**	1	4	[b]
AIC Rubber membrane	232	589	155.2**	32	27	0.4NS	109	167	12.1**	1	4	[b]
No AIC (control)	27	43	3.6NS	8	16	2.6NS	54	101	14.5**	3	4	[b]
1981												
AIC PVC membrane	30	93	32.2**	7	11	0.8NS	92	155	16.0**	0	1	[b]
AIC Rubber membrane	34	142	66.2**	3	5	[b]	66	136	24.2**	0	1	[b]
No AIC (control)	4	5	[b]	1	1	[b]	9	19	3.5NS	0	0	[b]

[a]Value of χ^2 statistic. Under H_a: No. of females captured greater than number of males. **, significant at 0.01 level.
[b]χ^2 test not performed due to Cochran's restriction.

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DEFENSIVE SECRETION OF THE PILL MILLIPEDE *Glomeric marginata* I. Fluid Production and Storage

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Abstract—Adult *Glomeris marginata* reloaded glandular defensive fluid slowly and fairly constantly at 15°C for 100 days after being milked to depletion. Female millipedes produced more sticky exudate than males, but the two sexes stored secretion at approximately the same absolute rate, 12 μg/individual/day. Hence, males, which weighed one third as much as females, accumulated disproportionately more secretion. Male *Glomeris* in the reloading treatments after 75 days yielded as much exudate as controls, millipedes given 100 days to supplement their field reserves, whereas experimental females always produced less secretion than controls. Projections from these data suggest that adult *Glomeris*, regardless of sex, require more than 4 months to replenish their defensive reserves after completely discharging them in an attack.

Key Words—millipede, *Glomeris marginata*, Glomerida, Diplopoda, defense, secretion, fluid, production, sexual dimorphism.

INTRODUCTION

Chemical defenses of many millipedes are well characterized with regard to their molecular constituents, glandular morphology, and antipredator actions. However, little is known about the synthesis, storage, or discharge of these fluids (Eisner et al., 1978; Blum, 1981). I here report on production of sticky exudate by adults of the European millipede *Glomeris marginata* (Villers) (Glomerida, Glomeridae) under controlled laboratory conditions.

When attacked, *Glomeris* coil into a sphere and secrete from one to eight viscid drops from segmentally arranged glandular openings along the dorsal midline. The fluid, which is stored in pairs of elongated sacs thinly lined with cuticle, contains two quinazolinone alkaloids in approximately

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equal proportions dissolved in a watery protein matrix (Eisner et al., 1978, and references therein). The alkaloids are potent antifeedants and toxins for spiders, insects, and vertebrates (Schildknecht et al., 1967; Eisner, 1970; Carrel, 1971; Carrel and Eisner, in preparation). The adhesive exudate also can entrap appendages of ants and possibly occlude their exteroceptors (Eisner and Meinwald, 1966; Schildknecht et al., 1967; Eisner et al., 1978).

Female *Glomeris*, because of their relatively large size, might be expected to accumulate defensive secretion more quickly than males of this species. Mature females weigh from 50 to 350 mg, mature males weigh from 20 to 100 mg, and within a cohort of *Glomeris* the female-male weight ratio typically is 3:1 (Heath et al., 1974). Both sexes molt annually for up to 8 years after reaching sexual maturity at the age of 3–4 years, but females continue to grow more than males (Heath et al., 1974). If the rate of biosynthesis and storage of defensive exudate are positively correlated with body size, then a sexual difference in secretion accumulation should exist. Alternatively, if it is selectively advantageous for adult millipedes, regardless of size, to have secretory droplets sufficiently large to deter certain predators, then regeneration of defensive reserves should be similar between sexes of *Glomeris*. The following experiment was designed to discriminate between these two alternatives.

METHODS AND MATERIALS

Millipede Collecting. Adult *Glomeris* were gathered at the Peak District, Derbyshire, England (National Grid Reference SK 152548, 197638, 165714, and 088724; elevation 250 m) in four woodlots closed to livestock and the public. They were collected in moist leaf litter and beneath moss and crumbled limestone in late March and early April.

Special care was taken to prevent millipedes from prematurely discharging their secretion. In the field they were gently rolled onto a cool spoon and then off the spoon into vials lined with moss and leaf mold. They were quickly transported to the laboratory where they were spooned individually into translucent 0.5-liter plastic beverage cups filled with moist leaf litter above 2 cm of sieved soil from their habitats. Under these circumstances, they never visibly released any exudate. Millipedes remained undisturbed in capped cups until they were transferred by spoon to a culture dish lined with filter paper just before their secretion was assayed.

Secretion Assay. Glomeris were milked singly to depletion under a dissecting microscope by squeezing them uniformly with slide forceps whose square jaws had been drilled out to form a template for a coiled millipede. The efficacy of the milking process was demonstrated when 20 millipedes remilked within 24 hr did not visibly release any exudate. Even when probed with a hot needle or blown on with a jet of hot air, stimuli known to be highly

effective, none of these animals secreted. Emptiness of glands was confirmed subsequently by dissecting several milked animals.

Exudate from a single millipede initially was aspirated into a volumetrically calibrated capillary (Bolab Microcaps) and the volume and weight of its contents were quickly determined. Secretion volume was calculated by multiplying the length of the continuous column of fluid in a glass tube, as measured microscopically in profile view to within 0.02 mm with calipers, by the tube's cross-sectional area. Secretion mass was weighed to within 0.1 mg on a semimicrobalance. This procedure was followed for 24 samples at 20°C, and the density of secretion was calculated.

Aspiration of secretion proved unacceptably inefficient. As a substitute, each millipede was weighed twice, just before and immediately after blotting its secretion with several small squares of clean filter paper, a process requiring less than 2 min. Secretion mass was presumed to equal the loss in body mass for a millipede. Secretion samples were individually preserved in ethanol for chemical analyses.

Experimental Design. Adult *Glomeris* in eight groups of 10 males and 10 females chosen at random were milked to depletion and then given from 7 to 100 days to replenish their secretion before being remilked. During this time millipedes were kept individually at 15 ± 2°C in capped plastic cups inside an air-conditioned room illuminated indirectly by sunlight through a north-facing window. The climatic conditions were designed to simulate average temperatures and photoperiod experienced by these millipedes across the United Kingdom during 9 months of their above-ground activity (Bocock and Heath, 1967; Mochlinski, 1970; Blower, 1974; Chandler and Gregory, 1976).

As a control to approximate maximal loading of defensive secretion, an additional 130 *Glomeris* were left undisturbed for 100 days to supplement their spring field reserves. They were placed unmilked in cups and were maintained under the same conditions as the other millipedes.

The data were analyzed statistically as a function of sex, time, and body size using general linear regression, ANOVA, Student's, and Duncan's parametric tests (Neter and Wasserman, 1974; SAS, 1979; Snedecor and Cochran, 1980). The hypotheses that one sex produced less secretion than the other sex, that the reloading treatments represented a temporal progression, and that the treatments were less than the controls were tested using Wilcoxson's rank sum, Jonckheere's, and Dunn's nonparametric procedures (Hollander and Wolfe, 1973).

RESULTS

Secretion Density. Density of *Glomeris* secretion at room temperature (20°C) was relatively constant regardless of the volume released individually

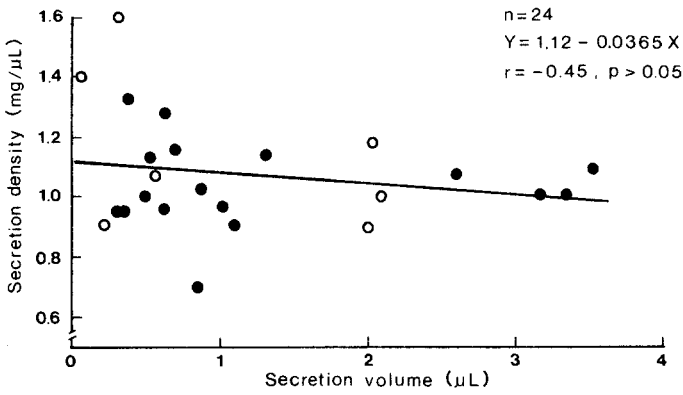


FIG. 1. Density of *Glomeris* secretion as a function of the amount discharged individually by 7 male (○) and 17 female (●) millipedes.

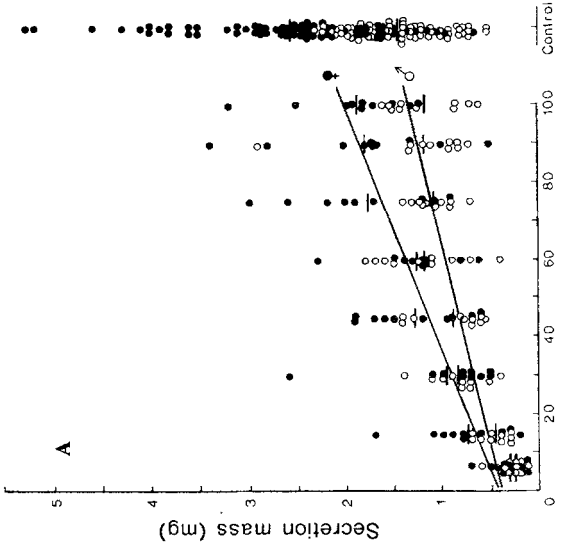
by adult millipedes (Figure 1). In addition, there was no significant difference in the density of secretion from male and female millipedes (Wilcoxson's rank sum test, $P > 0.10$). Because the slope of the linear regression in Figure 1 was not significantly different from zero (Student's t test, $P = 0.4$), all samples were lumped into one group. The average density ($\bar{X} \pm$ SE) for the 24 samples was 1.07 ± 0.04 mg/μl, slightly greater than the density of water.

Most data for *Glomeris* exudate in this paper are reported in gravimetric units, untransformed from the original measurements. To convert to precise volumetric units (μl), one need simply divide by the density of the secretion. As an approximation, the secretion density can be set at unity, making mass and volume easily interconvertible.

Secretion Reloading. Glomeris reloaded secretion slowly at almost constant rates during the 100-day experiment (Figure 2A). Female millipedes each tended to produce more secretion than males, but this difference was only slightly significant until more than 90 days had elapsed (Table 1).

A different view of reloading was obtained when secretion amounts were expressed as the proportion of individual body mass before remilking (Figure 2B). As before, rates of secretion production were highly linear throughout most of the 100-day experiment. However, male millipedes, by virtue of their small size, accumulated almost 1.6 times as much defensive fluid per unit body mass as females. During the entire experiment differences in proportionate amounts of defensive secretion between sexes were always highly significant (Table 1).

According to a two-way analysis of variance for the secretion reloading, sex and time were both highly significant variables (Table 2). The nonsignificant sex-by-time interaction indicated that although the two sexes dif-



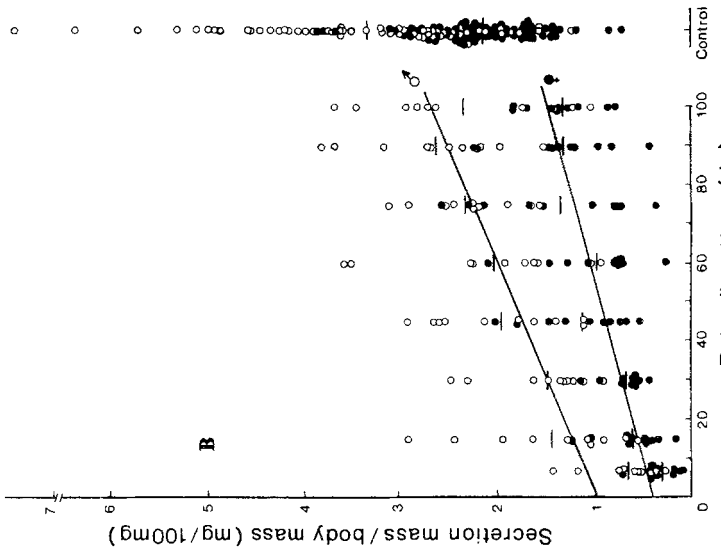


FIG. 2. Secretion yield of individual male (○) and female (●) *Glomeris* as a function for reloading time. Means (horizontal bars) and best-fit linear regression lines for absolute and proportionate yields are indicated. The exact reloading equations are (A): males, $y = 0.395 + 0.0093x$ ($F = 51.42$, $P < 0.0001$; $R^2 = 0.397$); females, $y = 0.430 + 0.0156x$ ($F = 63.51$, $P < 0.0001$; $R^2 = 0.449$); and (B): males, $y = 0.0097 + 0.00017x$ ($F = 46.85$, $P < 0.0001$; $R^2 = 0.375$); females, $y = 0.0040 + 0.00011x$ ($F = 44.54$, $P < 0.0001$; $R^2 = 0.363$). Yields of controls, millipedes given 100 days to supplement their field reserves, are shown on the right.

TABLE 1. TEST STATISTICS FOR SEXUAL DIFFERENCES IN AMOUNT OF SECRETION RELOADED BY ADULT *Glomeris*[a]

Reloading time (days)	Secretion mass (mg)				Secretion mass/body mass (mg/mg)			
	t	*P*	*W*	*P*	*t*	*P*	*W*	*P*
7	0.84	NS[b]	92.5	NS	2.77	<0.01	70.5	<0.005
15	2.10	<0.025	82.0	<0.05	3.08	<0.005	67.5	<0.005
30	0.43	NS	99.0	NS	4.07	<0.0005	58.0	<0.001
45	2.06	<0.05	78.5	<0.025	3.12	<0.005	71.5	<0.005
60	0.28	NS	101.5	NS	3.29	<0.005	67.0	<0.005
75	2.82	<0.01	77.5	<0.025	3.15	<0.005	74.0	<0.01
90	1.71	NS	78.0	<0.025	4.61	<0.0005	61.0	<0.001
100	3.04	<0.005	67.5	<0.005	3.13	<0.005	76.0	<0.025
Control	8.30	<0.0005	7.10[c]	<0.0001	6.57	<0.0005	6.07[c]	<0.0001

[a]Values for one-sided Student's *t* and Wilcoxson's *W* with their associated probabilities are given. $N_m = N_f = 10$ in all groups except for the controls, where $N_m = 58$ and $N_f = 68$.
[b]NS = not significant ($P \geq 0.05$).
[c]Large sample approximation.

fered overall in mean secretion accumulated, in the course of the study they produced secretion at approximately the same absolute rate. Males and females averaged 0.89 mg and 1.25 mg secretion, respectively; their mean proportionate amounts of secretion were 1.87% and 0.97% throughout the entire reloading experiment. In general, then, males stored only 71% as much secretion as females, but this amounted to twice the investment per unit body mass for males relative to females. Linear regression analysis, as shown

TABLE 2. TWO-WAY ANOVA SUMMARY OF SECRETION RELOADING IN ADULT *Glomeris*[a]

Source of variation	*df*	Secretion mass (mg)			Secretion mass/body mass (mg/mg)		
		Sum of squares	*F*	*P*	Sum of squares	*F*	*P*
Sex (*S*)	1	5.33	23.58	<0.0001	0.003128	89.77	<0.0001
Time (*T*)	7	27.63	17.46	<0.0001	0.003676	14.65	<0.0001
$S \times T$ interaction	7	2.67	1.69	NS[b]	0.000254	1.01	NS
Error	144	32.55			0.005161		

[a]See text explanation of the factors.
[b]NS = not significant ($P \geq 0.05$).

in Figure 2B, revealed that by 100 days secretion typically represented 2.7% of a male's and 1.3% of a female's body mass. More detailed comparisons of the regression curves by sex were not possible because their error variances were not equal ($F^* = 2.3$, $P > 0.10$).

Secretion production was positively correlated not only with sex and elapsed time, but also to a lesser degree with millipede body weight. As indicated in Figure 3, significant regressions of secretion mass as a function of

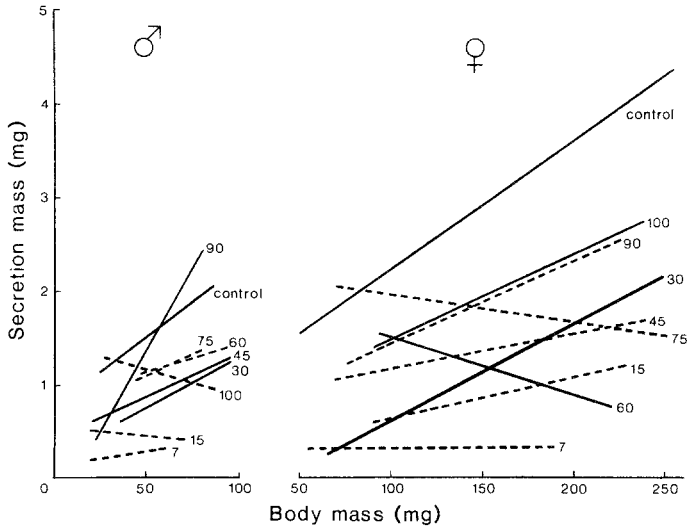


FIG. 3. Secretion yield of male and female *Glomeris* in reloading and control groups plotted against body mass. Linear regression equations for 10 males or 10 females, except controls which had 58 males or 68 females, are depicted by solid lines if significant or broken lines if nonsignificant at the $P = 0.10$ level (two-tailed F test). The exact equations for males are: 7 days, $y = 0.134 + 0.0030x$ ($F = 0.67$, $P = 0.44$; $R^2 = 0.078$); 15 days, $y = 0.544 - 0.0019x$ ($F = 0.34$, $P = 0.58$; $R^2 = 0.041$); 30 days, $y = 0.224 + 0.16x$ ($F = 8.31$, $P = 0.020$; $R^2 = 0.509$); 45 days, $y = 0.426 + 0.0092x$ ($F = 7.12$, $P = 0.028$; $R^2 = 0.471$); 60 days, $y = 0.839 + 0.0060x$ ($F = 0.34$, $P = 0.57$; $R^2 = 0.041$); 75 days, $y = 0.715 + 0.0077x$ ($F = 1.70$, $P = 0.23$; $R^2 = 0.175$); 90 days, $y = -0.404 + 0.0355x$ ($F = 20.29$, $P = 0.0020$; $R^2 = 0.717$); 100 days, $y = 1.343 - 0.0033x$ ($F = 0.10$, $P = 0.76$; $R^2 = 0.012$); control, $y = 0.768 + 0.0148x$ ($F = 10.91$, $p = 0.0017$; $R^2 = 0.163$). For females they are: 7 days, $y = 0.305 + 0.0001x$ ($F = 0.01$, $P = 0.93$; $R^2 = 0.001$); 15 days, $y = 0.197 + 0.0045x$ ($F = 1.47$, $P = 0.26$; $R^2 = 0.155$); 30 days, $y = -0.437 + 0.0105x$ ($F = 31.02$; $P = 0.0005$; $R^2 = 0.795$); 45 days, $y = 0.801 + 0.0037x$ ($F = 1.99$, $P = 0.19$; $R^2 = 0.181$); 60 days, $y = 2.153 - 0.0063x$ ($F = 3.52$, $P = 0.098$; $R^2 = 0.306$); 75 days, $y = 2.255 - 0.0028x$ ($F = 0.48$, $P = 0.51$; $R^2 = 0.065$); 90 days, $y = 0.575 + 0.0087x$ ($F = 2.78$, $P = 0.13$; $R^2 = 0.285$); 100 days, $y = 0.561 + 0.0092x$ ($F = 6.38$, $P = 0.036$; $R^2 = 0.444$); control, $y = 0.859 + 0.0138x$ ($F = 46.58$, $P < 0.0001$; $R^2 = 0.414$).

body mass were obtained for three of the eight reloading groups. The lack of a significant relationship at 7 and 15 days was not surprising because most *Glomeris* had marginally detectable exocrine loads. But the apparent disappearance of the positive relationship between secretion mass and body mass midway through the experiment was unexpected. This effect was more pronounced in females than in males. It coincided with the onset of molting in both sexes. The first molt among groups of 20 millipedes was detected at 60 days. The frequency of exuviae from both sexes increased steadily between 60 and 100 days. At the end of the experiment, all millipedes had molted at least once. Two females, initially weighing 51 and 105 mg, each gained nearly 50 mg and molted twice within 90 days, as indicated by the presence of two complete exuviae in their containers.

Secretion in Control Millipedes. Sexual dimorphism in defensive reserves was exhibited by 125 *Glomeris* in a control group that had 200 days to supplement their field loads of secretion before they were first milked (Figure 3). Fifty-eight males and 68 females each possessed an average of 1.43 mg and 2.56 mg of fluid, respectively, which amounted to 3.34% and 2.16% of their body weights. Both differencs between sexes were highly significant (Table 1).

Parametric and nonparametric methods were used to test the hypothesis that the amount of secretion reloaded during $\leqslant 100$ days by each sex was less than that held by the controls. The null hypothesis was that there was no significant difference between a reloading group and the control group of millipedes by sex with regard to the size of their defensive reserves. As indicated in Table 3, the absolute or proportionate mass of secretion reloaded by male *Glomeris* after 60 or 75 days, respectively, was not significantly different from the controls according to both statistical methods. However, for females there was a slight discrepancy between the outcomes of the two tests. Dunn's nonparametric test showed no significant difference in the absolute mass of fluid discharged by experimental females after 75 days of reloading and that given off by the control females, but the other three statistical analyses showed the secretion reloaded by females was always significantly less at $P = 0.05$ than that exuded by control females.

The temporal orderliness of the reloading and control secretion data was demonstrated using the large sample approximation for Jonckheere's distribution-free statistic (J^*). For each sex, all possible pairs of observations on the absolute or proportionate secretion loads in each group were ranked wherein the controls were considered the last sample date. In all cases the results were highly significant ($J^* > 5.7$, $P < 0.0001$), even when the control data were omitted from the calculations. Results of Duncan's multiple-range test, shown in Table 3, confirm that the samples came from populations whose medians were in a specified temporal progression.

Assuming the control means for each sex represented nearly maximal

TABLE 3. SUMMARY OF STATISTICS COMPARING DEFENSIVE FLUID RELEASED BY RELOADING AND CONTROL (C) *Glomeris*

Sex	Variable	Statistical test	Reloading time (days) 7	15	30	45	60	75	90	100	C
Male	Secretion mass	Dunn's					—	—	—	—	—
		Duncan's					—	—	—	—	—
					—	—	—	—	—	—	
			—	—							
	Secretion mass / Body mass	Dunn's						—	—	—	—
		Duncan's						—	—	—	—
						—	—	—	—	—	
				—	—	—	—				
Female	Secretion mass	Dunn's						—	—	—	—
		Duncan's				—	—	—	—	—	
				—	—	—	—				
			—	—	—						
	Secretion mass / Body mass	Dunn's									
		Duncan's				—	—	—	—	—	
				—	—	—	—				
			—	—	—						

[a]In each row Dunn's nonparametric test compares single treatments to the controls, whereas Duncan's parametric test compares all groups on a pairwise basis. Groups connected by a line are not significantly different at $P = 0.05$.

secretion storage, the two sets of linear regression equations in Figure 2 were used to project the time needed for *Glomeris* to achieve repleteness after totally exhausting their defensive reserves. Males and females were estimated to require 110–140 days and 140–170 days, respectively, to refill their glands fully at 15°C.

DISCUSSION

During the first 90 days of secretion reloading, male *Glomeris* had only slightly less fluid than females and this difference usually was not highly significant ($P > 0.01$). Hence, from a predator's perspective, both sexes might not only share a common appearance, but on contact they might also present comparable chemical defenses.

Might male *Glomeris* invest disproportionately in sticky exudate because they are preyed upon more intensively than females? Size and behavioral attributes would seem to make adult males more vulnerable to pre-

dation than adult females. Many European arthropods as well as vertebrates consume prey as large as adult male *Glomeris*, but few sympatric spiders, centipedes, ants, and ground beetles can extend their mouthparts wide enough to bite adult female *Glomeris* once they have coiled up (J. Carrel, unpublished observations; Carrel and Eisner, in preparation). Male *Glomeris*, because of their greater mobility (Heath et al., 1974), may also be exposed to more predatory encounters than females.

Adult *Glomeris* retained some, if not most, of their secretion during molts (Figures 2 and 3). The spiroboloid millipede *Narceus annularis* also conserves much of its defensive benzoquinones when shedding its skin (Eisner et al., 1978). This seems remarkable considering that the defensive glands of all diplopods examined so far are lined with cuticle (Eisner et al., 1978), the wholesale removal of which should carry along fluid reserves.

The possibility that adult *Glomeris* might require more than 4 months to reload their empty glands agrees with the results of two previous studies of this kind. Eisner et al. (1967) reported that the millipedes *Apheloria corrugata* and *Pseudopolydesmus serratus* from upstate New York slowly and variably regenerate their cyanogenic capacity. But these authors cautioned that their findings may be subnormal, reflecting the failing health of a limited number of animals maintained under laboratory conditions of high mortality. This was not the case in my experiments: less than 3% of adult *Glomeris* in both reloading and control groups died, and observations on feeding, growth, and oviposition indicated that the animals generally were healthy. Plattner et al. (1972) also found that nymphs of the cockroach *Blatta orientalis* accumulate sticky secretion on their terminal tergites more or less constantly but with a high degree of individual variation for 80 days before molting to adults. Perhaps, as suggested by Eisner (1981), biosynthetic activity in millipedes and their kin is limited by a pervasive shortage of utilizable nitrogen in their diets. The effect could be particularly acute on *Glomeris* whose exudate is rich in nitrogenous compounds.

Based on their life history, one wonders whether adult *Glomeris* often achieve a full reserve of defensive fluid in the field. They are active in the United Kingdom from mid-March to to early November except for about 3 weeks in the summer when they coil up to molt (Bocock et al., 1967; J. Carrel, unpublished observations). From November to March they remain buried in the soil, seldom feed, and grow little (Bocock and Heath, 1967; Bocock et al., 1967). If secretion synthesis is confined yearly to a 230-day period of activity, millipedes may need most of this time to replace the varying amounts of fluid they have discharged during the normal course of encounters with predators.

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FILBERTWORM SEX PHEROMONE
Identification and Field Tests of (*E,E*)- and (*E,Z*)-8,10-Dodecadien-1-ol Acetates[1]

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Abstract—(*E,E*)- and (*E,Z*)-8,10-Dodecadien-1-ol acetates were identified in a 1:4.3 ratio in the extract of abdominal tips of female filbertworm moths, *Melissopus latiferreanus* (Walsingham). The identifications were based on electroantennogram (EAG) analysis, gas chromatography, mass spectrometry, ozonolysis, and synthesis. The *E,Z* isomer produced the stronger EAG response. In the field tests of various ratios of *E,E*:*E,Z*, the ratio found in the extract captured the most males. The pure *E,E* isomer initially was not attractive by itself (<0.1% *E,Z*) but became attractive after a few days, presumably because of isomerization. The *E,Z* isomer (<0.1% *E,E*) was attractive initially, but this compound might have isomerized faster than the *E,E* isomer. A study of the isomerization showed that regardless of the initial mixture of 8,10-dodecadien-1-ol acetate isomers, almost complete equilibration existed after one month. The equilibrium mixture consisted of 9% *Z*8,*E*10, 65% *E*8,*E*10, 23% *E*8,*Z*10, and 3% *Z*8,*Z*10. Concentrations in rubber septa (1:4 ratio of *E,E* to *E,Z*) of 0.03–3.0 mg/septum produced equivalent trap catches.

Key Words—*Melissopus latiferreanus*, Lepidoptera; Tortricidae, Olethreutinae, filbertworm, sex pheromone, sex attractant, (*E,E*)-8,10-dodecadien-1-ol acetate, (*E,Z*)-8,10-dodecadien-1-ol acetate, conjugated diene isomerization.

[1]This paper reports the results of research only. Mention of a commercial product in this paper does not constitute a recommendation by the U.S. Department of Agriculture.

INTRODUCTION

The filbertworm, *Melissopus latiferreanus* (Walsingham), is a pest of filberts in Oregon and Washington and walnuts and pomegranates in California. Previously, from field screening trials, we identified (*E*,*E*)-8,10-dodecadien-1-ol acetate (*E*8,*E*10–12:Ac) as an attractant for male *M. latiferreanus* (Davis and McDonough, 1981). Subsequently, we undertook a chemical study of the electroantennogram (EAG) active components extracted from female abdominal tips and identified two components which were then evaluated in field tests. These laboratory and field studies are the subject of this report.

METHODS AND MATERIALS

Insects. Filbert nuts were collected from the ground in an abandoned orchard near Dundee, Oregon, on September 14, 15, and 16, 1981. The nuts were maintained in a rearing room at a temperature of 21°C, 65% relative humidity, and a 16-hr day length. The day length was reduced by 2 hr per week until a 12-hr day length was reached. Corrugated cardboard strips (2 cm wide) were provided for the insects to diapause in.

On November 19, strips containing 1868 cocoons were placed in an environmental chamber to induce diapause. The chamber was set at 2.8°C, a relative humidity of 38–40%, and with total darkness.

On January 6, 1982, 592 cocoons were removed from the chamber and returned to the rearing room at a temperature of 18°C, 50–55% relative humidity, and a photophase of 12 hr. After one week the temperature was raised to 21°C and the photophase was increased to 14 hr. After the second week the temperature was raised to 24°C and the photophase was increased to 16 hr.

The first emergence occurred on February 25, 1982, and subsequently a total of 309 adult moths (52.7% males and 47.3% females) emerged.

On March 1 the remaining strips with 1276 cocoons were placed in a rearing room with conditions identical to those previously described. The first emergence for this group occurred April 15, 1982, and a total of 733 moths (50.7% males and 49.3% females) subsequently emerged.

Collection of Pheromone. Female moths (2–5 days old) were collected 1 hr after the beginning of scotophase and placed in a refrigerator to inactivate them for at least 10 min prior to dissection. Severed abdominal tips were allowed to steep 15 min in dichloromethane. Then the solution was removed with a syringe. The amount of pheromone obtained per female was usually ca. 2 ng (range 0.5–4.5 ng).

Gas Chromatography. The following gas chromatographic columns were used: (A) silanized glass column (1.8 × 2.3 mm OD) packed with 3%

dimethyl silicone (SE-30) on 80/100 mesh Gas Chrom Q and operated at 150°C for 2 min and then temperature programed at 4°/min to 210°C and held at this temperature; (B) 30 m × 0.25 mm ID glass capillary coated with polyethylene oxide (Carbowax 20 M) operated at 80° for 2 min and programed at 32°/min to 150°C and held at this temperature; (C) 56 m × 0.25 mm ID glass capillary coated with dimethyl silicone (SE-30) operated at 80° for 2 min and programed at 32°min to 180°C and held at this temperature; (D) 60 m × 0.25 mm ID fused silica with crosslinked methyl silicone bonded to the column wall (DB-1) operated at 80° for 7 min and programed at 6°/min to 200°C and held at this temperature.

Purification of Sex Pheromone. The pheromone was purified by collection from a gas chromatograph equipped with a flame ionization detector and a column effluent splitter (1 part of effluent to the detector and 10 parts to the collection trap). Column A was used. Effluent was collected in glass tubing cooled in a mixture of Dry Ice and acetone.

Electroantennogram (EAG) Analysis. The technique used here has been described previously (McDonough et al., 1980).

Gas Chromatography–Mass Spectrometry (GM-MS). A quadrupole mass spectrometer (model 4000, Finnigan Corp., Sunnyvale, California) with computerized data collection and a GC inlet was used. A dimethyl silicone (DB-1) capillary column (0.25 mm ID, 60 m) was held at 120°C for 0.6 min and then programed at 25.5°C/min to 225°C and held at this temperature. All spectra were taken in the electron impact mode.

Ozonolysis. Purified pheromone in 25 μl of heptane and cooled in a Dry Ice–Acetone bath was ozonized with an ultraviolet ozonizer (model 03VI, Orec Co., Phoenix, Arizona) and the ozonide was reduced with triply recrystallized triphenylphosphine (Beroza and Bierl, 1967). The product was analyzed on column D. The control experiments were conducted in the same manner. The control substances were: (1) synthetic *E*8,*E*10–12:Ac collected from the gas chromatograph under the same conditions used for purification of the pheromone, and (2) column bleed from the gas chromatograph.

Synthesis of (E,E)- *and* (E,Z)-*8,10-Dodecadien-1-ol Acetates.* A 1:1.6 mixture (*E,E*:*E,Z*) was produced by the same procedure used for the synthesis of (*E,E*)- and (*E,Z*)-10,12-tetradecadien-1-ol acetates for *Amorbia cuneana* (McDonough et al., 1982) except that 1,8-octanediol was substituted for 1,10-decanediol.

Liquid Chromatography. Separation and purification of the *E,E* and *E,Z* isomers of synthetic 8,10–12:Ac were carried out by HPLC (Hewlett-Packard, model 1084B) with an 0.5-cm-OD × 20-cm-long column packed with octadecyl reverse phase support (RP-18; 10 μm). The isomers were eluted with methanol–water (70:30) at a flow rate of 2 ml/min. The eluants were monitored with a variable wavelength ultraviolet detector set at 240 nm. The *E,Z* isomer eluted at 23.0 min and the *E,E* at 27.4. After one purifi-

cation, the *E,Z* isomer contained 1.5% *E,E* (by capillary GC analysis) and the *E,E* isomer contained 2.5% *E,Z*. For the field tests of the pure *E,E* and *E,Z* isomers, the compounds were subjected to a second purification and contaminating isomers were undetectable (less than 0.1%).

Field Tests. All the field tests were conducted in abandoned filbert orchards at two locations in the Williamette Valley of Oregon. Pherocon IC sticky traps were attached to the interior of each of the trees at a height of approximately 3.7 m. Trees were about 8 m apart. Traps were placed at this height because tests conducted in pomegranate orchards (Davis et al., 1983) showed that trap catch increased with height. Traps were arranged in a randomized complete-block design. For each test, traps were rotated every other day, and each replicate occupied each location once.

One row of trees was unused on all sides of the test area to serve as a buffer zone to prevent a border effect, and all traps used in the experiment were randomly selected for the initial trap location. With the exception of the dosage test, all rubber septa containing pheromone treatments (blank septa served as controls) were charged at the rate of 0.5 mg/septum (total of both components).

Analysis of Field Aged Lures. The lures were extracted with dichloromethane as previously described (Butler and McDonough, 1979). The extract was evaporated to near dryness with a rotary evaporator and the residue was made up to a volume of 5 ml in dichloromethane. Then 200 μl of the dichloromethane solution was made up to 1 ml in heptane. This solution was analyzed by capillary gas chromatography (column C).

RESULTS AND DISCUSSION

Structure Determination. An extract of 105 female abdominal tips was injected onto column A and fractions were collected every 2.5 min for 22.5 min. Standards of (*E,E*)-8,10-dodecadien-1-ol acetate (*E*8,*E*10–12:Ac) and octadecyl acetate eluted at 7.9 and 21.0 min, respectively. Each fraction was subjected to EAG analysis and only fraction 4 elicited a significant response: 1.3 mV vs. 0.20–0.35 mV for the other fractions; air, 0.15 mV. The GC trace of fraction 4 showed a peak with the same retention time as *E*8,*E*10–12:Ac. Fraction 4 was reinjected onto column A, and a fraction containing the peak with the retention time of *E*8,*E*10–12:Ac was collected plus two other fractions containing eluate for 2 min preceding and 2 min following the peak with the retention time of *E*8,*E*10–12:Ac. When these two fractions were analyzed by EAG, only the one containing the peak with the retention time of *E*8,*E*10–12:Ac was active: 1.1 mV vs. 0.4 and 0.5 mV; air, 0.5 mV.

Next, the purified active material was injected onto polar and nonpolar capillary columns (columns B and C, respectively). On column C two peaks

were obtained in the ratio of 1:4.3. The earlier eluting peak had the same retention time as *E*8,*E*10–12:Ac (28.25 min), while the second peak eluted at 28.60 min. On column B two peaks were also found in the ratio of 1:4.3, and the first peak again had the retention time of *E*8,*E*10–12:Ac (31.20 min) and the second peak eluted at 31.90 min.

The mass spectra of these two candidate pheromone components were identical to each other and to *E*8,*E*10–12:Ac. These compounds showed diagnostic peaks at $m/e = 61$ ($CH_3CO_2H_2^+$), $m/e = 224$ (M^+), and $m/e = 164$ ($M\text{-}60^+$).

In two separate determinations on 50 and 215 ng of purified pheromone, ozonolysis gave only one product detectable by GC and its retention time was the same as 8-acetoxyoctanal produced by ozonolysis of *E*8,*E*10–12:Ac. At least one ozonolysis product would have been detected for any position of the double bonds.

The foregoing data establish that the two compounds isolated from the female sex pheromone glands are two of the four geometric isomers of 8,10-dodecadien-1-ol acetate. Because the capillary columns which were used will separate the four isomers (McDonough et al., 1982), the first eluting isomer has the *E*8,*E*10 configuration. Based on the known relationship of retention time to double-bond configuration for this type of compound (Roelofs et al., 1972; McDonough et al., 1982), the second eluting component must have the *E*8,*Z*10 configuration. When injected on the capillary columns B and C, the synthetic mixture of *E*8,*E*10- and *E*8,*Z*10–12:Ac had retention times identical to the pheromone gland components, therefore, substantiating this assignment.

EAG of EE *and* EZ *Isomers.* EAG responses to various doses of *E,E* and *E,Z* isomers were compared. At 5, 10, and 100 ng, the *E,E* isomer produced 0, 0, and 0.40 mV response, while the *E,Z* isomer produced 0.70, 0.75, and 1.15 mV response. Thus, the *E,Z* isomer clearly evoked the stronger response.

Field Tests. Because traps baited with septa containing *E*8,*E*10–12:Ac had been previously shown to be attractive to *M. latiferreanus*, the first tests were designed to determine the effect of *E,E* : *E,Z* ratio on trap catch (Table 1). Two successive tests of two weeks each were conducted. During the first two weeks, the ratio found in the abdominal tip extract (20:80, *E,E*:*E,Z*) produced the highest trap catch. During the second two weeks, all baits except the highest ratio of *E,E*:*E,Z* produced equivalent trap catches.

In a separate test, the pure *E,E* and *E,Z* isomers (less than 0.1% contaminating isomer) were compared in three traps baited with each isomer. Total catches during the first 2 days were: *E,E*, 1; *E,Z*, 18; and blanks, 0. Totals after the first 4 days were: *E,E*, 1; *E,Z*, 31; blanks, 0. And totals after the first 6 days were: *E,E*, 8; *E,Z*, 78; blanks, 0. It is known that conjugated dienes isomerize in rubber septa (Fujiwara et al., 1976; Shani and Klug, 1980).

TABLE 1. CAPTURES OF *M. latiferreanus* IN FILBERT ORCHARDS BY DIFFERENT RATIOS OF (*E,E*)- AND (*E,Z*)-8,10-DODECADIEN-1-OL ACETATES (JULY 27–AUGUST 24, 1982; 5 REPLICATES; TOTAL DOSAGE 0.5 MG/TRAP)

Ratio, *EE*:*EZ*	Captures per trap[a]	
	July 27–Aug. 10	Aug. 11–Aug. 24
97:3	11.2 b	6.8 b
80:20	11.4 b	10.2 ab
60:40	15.4 b	7.2 ab
40:60	7.8 b	14.8 a
20:80	25.6 a	13.2 ab
2:98	15.4 b	11.8 ab

[a]Means followed by same letter are not significantly different (Duncan's multiple-range test, $P < 0.05$).

The *E,E* isomer appears to be unattractive by itself. Probably the *E,E* captures on days 5 and 6 are a result of formation of *E,Z* isomer. The *E,Z* isomer could be attractive by itself. On the other hand, perhaps it isomerizes faster (being less stable than the *E,E* isomer) and some *E,E* isomer was present even on the first day of the test.

The effect of attractant dosage on trap catch for septa baited with the *E,E* isomer has been reported elsewhere (Davis and McDonough, 1983). Because of a possible interdependence between optimum dosage and ratio of pheromone components on trap catch (Roelofs 1978), the effect of dosage of the 1:4 ratio (*E,E*:*E,Z*) was evaluated (Table 2).

Over the 100-fold range tested, there was no change in attractancy with dosage. These dosages correspond to evaporation rates of 0.019–1.9 μg/hr at 23°C (Hathaway et al., 1979). This result is in contrast to our earlier study (Davis and McDonough, 1983) in which a dosage of 0.64 mg was superior to lower dosages.

Analysis of Field-Aged Lures. Because conjugated dienes are known to isomerize, the ratio of components used in our field tests might have been changing during the test. To obtain some estimate of the degree of change which might have occurred, the septa were collected at the end of the tests and stored in a freezer (−18°C) and subsequently analyzed. The data are summarized in Table 3.

The degree of isomerization was surprisingly extensive. Regardless of the initial ratios, the final ratios were very similar. Those with the initially high *E,E* content ended at ca. 70% *E,E* and those with initially low *E,E* content ended at about 60% *E,E*. Also, the high *E,Z* baits ended at about 30% *E,Z* and the low at about 20% *E,Z*. Because of the initial ratios were symmetrical with respect to the tested isomers and all baits ended with sim-

TABLE 2. EFFECT OF DOSAGE OF 1:4 RATIO (*EE*:*EZ*) OF PHEROMONE COMPONENTS ON TRAP CATCH (5 REPLICATES)

Dosage (mg)	Males caught/trap[a] (Aug. 6–16, 1982)
0.03	19.8 a
0.10	18.2 a
0.30	21.8 a
1.00	18.0 a
3.00	14.4 a
Blank	0.0 b

[a]Means followed by the same letter are not significantly different (Duncan's multiple-range test, $P < 0.05$).

ilar ratios, the average should give a good estimate of the equilibrium values of these isomers in rubber. The averages were 9% *Z,E*, 65% *E,E*, 23% *E,Z*, and 3% *Z,Z*. These averages are similar to those found by Ideses et al. (1982) for *E*7,*E*9-12:Ac except that they found the *E,Z* and *Z,E* isomer content to be about equal (ca. 15% each). In our case the greater amount of *E,Z* over *Z,E* could be due either to the difference in compounds or the difference in media—rubber septa versus cyclohexane.

These results may explain the ratio test results (Table 1). During the first two weeks of the ratio test, the traps with the natural ratio (1:4, *E,E*:*E,Z*) captured the most males, but the preference was not large probably because significant isomerization had already occurred. During the second

TABLE 3. RATIOS OF ISOMERS OF 8,10-DODECADIEN-1-OL ACETATE IN RUBBER SEPTA FROM FIELD TESTS OF TABLE 1 AFTER 4 WEEKS OF FIELD TESTS AND 2 MONTHS' STORAGE AT −20°C[a]

Original %		% After aging			
EE	*EZ*	*ZE*	*EE*	*EZ*	*ZZ*
97.5	2.5	9.9	69.7	17.5	2.9
80.0	20.0	8.4	69.4	18.9	3.2
60.0	40.0	10.4	66.6	20.5	2.5
40.0	60.0	8.4	64.9	24.1	2.5
20.0	80.0	7.9	58.1	30.9	3.1
1.5	98.5	8.9	59.6	28.5	3.0
Average		9.0	64.7	23.4	2.9

[a]Total dosage per septum was 0.50 mg. Each value is the average of 2 septa.

two weeks of the test, the isomerization was so extensive that all baits except one were equivalent.

The fast rate of isomerization of *E*8,*E*10- and *E*8,*Z*10-12:Ac has implication for other conjugated dienes. Even for single-component pheromones, isomerization could be important because isomers can be inhibitory (Roelofs, et al., 1972). This may explain the observation of Greenway and Wall (1981) that the pea moth attractant, *E*8,*E*10-12:Ac is initially highly attractive, but looses attractiveness in the field in a few days.

*E*8,*E*10-12:Ac has been reported to be a sex attractant or sex pheromone for three other species of Lepidoptera, all of which are also members of the Olethreutinae subfamily of the Tortricidae: the pitch pine tip moth, *Rhyacionia rigidana* (Hill et al., 1976); the pea moth, *Cydia nigricana* (Wall et al., 1976); the green budworm moth, *Heyda nubiferana* (Frerot et al., 1979). In contrast, this is the first report of *E*8,*Z*10-12:Ac being a sex pheromone component for any species.

Roelofs and Cardé (1974) have pointed out that Tortricinae use 14-carbon compounds in their sex pheromones whereas Olethreutinae use 12-carbon compounds. The sex pheromone of the Western avocado leafroller (*E*10,*Z*12-14:Ac and *E*10,*E*12-14:Ac; McDonough, et al., 1982) which is a Tortricinae, and the filbertworm which is an Olethreutinae are examples of this relationship. These pheromones have identical functional groups, double-bond configurations, and double-bond position (from the nonpolar end of the molecule) and differ only in the number of carbon atoms—14 and 12 uninterrupted carbon atoms, respectively.

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CONCENTRATION AND PRELIMINARY CHARACTERIZATION OF A CHEMICAL ATTRACTANT OF THE OYSTER DRILL, *Urosalpinx cinerea*

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Abstract—Predatory muricid gastropods, *Urosalpinx cinerea*, respond to specific chemical stimuli by creeping upcurrent. Attractant substances originate from living barnacles. Newly hatched snails have no prior predatory experience but respond strongly to attractants. We report here methods for rapidly extracting and desalting attractants from seawater. Attractants from living barnacles are relatively large, at least partially proteinaceous, heat-stable molecules (>1000 but <10000 daltons) that adsorb onto Amberlite XAD-7, a polyacrylate water purification resin, at neutral pH. Attractants remain adsorbed to the resin during a wash with deionized water and can be eluted in a small volume with 100% methanol. Attractant substances are effective in the bioassay in μg/liter concentrations (octa- to nanomolar range). Potency is destroyed by nonspecific proteases (carboxypeptidase and pronase) but not by trypsin. Attractant is not sequestered within barnacles.

Key Words—Attraction, muricid gastropods, barnacles, chemical sensing, *Semibalanus balanoides, Balanus eburneus, Urosalpinx cinerea,* oyster drill, snail, predator–prey relationships.

INTRODUCTION

The majority of studies performed on the chemical nature of attractants for aquatic organisms have been on feeding attractants. These studies have been

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of two types: (1) tests and analyses of extracts or homogenates of prey (Shelton and Mackie, 1971; Atema et al., 1980; Carr, 1967; Townsend, 1973; Castilla, 1972; Gurin and Carr, 1974; Collins, 1975); and (2) characterization of molecular constituents of washings (Shelton and Mackie, 1977; Atema et al., 1980; Carr, 1967b; Hanscomb et al., 1976) or extracts of living animals and molecular analysis of individual compounds in synthetic mixtures (for review see Bardach, 1975; Atema, 1980). Both approaches indicate the presence, and some of the types of, molecules composing prey that are attractive to predators or scavengers (mainly amino acids and proteins). However, behavioral and biochemical identification of highly specific attractants such as those indicating specific prey are obscured by either approach. Homogenization and extraction generate complex organic mixtures from which trace organic molecules are not easily retrieved. Reconstitution of active mixtures is limited to those molecules readily detected, characterized, and commercially obtainable. Neither approach answers questions on the nature of a native attractant released from intact prey.

To paraphrase William Herrnkind (personal communication), in nature predators do not usually encounter extracted or homogenized prey, nor do they locate mixtures of readily available shelf chemicals. Furthermore, a specific behavioral response to molecular stimuli suggests that specific information must have been conveyed. Specific information requires a unique molecular structure to code that information. It might be expected that specific attractants would be similar in size and complexity to other information-conveying molecules, for example, hormones. Therefore, a basic premise in the investigation reported here is that specific attractants can be purified and characterized from the seawater bathing living intact prey.

Oyster drills, *Urosalpinx cinerea* (Say), have a well-documented ability to locate intact prey from a distance by creeping upcurrent in response to chemical cues (for a thorough review of the early literature see Carriker, 1955; Blake, 1962; Wood, 1968; Pratt, 1974, 1976; Ordzie and Garafalo, 1980). Newly hatched *Urosalpinx cinerea* from the Delaware Bay region have a specific propensity for a molecule(s) from barnacles (Rittschof et al., 1983). This propensity is maintained in adults even after they have fed exclusively upon oysters for over a year (Rittschof and Gruber, unpublished data). Indeed, if barnacles were a commercially important shellfish, oyster drills would probably be commonly called barnacle drills.

As part of an ongoing project with the objective of assessing the potential of chemical biocontrol measures for oyster drills, we reported (Rittschof et al., 1983) the development of a bioassay useful in monitoring attractants from prey. The assay uses newly hatched drills and their response to attractants in seawater. The assay was developed specifically for use in the determination of the chemical nature of attractant molecules. Here we report on the concentration, purification, and initial characterization of the attractant originating

from balanoid barnacles. Identification of the active substance(s) is in progress.

The rapid, simple procedure reported here for purifying and concentrating attractants as well as preliminary information on the nature of the molecule have applications in many areas of chemosensory inquiry. Our bioassay has both a defined response and a highly concentrated and partially purified stimulus and is being put to other uses. The concentration and desalting step that uses Amberlite XAD-7 may have general applicability for extraction and desalting of other complex bioactive molecules. Several coworkers have already communicated positive results with this technique.

METHODS AND MATERIALS

Reagents. Deionized water was produced by a Millipore Corporation Milliq system. The Amberlite XAD-7, XAD-4, and XAD-2 resins used were generous gifts of the Rohm and Haas Corporation. Cellulose was Sigmacel 50 from Sigma Chemical Corporation. Methanol was HPLC grade (Bodman Chemicals, Media, Pennsylvania). All other reagents were reagent grade. Enzymes were purchased from Sigma immediately prior to use. Trypsin was from bovine pancreas (Sigma T-2884), carboxypeptidase was from bovine pancreas (Sigma C-0386), and pronase E (Sigma P-5147) was from *Streptomyces griseus.*

Preparation of XAD Resins and Cellulose. XAD resins were washed thoroughly with water and then with methanol. Methanol removed a flocculent white material (antistatic agent, according to the manufacturer) and was a necessary step in their preparation. Columns were prepared by back-flushing as recommended by the manufacturer. Batch treatment of resins was by the same washing procedure. Cellulose, batch washed in a Buchner funnel, was washed extensively with water, ethanol, and either acidified or basified ethanol. After washing and sequentially rewashing the material with methanol and water, it was dried under vacuum.

Column Procedures. XAD columns were run at rates from 1 ml/min to >1 liter/min. Slow flow rates were used in analytical procedures. High flow rates were used in preparative procedures.

Pressure Dialysis. Pressure dialysis was performed with an Amicon Corporation 180-ml ultrafiltration cell with or without a 2-liter reservoir. Membranes were prepared and handled according to the instructions of the manufacturer. Filtrations requiring longer than 30 min were conducted at 4°C. Short-term dialysis was performed at ambient temperatures (20–23°C).

Test Snails. Newly hatched oyster drills, *Urosalpinx cinerea* (Say), were harvested from our laboratory hatchery as previously described (Rittschof et al., 1983). They were stored at 8–13°C without food. Snails used one day for

one experiment were sometimes reused another day in a separate experiment as described in Wood et al. (1983). Williams et al. (1983) showed that *U. cinerea* exposed to attractants for several hours may have a slightly reduced response to that attractant on a subsequent day. Snails were not exposed to attractant for more than 15 min on any particular day. Snails were stored in aged seawater when not being tested.

Barnacles. Semibalanus balanoides and *Balanus eburneus* were sources of attractant. *B. eberneus* were obtained locally and stored in a 16-liter polycarbonate container with aerated seawater. Bricks and rocks on which *S. balanoides* had set were collected from the inner breakwater of Delaware Bay and Indian River Inlet. In all, several hundred living intact *B. eburneus* averaging over 1 g total weight each (including shell) and many thousands intact living *S. balanoides* weighing about 0.1 g each were used. Rocks encrusted with *S. balanoides* were maintained in a 40-liter glass aquarium with aeration and fed several times a week with algae from the University of Delaware's Mariculture Facility. Attractant was in seawater in which barnacles were living independent of whether or not they were fed.

Preparation of Attractant Water. Attractant-free seawater was 1-μm-filtered seawater that had aged by standing for several days. The response to this water in the bioassay is less than 15%.

Attractant was prepared in two ways: (1) intact living barnacles were bathed in aged, 0.4-μm polycarbonate-filtered seawater (30–32 ppt.) for a minimum of 3 hr; (2) less-crude stimulus was produced by first 0.2-μm-polycarbonate filtering and then purifying the aged seawater by passing it through XAD-7 (Rohm and Haas resin). Barnacles were rinsed in aged filtered seawater, then in three changes of XAD-7 "scrubbed" water, and finally bathed in fresh polycarbonate-filtered and XAD-7-scrubbed seawater. After a 3-hr incubation, the water surrounding the barnacles was filtered through glass fiber filters (1 μm nominal pore size) and 0.4 μm polycarbonate filters. This was the starting material. Preparations were bioassayed at several log dilutions to determine potency.

Bioassay. Bioassay procedures used were those described by Rittschof et al. (1983) and Wood et al. (1983) with the exception of a microbioassay. The latter assay was used when only small volumes of attractant were available. The microassay apparatus was designed for use with microliter amounts of stimulus and milliliter volumes of seawater. It consisted of a Desage syringe pump modified to hold six disposable 10-ml syringes. Each syringe was fitted with a 0- to 200-μl disposable automatic pipetter tip. Borosilicate glass 1-ml pipets were cut in half and bent as previously described (Rittschof et al., 1983). The response criteria were the same as previously described for the larger assay apparatus, but the total volume of seawater necessary to run a 10-min assay was 1.7 ml.

Temperature Treatments. In a first series of temperature experiments,

stimulus water to be tested for temperature effects on activity was glass-fiber-filtered and 0.2-μm polycarbonate-filtered. Untreated attractant was placed immediately in an ice bath. When incubation temperature was 70°C or less, undiluted attractant was incubated in an Erlenmeyer flask in a water bath set at the test temperature. A flask of equal volume containing a thermometer was incubated simultaneously. When the solution in the parallel container reached the test temperature, timing was started. Solutions treated at 30, 50, and 70°C were incubated for 30 min and then cooled to ambient temperatures with ice. Attractant to be boiled was placed in an Erlenmeyer flask with a loose glass lid to retard evaporation. The flask was placed in a boiling water bath and timing started when the solution began to visibly boil. After 5 min of boiling, the solution was cooled on ice. Aged filtered seawater was treated similarly and tested with and without addition of unheated attractant to determine the effects of boiled seawater on the snail responses. In a second series of experiments, concentrated and size-fractionated attractant was boiled in both open and sealed ampules from 0 to 90 min. Attractant was diluted in aged seawater and potency compared to that of unheated attractant.

Reagent Experiments. Reagents other than aged seawater used in experiments were high-performance liquid chromatography (HPLC)-grade methanol, deionized water, and boiled seawater. Effects of each reagent in the bioassay were tested by adding a constant small volume of cencentrated attractant and performing dilution series experiments on the reagent over the concentration range to be added to the assay. Control experiments included aged seawater alone and reagent without added attractant.

Matrix-Binding Experiments. A series of matrix-binding experiments was conducted to determine if attractant associated with barnacles could be removed from seawater. Cellulose and commercially available water treatment resins were tested.

Recovery Experiments. Experiments were conducted to determine if the biological activity removed from seawater in matrix-binding experiments could be recovered. Solvents tested for effective removal of stimulus from the resin were deionized water and HPLC-grade methanol.

Homogenization Experiments. Experiments were conducted to determine if homogenization of barnacles would improve the yield of chemoattractant. In these experiments: (1) barnacle stimulus was first prepared by our normal procedures, that is, by letting living barnacles stand in seawater and extracting the attractant from seawater with XAD-7 resin; and (2) the same barnacles that produced the attractant in step 1 were scraped from the rocks and weighed. The 125 g of barnacles was ground fine for 5 min in a mortar and pestle in 100 ml of deionized water. This homogenate and three additional 100-ml washes of the residue with distilled water were centrifuged at 3000 *g* for 5 min in a Sorval ss-34 rotor. The supernatant was filtered through a glass fiber filter and 0.4-μm polycarbonate filter. The filtrate was diluted to 3 liters

with XAD-7-treated 0.4-μm polycarbonate-filtered seawater and subjected to the same stimulus extraction, washing, and elution procedures as the stimulus prepared in step 1. All subsequent bioassays and biochemical operations were performed simultaneously on both preparations.

Enzyme Experiments. Enzyme experiments tested the ability of trypsin, carboxypeptidase, and pronase to destroy biological activity. Trypsin was tested at 200 and 2000 BAEE units/ml concentrated attractant. Carboxypeptidase was tested at 10 and 1 units/ml concentrated attractant (1 unit hydrolyzed 1.0 μmol hippuryl-L-phenylalanine/min at 25°C and pH 7.5). Pronase was tested at 1 and 0.1 units/ml concentrated attractant (1 unit hydrolyzed casein to produce color equivalent to 1.0 μmol of tyrosine/min at 37°C and pH 7.5). Separate aliquots were prepared for each incubation at 21°C (0, 0.5, 2, 4, 6, 8 hr). At each time interval an aliquot was diluted 1:250 and assayed. Controls were: seawater with no additions; seawater with the addition of each enzyme incubated for each time interval; seawater and attractant; and seawater, attractant, and each enzyme assayed immediately upon mixing.

Definition of Activity Unit. Units are defined arbitrarily based upon the known response of test snails (Rittschof et al., 1983; Williams et al., 1983; Wood et al., 1983). One unit is the attractant required per milliliter to evoke a 37% response in the assay. Because of the nonlinear nature of the cumulative percent response assay (Williams et al., 1983), 0.1 units evoke a 13% response. Dilution series bioassays were performed on preparations throughout purification procedures in order to determine location, amount, concentration, percentage recovery, and units per milligram dry weight of stimulus. When assaying preparation of unknown potency, we made and tested dilutions until responses between 13 and 38% were obtained. Units were estimated from plots of percentage response against the log of the dilution.

RESULTS

Manipulations. Biological activity was always observed in water bathing either living *Semibalanus balanoides* or *Balanus eburneus*. Relatively potent preparations (1–5 units) were used to examine the effects of manipulations on stimulus activity. Stimulus could be centrifuged at 10,000 *g* for 20 min at 4°C with no detectable loss of activity. Vacuum aspirator filtration through 1-μm glass-fiber filters also had no appreciable effect on potency. However, filtration through polycarbonate filters (0.2 μm) with vacuum filtration sporadically produced marked increases in potency (often more than doubling in apparent number of activity units). The same result could be obtained by a period of vacuum treatment without filtration. This phenomenon was observed only with crude attractant preparations that could be diluted and still retain activity but that did not evoke high levels of response at any

dilution. The normal log relationship between stimulus concentration and snail activity (Rittschof et al., 1983) was not observed. That is, dilution of the stimulus resulted in much higher estimates of the number of units in a preparation than did assay of the concentrated material. Assay of the material after vacuum treatment resulted in the normally observed relationship.

Water that had not been polycarbonate-filtered to remove bacteria rapidly lost potency upon standing at room temperature. One-micron-filtered attractant lost 50% of its potency in 6 hr at room temperature. The 0.2-μm polycarbonate-filtered attractant retained potency for at least 6 hr at room temperature.

Molecular Size Determination. Two types of dialysis experiments were employed to estimate the size of molecule of the barnacle stimulus. First, 100 ml of approximately 5 activity units of stimulus were placed in a dialysis bag (20 Å pore size, approximately 10,000-dalton exclusion limit) and dialyzed against 300 ml of 0.2-μm polycarbonate-filtered seawater for 4 hr. Bioassays of the dialysate showed stimulus activity ($P < 0.005$). Second, a series of experiments employing Amicon pressure dialysis membranes was conducted. Bioassayable activity passed through 10,000- and 5000-dalton membranes and was retained by the 1000-dalton membrane and by the 500-dalton membrane (Figure 1).

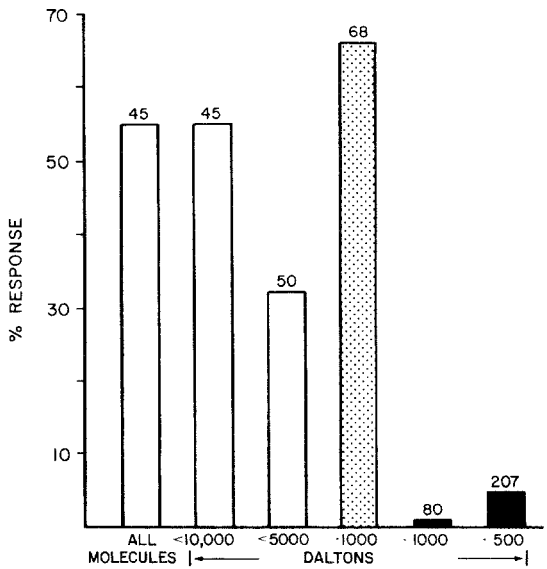

FIG. 1. Molecular size of barnacle attractant. Pressure dialysis with Amicon membranes followed by bioassay indicates that attractant is approximately 5000 daltons. Numbers above bars are numbers of snails tested.

Matrix-Binding Experiments. Experiments were conducted to determine a nonionic matrix that binds stimulus activity. Following Blake's lead (Blake, 1962, observed stimulus was bound to cellulose-containing products), we tried cellulose matrices. Additionally, we tested two water-purification resins, Amberlite XAD-2 that adsorbs nonpolar substances and Amberlite XAD-7 that adsorbs both polar and nonpolar substances. Significant ($P < 0.005$) amounts of activity were removed by untreated Sigmacel cellulose and base-washed cellulose, but not by acid-washed cellulose. Although there was significant retention of the attractant, cellulose matrices had low capacity. Of the Amberlite resins, XAD-2 had low apparent capacity, while XAD-7 was very effective (>95% retention) (Figure 2). Subsequent tests showed XAD-7 resins had a high capacity for attractant while XAD-2 and a second XAD-4 had limited capacity. We passed 1800 units of activity through a column containing 0.3 g of XAD-7 resin with less than a 5% leak rate at flow rates of 4 ml/min. XAD-7 resin was used in subsequent testing.

Once attractant was adsorbed to the XAD-7 resin, washing to remove salt and recovery of the activity was attempted. A larger column that had a theoretical capacity of over 230,000 units was constructed of approximately 40 g of XAD-7 resin, and run at flow rates of 300 ml/min. We passed 18,000 units through this column and assayed the flow in fractions and found no detectable activity. Gradual decrease in salinity and distilled water washing produced no detectable release of activity. However, 100% methanol applied

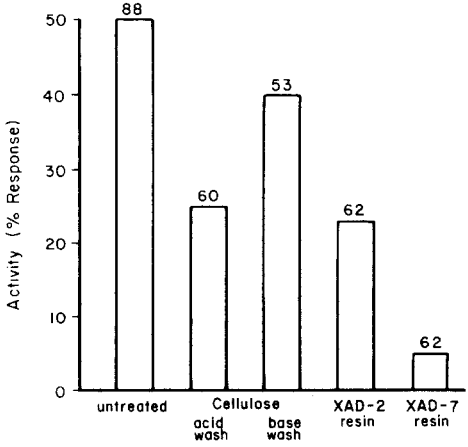

FIG. 2. Matrix binding of attractant. A known amount of activity was passed through different matrices and assayed for activity. Acid-washed cellulose and XAD-2 and XAD-7 resins removed at least some activity (compare to untreated control). Amberlite XAD-7 reduced assay responses to levels observed in attractant-free seawater. Numbers above bars are numbers of snails tested.

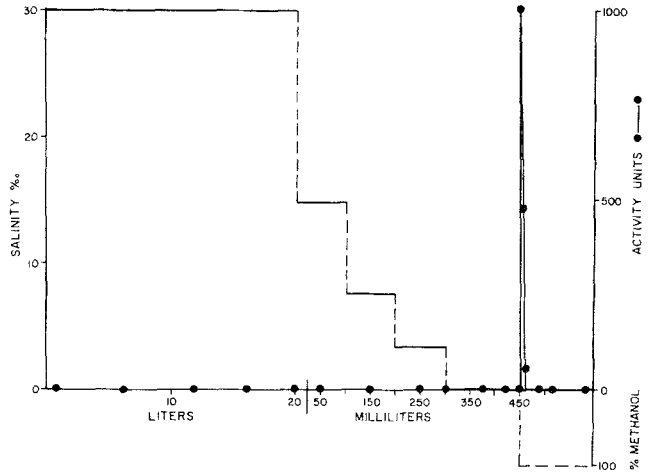

FIG. 3. Concentration and desalting of specific attractant with Amberlite XAD-7 resin. Attractant is adsorbed onto XAD-7 resin (100 ml/min onto a 2-cm ID × 25-cm bed), salinity is reduced to 0, and attractant is stepped off column at interface between deionized water and 100% methanol.

to the column released at least 65% of the units loaded on the column attractant eluted at the interface between the water and methanol (Figure 3, Table 1). About 30 min was required to pass 20 liters of seawater through the column, 10 min to wash away salts and nonadsorbed material, and 5 min to collect concentrated attractant.

Temperature Experiments. Temperature stability of the attractant was tested prior to further purification and characterization. First experiments were conducted with crude preparations that had been 0.2-μm polycarbonate-filtered. These experiments demonstrated that biological activity was relatively heat stable. There was no detectable change with 30 min of heating at 30, 50, or 70°C. Boiling crude attractant for 5 min reduced assay potency at high concentrations but had no effect at low concentrations. Boiling seawater alone inhibited assay responses.

Next a series of experiments was conducted on concentrated attractant. Attractant was heated at 100°C in sealed and open vials for 30, 60, and 90 min. Biological assays compared the activity of the different boiled samples to that of unboiled stimulus and to aged seawater controls. Whereas there was significance reduction of biological activity in samples boiled open to the air (G statistic $P < 0.005$), samples heated in sealed ampules showed full biological activity.

Homogenization Experiments. Attractant extracted from seawater was compared to stimulus prepared by homogenizing and extracting tissues of the

TABLE 1. PURIFICATION OF BARNACLE STIMULUS

Fraction	ml	U/ml	mg/ml	U/mg	Total units	Recovery (%)	Purification	13% A[a] (M)	37% A (M)
Starting	4000	3.0	34.800	0.086	12,000	100	1	10^{-4}	10^{-3}
XAD-7	47	150.0	0.560	267.140	6,964	58	3,106	10^{-7}	10^{-6}
YM5 FT	43	100.0	0.083	1204.820	4,300	36	14,009	10^{-9}	10^{-8}
UM2 Ret[b]				1266.666	2,406		14,728	10^{-9}	10^{-8}

[a] Molar concentrations were estimated assuming a minimum molecular weight of 1000 and using the dry weight of each fraction.

[b] Material tested was from a separate purification and was passed through a YM5 membrane prior to concentration on the UM2 membrane. This material was assayed and then dried and weighed as were all the other preparations.

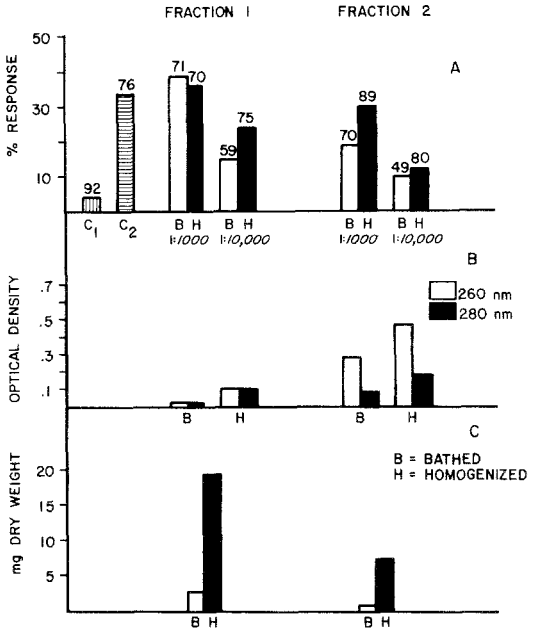

FIG. 4. Comparison of homogenization and seawater extraction of attractant. (A) Bioassay of active material from each preparation. C1 is attractant-free seawater control, C2 is response to a standard preparation. Fractions 1 and 2 account for 80–95% of the recoverable activity in the preparations. Numbers above bars indicate number of snails tested. (B) Relative absorbance at 260 and 280 nm of each of the active fractions for each preparation. (C) Total dry weights for each of the active fractions.

same barnacles. Both preparations contained activity. Relative potencies were similar in the two preparations (Figure 4). Both preparations had similar OD 260/280 (Figure 4B) ratios and showed consistent differences between fractions 1 and 2. Absorbances for the homogenized preparation were markedly higher in each fraction.

Homogenization resulted in approximately a 10-fold decrease in the specific activity of the preparation as a result of the increase in inactive material from soft tissues of the barnacle copurifying with the stimulus (Fig. 4C).

Reagents and Bioassay. Discovery that attractant could be bound to XAD-7 resin, desalted, and eluted in concentrated form suggested that the bioactive compounds were stable after treatment with methanol and to low ionic strength solutions. However, since reagents can alter snail bioassay behavior, experiments were designed to determine their effects on the assay. Potency was examined in both a dilution series of the reagent alone and with a constant addition of barnacle stimulus and a dilution series of the reagent to

be tested. The three additions to the bioassay during test procedures were boiled water, deionized water and methanol. Reagents were tested separately at dilutions likely to be encountered during subsequent steps in the purification. As the stimulus was concentrated, maximum concentrations of additives in the bioassay were likely to range downward from a 1:100 ($\simeq$0.3 M) dilution, in all probability to a range less than a 1:1000 ($<<$ 0.03 M) dilution. Of the reagents tested, none stimulated drills to creep. Methanol, however, had a negative effect on the assay at higher concentrations (G statistic $P << 0.005$). There was an inverse relationship between the amount of methanol and the response to attractant. Dilutions of methanol greater than 1:500 (0.06 M) had insignificant effects on responses (Figure 5).

Response to Partially Purified Concentrated Attractant. Partially purified and concentrated attractant was tested for biological activity over a range of dilutions (example, Figure 6). Response of snails was optimal at dilutions of attractant that corresponded to approximately 10^{-8} m to 10^{-9} m concentrations of attractant by weight. Concentrations above the optimum evoked lower percentage responses as did concentrations that were less than the optimum. Responses to optimal concentrations approached 100% when assay conditions (temperature, care of snails) were attended to carefully.

Enzyme Experiments. Responses of snails to solutions containing enzymes were the same as those to solutions containing only seawater (0 to 4%). Responses to controls containing attractant were between 35 and 40%

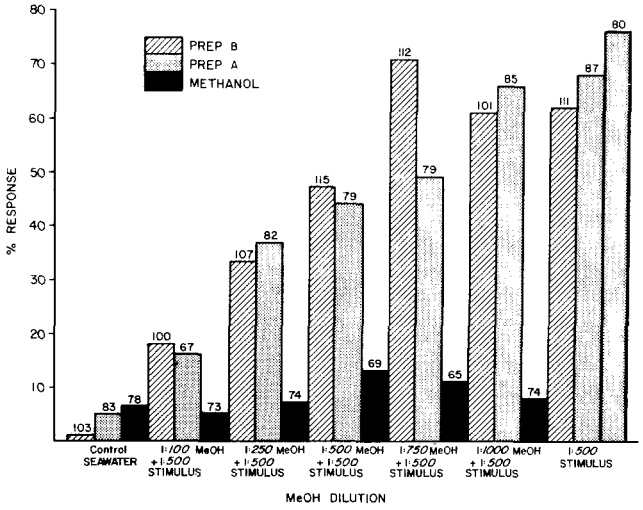

FIG. 5. Effect of methanol on activity evoked by standard attractant. Slashed bars represent one complete experiment, stippled bars represent a second replicate experiment. Solid bars are the effect of methanol in the absence of attractant on the assay. Numbers above bars indicate numbers of snails tested.

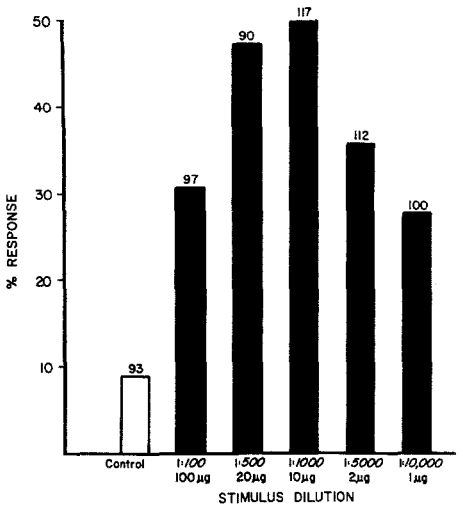

FIG. 6. Representative response to dilutions of concentrated, partially purified attractant. Attractant was concentrated and desalted with XAD-7 resin, rotary evaporated to near dryness to remove methanol, size fractionated by pressure dialysis, and tested for activity in the bioassay. Numbers above bars indicated numbers of snails tested at each dilution for that experiment. If care is taken to control temperature and absolute age treatment of snails, 90–98% response to optimal concentrations of attractant can be obtained.

(seawater plus attractant and seawater plus attractant and each enzyme assayed immediately). Trypsin had no effect on potency (experimental vs. attractant control, $G = 0.009$ NS) after 8 hr at 2000 units/ml. At all concentrations tested, carboxypeptidase and pronase E rapidly reduced potency. At 10 units carboxypeptidase significantly decreased attractant potency after 2 hr ($G = 6.88$, $P < 0.001$). Similarly, pronase E at 1.0 unit/ml significantly decreased potency after 2 hr incubation with concentrated attractant (experimental vs. attractant control, $G = 4.75$, $P < 0.05$). After 4 hr of incubation, responses to carboxypeptidase- and pronase-treated attractant were indistinguishable from responses to seawater alone.

DISCUSSION

To our knowledge, oyster drill attractant is the first marine attractant to be isolated as a by-product of living intact prey. Attractant molecules are relatively complex and temperature stable, having molecular weights between 1000 and 5000 daltons. Attractant can be destroyed rapidly by proteolytic enzymes that attack carboxy termini and or that degrade peptides to amino acids. Trypsin, a more specific serine protease that attacks only linkages

containing lysine and arginine, had no effect on attractant activity, even at enzymatic activities 200 to 2000-fold higher than those effective for the other enzymes. We conclude from these experiments that attractant is at least partially proteinaceous.

The key to isolation and concentration of attractant is its affinity for XAD-7 resin at near-neutral pH and relatively high ionic strength. This promotes virtually complete removal of stimulus even by batch procedures. Amberlite XAD-7 is a polyacrylate resin with affinity for both polar and nonpolar substances and has flow and stability characteristics that enable rapid extraction and concentration of the dilute active molecule from seawater. A second very important factor is that, once adsorbed to the resin, attractant remains bound tightly to the resin even with removal of salt by distilled water washing.

XAD resins have been used previously to extract humic substances from natural waters (Jolley, 1981). These substances compose a majority of organics in seawater and are retained poorly or not at all by XAD-7 at neutral pH. Thus extraction of seawater at neutral pH and washing of resin with deionized water selectively avoids contamination by humics. Additional reduction of contaminants is accomplished by XAD-7 extraction of seawater prior to its exposure to barnacles. This step removes inactive molecules with affinity for XAD-7 that are present in seawater.

Temperature stability and molecular size of the attractant facilitate further purification by heating under vacuum to remove methanol introduced at the resin elution step. At this step, temperature-sensitive contaminants precipitate. Molecular sizing of the temperature-treated material removes the precipitate as well as contaminants such much larger and much smaller than attractant. This partially purified material is stable and potent at a 10^{-9} M concentration by weight.

Comparison of attractant extraction by XAD-7 from seawater with attractant extraction by XAD-7 from barnacle homogenate suggests that attractant is not a major component of living barnacles and is not extensively sequestered in the organism. There are much higher levels of contamination if the starting material is homogenized soft tissues of barnacles than if it is seawater extract. This is what would be expected if levels of potentially contaminating organics are taken into account. Whereas levels of organics in seawater are on the order of milligrams per liter (Fox, 1981), organics within living organisms are many grams per liter. Thus, beginning with a homogenate can be expected, in the case of a nonsequestered or minor organic component, to elevate levels of contamination from 10^3 to 10^5 times. Further study may show that a specific tissue of barnacles is rich in attractant. Should this be the case, it could provide an acceptable alternative to seawater extraction, especially when possible copurification of degradation products by seawater extraction is taken into consideration.

With the exception of the protein detection system of *Ilyanassa obsoleta* described by Gurin and Carr (1974) and the food-finding behavior of *Aplysia californica* (Jahan-Parwar, 1975), the molar activity of even our crudely purified attractant exceeds that of published snail feeding attractants by about three orders of magnitude. *Urosalpinx cinerea* attractant appears to be similar in activity to chemoattraction systems that are mediated by peptides or proteins (Gurin and Carr, 1974; Fernandez, 1978; Schiffman and Gallin, 1979). It is apparent that *U. cinerea* attractant is not a chemical picture (Atema, 1980) because we have succeeded in discarding over 99.999% of the molecules in the preparation. This is not to say, of course, that the chemical milieu within which the molecule is solvated is not important to its activity, but rather that a relatively minor component of the chemical milieu carries specific information.

There is a good likelihood that many molecules that are detected and used in location of living, intact prey may be similar to attractant from barnacles. Straightforward adsorption techniques may therefore prove useful in concentrating and purifying other biologically active molecules. As such, adsorption techniques present an exciting perspective for the study of compounds dissolved in natural waters.

In its partially purified state, *Urosalpinx cinerea* attractant is a powerful probe. Large amounts of stable, highly concentrated attractant enable its use as a meterstick for assessment of virtually any perturbation upon snail attraction. We are using attractant and the bioassay to test and quantify additions of reagents and the effects of exposure of embryos to media from living intact prey, and to measure ambient environmental levels of attractants.

It is apparent from Rittschof et al. (1983), Wood et al. (1983), Williams et al. (1983), and this report that *U. cinerea* attractant might also be considered the molecular essence of barnacle. To withstand selection pressures resulting from millions of years of barnacle predation by oyster drills, the attractant must perform some important function for barnacles, or it is an unavoidable excretory product. We are beginning investigations on the role(s) of this molecule in the biology of barnacles. It may, for example, function in the settlement of spat (Crisp and Meadows, 1962), in the molting process of barnacles, or it may be a component of barnacle cement. We believe that discovery of the site of production of the molecule will shed light on additional functions. We agree with Pratt's (1974) conclusion that, for *Urosalpinx cinerea*, detection is the first step in a predatory act that from all indications is a sophisticated, complex, and highly redundant resource verification system.

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TOXICITY OF NITRO COMPOUNDS FROM *Lotus pedunculatus* TO GRASS GRUB (*Costelytra zealandica*) (COLEOPTERA: SCARABAEIDAE)

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Abstract—Crude extracts of root of the forage legumes *Lotus pedunculatus* and *Coronilla varia* (crownvetch) were toxic when administered orally to 3rd instar *Costelytra zealandica* larvae. A group of 3-nitropropanoyl-D-glucopyranoses was isolated from active fractions of the crude extracts. These toxins, some of which were already known from *C. varia*, have not previously been reported from *L. pedunculatus*. The compounds were present in root tissue of this species at a concentration of about 1% dry weight. They include the triester karakin and the diesters coronarian and cibarian, all of which were toxic to larvae of *C. zealandica* at levels which could account fully for the activity of the crude extracts.

Key Words—*Costelytra zealandica, Lotus pedunculatus*, insect resistance, insect toxins, nitro compounds, karakin, coronarian, cibarian.

INTRODUCTION

Some pasture and crop legumes, notably *Lotus pedunculatus* Cav. and *Medicago sativa* L., lucerne, are resistant to the subterranean pasture pest *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae) (Farrell and Sweney, 1972, 1974; Farrell and Stufkens, 1977). In both these species, the presence of strong feeding deterrents, vestitol and saponins, respectively, suggested nonpreference resistance mechanisms (Sutherland et al., 1975a,b; Russell et al., 1978). However, we also found crude extracts of *L. pedunculatus* and lucerne were toxic to larvae of *C. zealandica* and to another scarab pasture pest, *Heteronychus arator* (Fabricius) (Sutherland and Greenfield

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1976, 1978). Purified lucerne saponins and *Lotus* tannins were subsequently tested for toxicity to larvae of *C. zealandica* and while the saponins were sufficiently toxic to account for all the activity of the crude extract of lucerne root, the tannins from *L. pedunculatus* did not affect larvae at all and apparently played no part in the toxicity of this plant (Sutherland et al., 1982a). In the course of screening other pasture legumes reported to have some degree of resistance to the insects, we found that *Coronilla varia* L., crownvetch, root extracts were more toxic than those of *L. pedunculatus*. Preliminary fractionations of crude extracts of the two species indicated a close chemical similarity between their toxins. We report here the identification of toxins in *C. varia* and *L. pedunculatus* as 3-nitropropanoic acid compounds.

METHODS AND MATERIALS

Plants and extracts. L. pedunculatus 'Grasslands Maku', *C. varia* cv. Penngift and *Trifolium repens* L. 'Grasslands Huia' (white clover) plants were grown outdoors in 15-cm flowerpots. Root of 6-month-old plants was washed thoroughly in cold water, dried between paper towels, and immediately homogenized in 50% ethanol. The extract was filtered, reduced to dryness under vacuum at 45° C, and redissolved in sufficient distilled water to return it to a concentration of 10 g fresh weight root/ml for testing with 3rd instar grass grubs. All extracts and fractions as outlined in the scheme (Figure 1) were tested at this concentration.

Bioassay. Vigorous, healthy specimens were selected from field-collected 3rd instar larvae of *C. zealandica* which had been starved for 24 hr. Test insects weighed 100–150 mg. Solutions of test extracts, fractions, and pure compounds were administered orally in 10-μl doses from a glass syringe mounted in a microapplicator. The blunted 30-gauge hypodermic needle was inserted between the mandibles and the unanesthetized grub gently slipped onto it until the needle reached the foregut (Sutherland et al., 1982a).

Crude root extracts and fractions were administered in aqueous solution. However, pure karakin, coronarian, and cibarian were not sufficiently water-soluble to remain in solution at all the test concentrations. They were instead dissolved by gentle warming in a 10% aqueous solution of dimethyl sulfoxide (DMSO). These solutions were then cooled to 23 ± 2° C and the syringe and its mount maintained at that temperature while dosing was in progress. Although it was not isolated from *L. pedunculatus* in this study, 3-nitropropionic acid (3-NPA) was also tested. It was prepared according to the method of Hass et al. (1951) and, although water soluble, was tested in solution in 10% DMSO together with the other pure compounds.

Crude extracts of roots and fractions were administered to 15 larvae, while the pure compounds were administered to 20 larvae at each concentration. Fifteen (or 20) control larvae were included on each dosing occasion

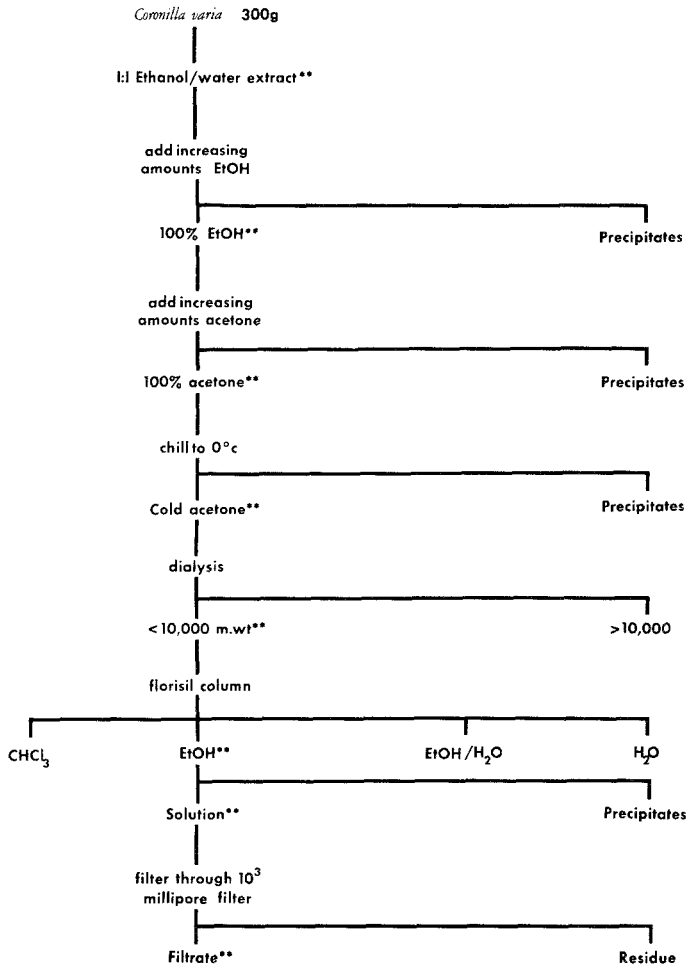

FIG. 1. A procedure for isolating toxins from a crude $EtOH/H_2O$ root extract of *C. varia*. Toxic activity is indicated by asterisks.

and were handled identically to the test insects. They were dosed with 10 μl distilled water or 10% DMSO. Dosed larvae were held individually in empty plastic dishes wrapped in damp towels and kept in the dark at room temperature. Larvae given crude extracts and fractions were examined after 24 hr and classified as dead if tactile stimulation elicited no observable movement of the body or any appendage. Those dosed with pure compounds were also examined at 24 hr but were classified into one of four categories: 0 = unaffected and responsive to tactile stimulation; + = lethargic and slow to respond; ++ = moribund, immobile but able to move one or more legs slightly or to contract the abdominal muscles; and dead.

Chemical Methods. A preliminary fractionation of root of *C. varia* was undertaken as in the scheme (Figure 1). Four fractions were collected from the millipore filter. The first (F1) comprised the filtrate obtained by filtration of the active solution. Further solvent was passed through the filter and a second filtrate (F2) was obtained. F3 was an aqueous suspension of the solid precipitate retained by the filter and F4 the residual liquor. Thin-layer chromatography of the toxic fraction F1 was performed on cellulose plates with a solvent of 25% ether in acetone.

A 1-kg sample of root of *L. pedunculatus* was extracted with 7.5 liters of acetone, the solvent then removed, and the aqueous phase washed with hexane and ethyl acetate (Moyer et al., 1979). It was then chromatographed on a silica gel column according to Moyer et al. (1979), and 50-ml collections of eluates were taken using a fraction collector. These were then characterized on silica gel thin-layer plates in a solvent system of EtOH–$CHCl_3$ (10:90); fractions containing esters of 3-NPA were detected by the diazotized sulfanilic acid spray system of Majak and Bose (1974).

Those fractions that had the same R_f values as the three nitro ester standards (karakin, coronarian, and cibarian) were each allowed to crystallize (toxin I, II, and III, respectively). The crystals of each were decanted from the mother liquor, recrystallized from 10% EtOH–$CHCl_3$ and examined by infrared spectroscopy, mass spectrometry, and nuclear magnetic resonance ([^{1}H]NMR).

Infrared spectra were obtained from a Perkin-Elmer model 237 spectrophotometer.

Mass spectral analyses were performed on an AEI MS30 spectrometer at 20 eV and a source temperature of 180° C. The samples were introduced by the in-beam technique of Ohashi et al. (1980).

[^{1}H]NMR spectra were obtained on a 200-MHz Varian XL-200 FT spectrometer. The samples were dissolved in acetone-d_6 containing a little H_2O-d_2. Chemical shifts were calculated in ppm (± 0.01) from a tetramethylsilane internal standard and coupling constants (J) in Hz (± 0.2).

RESULTS

Larval mortality following dosing with crude extracts of *C. varia*, *L. pedunculatus*, and *T. repens* roots is given in Table 1. *C. varia* was active at all concentrations tested, with the mortality reaching 100% at a concentration of 10 g/ml. The *C. varia* extract was consistently more active than *L. pedunculatus* extract which nevertheless caused considerable mortality at the highest concentration. Both extracts at the two higher concentrations severely affected surviving larvae, most of which were alive but moribund. Several larvae which received the highest concentration of *T. repens* extract were

TABLE 1. %MORTALITY AMONG GROUPS OF 15 3RD INSTAR LARVAE OF *C. zealandica* DOSED ORALLY WITH 10 μl OF CRUDE LEGUME ROOT EXTRACT.

Concentration (fresh g/ml)	Mortality (%) 0.5	1.0	5.0	10.0
Plant species				
Coronilla varia	7	7	73	100
Lotus pedunculatus	0	7	20	87
Trifolium repens	0	0	0	0
Control				0

sluggish 24 hr after the treatment, but there was no mortality among them. Control larvae were unaffected.

A crude ethanolic extract of *C. varia* root was further extracted and fractionated as shown in the scheme (Figure 1). Four fractions, F1–F4, were collected from the millipore filter. F1 and F2, comprising approximately 80% and 16%, respectively, of those compounds from the original solution which had passed through the filter, were highly toxic to *C. zealandica* larvae (Table 2). Thin-layer chromatography of F1 produced a single band at the front. This area of the plate (T1), the remainder of the plate (T2), and the origin (T3) were then extracted with water. Of these, only T1 was toxic to the test larvae (Table 2).

This single, active band was then extracted with water and freeze-dried, yielding a pale yellow powder. The infrared spectrum of this material (Figure 2) suggested the presence of a nitropropanoyl ester. This indicated that the insect toxin could be similar in structure to a group of 3-nitropropanoyl esters of D-glucose previously isolated from *C. varia* by Majak and Bose (1976) and Moyer et al. (1977).

In order to verify this, the final filtrate F1 was column chromatographed by the method of Moyer et al. (1977). When the fractions which were eluted from the column with 5% and 10% EtOH in $CHCl_3$ were analyzed by TLC, several compounds giving the specific red coloration formed by nitropropanoyl esters were found to be present. The toxicity of the F1 fraction of *C. varia* could then be due to the presence of these.

TABLE 2. % MORTALITY AMONG GROUPS OF 15 3RD INSTAR LARVAE OF *C. zealandica* DOSED ORALLY WITH 10 μl OF VARIOUS FRACTIONS OF ROOT EXTRACT FROM *C. varia* (SEE TEXT)

Fraction	Control	F1	F2	F3	F4	T1	T2	T3
Mortality (%)	0	100	93	0	47	80	0	0

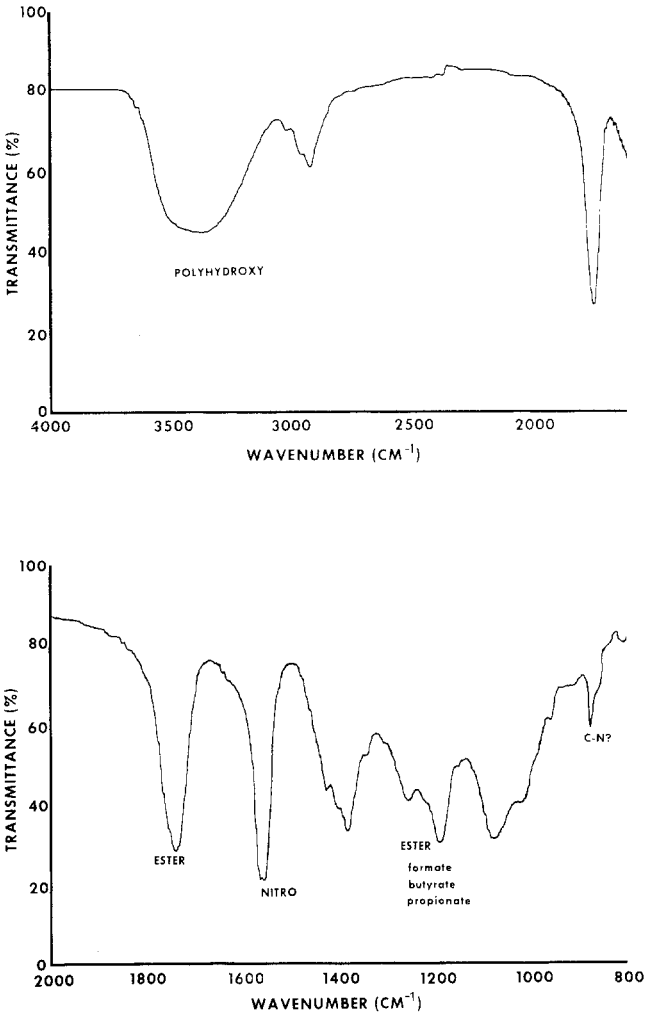

FIG. 2. Infrared spectrum of toxic fraction of roots from *C. varia*. [According to Bellamy (1958) the ester peak at 1190 could be due to the presence of any of the three esters listed.]

When the F1 fraction from root of *L. pedunculatus* was column chromatographed identically with that of *C. varia* and the eluates analyzed simultaneously by TLC, with standard compounds as markers, nitropropanoyl esters corresponding to three of those found in *C. varia* were found to be present in *L. pedunculatus* (Figure 3). The compounds were karakin, coronarian, and cibarian. Together with other unidentified nitro compounds, they

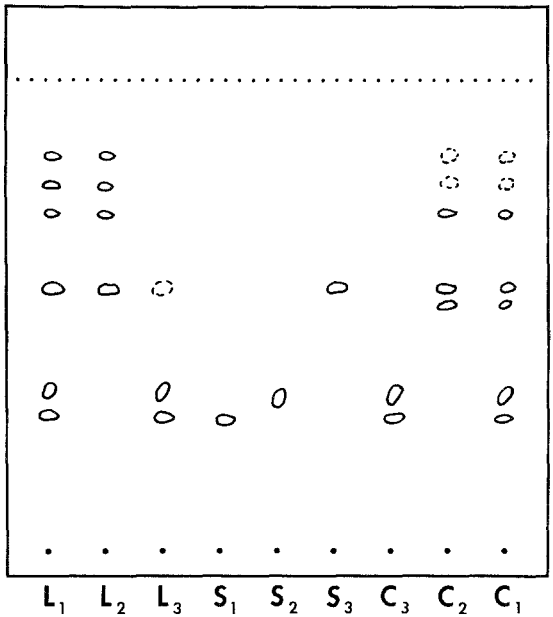

FIG. 3. Diagramatic representation of TLC of crude extracts of roots from *L. pedunculatus* and *C. varia* and fractions thereof sprayed with a three-reagent system specific for 3-nitropropanoyl-D-glucopyranoses. L_1, C_1, crude extracts of *L. pedunculatus* and *C. varia*, respectively; L_2, C_2, fractions from columns eluted with 5% EtOH in $CHCl_3$; L_3, C_3, fractions from columns eluted with 10% EtOH in $CHCl_3$; S_1, S_2, S_3, standards of cibarian, coronarian, and karakin, respectively.

constituted 1.02% dry weight of the root. The three compounds (toxin I, II, and III), which were crystallized from further fresh root extract, were then subjected to mass spectral analyses. The spectrum of toxin I showed the loss of three nitropropionic acid moieties at m/z 365, 246 and 127, whereas toxins II and III exhibited peaks at m/z 264 and 145, due to the presence of only two nitropropionic ester groups. Although the molecular ions of both the standards and samples were not apparent, the breakdown patterns of toxins I, II, and III corresponded to those of karakin, coronarian, and cibarian.

The [^{1}H]NMR spectra of these toxins, together with those of the standards and the literature values for the 3-nitropropanoyl esters of D-glucose, are presented in Table 3. From these data it can be seen that the spectrum of toxin I was consistent with that of karakin, toxin II with that of coronarian, and toxin III with that of cibarian. These identifications were finally confirmed by the identity of the IR spectra of the toxins with those of authentic reference spectra of karakin, coronarian, and cibarian.

Karakin comprised approximately 80% of the total nitropropanoyl

TABLE 3. [^{1}H]NMR FOR TOXINS I, II, AND III COMPARED WITH LITERATURE VALUES FOR KARAKIN, CORONARIAN, AND CIBARIAN

Compound	H-1	H-2	H-3	H-4	H-5	H-6	—CCH$_2$CH$_2$NO$_2$		
Toxin I	5.73	4.90	3.77	3.52	3.79	4.27 and 4.46	(4H) 3.09	4.80	J = 5.8
							(2H) 3.10	4.81	J = 5.8
	$J_{1,2}$ = 8.4,	$J_{2,3}$ =9.6,	$J_{3,4}$ = 9.6,	$J_{4,5}$ = 9.0,	$J_{5,6A}$ = 5.7,	$J_{5,6B}$ = 2.2, J_{AB} = 12.0			
Karakin[a]	5.78		3.75	3.25	3.85	4.42	3.05–3.14	4.76	J = 5.8
								4.81	J = 5.8
								4.87	J = 5.8
	$J_{1,2}$ = 8.4,		$J_{3,4}$ = 9.5,	$J_{4,5}$ = 10.0,	$J_{5,6A}$ = 5.5,	$J_{5,6B}$ = 2.4, J_{AB} = 12.0			
Toxin II	5.25	4.60	3.95	3.43	4.02	4.25 and 4.41	3.07	4.79	J = 6.0
							3.10	4.80	J = 6.0
	$J_{1,2}$ = 3.5,	$J_{2,3}$ = 10.0,	$J_{3,4}$ = 9.0,	$J_{4,5}$ = 10.0,	$J_{5,6A}$ = 5.4,	$J_{5,6B}$ = 2.2, J_{AB} = 11.8			
Coronarian[b]	5.30	4.65	3.95	3.45	3.95	4.35	3.07	4.85	
							3.17		
	$J_{1,2}$ = 3.5,	$J_{2,3}$ = 9.5,	$J_{3,4}$ = 9.5,	$J_{4,5}$ = 10.0,	$J_{5,6A}$ = 5.5,	$J_{5,6B}$ = 2.0, J_{AB} = 12.0			
Toxin III	5.52	3.36	3.50	3.36	3.63	4.27 and 4.40	3.05	4.78	J = 5.8
							3.14	4.81	J = 5.8
							3.15		
	$J_{1,2}$ = 7.8,	$J_{2,3}$ = 9.0,	$J_{3,4}$ = 9.0,	$J_{4,5}$ = 10.0,	$J_{5,6A}$ = 5.5,	$J_{5,6B}$ = 2.4, J_{AB} = 12.2			
Cibarian[c]	5.54		3.35–3.60		3.72	4.36	3.05	4.75	J = 5.8
(in H_2O-d_2)							3.14	4.79	J = 5.8
	$J_{1,2}$ = 8.0,			$J_{4,5}$ = 10.0,	$J_{5,6A}$ = 5.5,	$J_{5,6B}$ = 2.4, J_{AB} = 12.5			

[a]Harlow et al., 1975.
[b]Moyer et al., 1977.
[c]Stermitz et al., 1972.

TABLE 4. % MORTALITY AMONG GROUPS OF 20 3RD INSTAR LARVAE OF *C. zealandica* DOSED ORALLY WITH 10 μl OF SOLUTIONS OF KARAKIN, CORONARIAN, AND CIBARIAN AT THREE CONCENTRATIONS IN 10% DMSO

Compound	Conc. (%)	24 hr				ED_{50} (*c*)
		0	+	++	dead	
Karakin	0.1	100	0	0	0	
	0.5	0	30	50	40	
	1.0	0	5	20	75	0.78 (1.11)
Control (DMSO)		90	0	0	10	
Control (H_2O)		95	5	0	0	
Coronarian	0.1	65	10	5	20	
	0.5	35	30	5	30	
	1.0	15	10	5	70	0.74 (1.37)
Cibarian	0.1	85	0	10	5	
	0.5	55	10	15	20	
	1.0	0	10	35	55	0.97 (1.20)
Control (DMSO)		90	0	0	10	
Control (H_2O)		100	0	0	0	
3-NPA	0.1	90	0	10	0	
	0.5	20	45	25	10	
	1.0	0	5	45	50	
	2.0	0	0	5	95	1.04 (1.11)
Control (DMSO)		100	0	0	0	
Control (H_2O)		100	0	0	0	

[a]Because the ED_{50} (the concentration estimated to cause 50% mortality) is calculated on a logarithmic scale, its upper and lower standard error bounds are obtained by multiplying and dividing it by *c* rather than adding and subtracting. Here *c* = antilog (SE on log scale).

esters, the remainder including several unidentified esters besides coronarian and cibarian.

Authentic samples of karakin, coronarian, and cibarian in solution in 10% DMSO were all toxic to 3rd instar grass grubs (Table 4). The ED_{50}s of the three compounds were not distinguishable by probit analysis. Nor did the toxicity of 3-NPA differ significantly from that of its three esters and much the same proportion of larvae showed some physiological effect due to treatment with this compound.

On two of the three dosing occasions, 10% control larvae dosed with the solvent 10% DMSO died within 24 hr. These results were augmented with data from another eight experiments to obtain an estimated DMSO control mortality of 6.9%. None of those larvae receiving distilled water was substantially affected. The dosing procedure in itself thus has little effect on larvae given distilled water but occasional deaths due to handling can occur with this

technique (Sutherland et al. unpublished); 10% DMSO may increase this effect slightly.

DISCUSSION

Root of *Lotus pedunculatus* and *Coronilla varia* was toxic to larvae of *C. zealandica* and a mixture of 3-nitropropanoyl-D-glucopyranoses was isolated from an active fraction of each species. Three of these compounds, viz., karakin, coronarian, and cibarian, have been identified from *L. pedunculatus*. Authentic samples of these three esters were toxic when administered orally to 3rd instar larvae. This study has shown that these compounds, together with further minor components of the nitro compound mixture, were present in fresh root of *L. pedunculatus* at a concentration of about 0.2%. The standard 10-μl dose of crude root extract (conc. 10 g fresh wt/ml) thus contained approximately 0.2 mg nitro compounds and was toxic to larvae. Pure solutions of the individual esters were similarly toxic when administered at a concentration of 1%. As a 10-μl dose of such a solution contains 0.1 mg nitro compound, the compounds are quite active enough to account for all the toxicity of the crude extract of *L. pedunculatus* root and that of *C. varia*.

Preliminary assays of the toxicity of pure karakin, coronarian, and cibarian in 10% DMSO to grass grub gave inconsistent results and, in the case of karakin particularly, apparently less activity than reported here (Sutherland, 1982b). We believe the reason for this was our failure to warm the test solutions during the dosing period. The nitro esters gradually crystallized out, occasionally blocking the syringe needle, thus diminishing substantially the concentration of the test solutions. The dosing procedure adopted in this study overcame this problem.

As DMSO is a potent surfactant, the question arises whether it modifies in any way the toxicity of the nitro compounds in solution or whether the mortality we observed was an artifact. It seems likely that the presence of DMSO in the larval gut would facilitate the passage of the nitro compounds across the gut membrane, but we do not consider it would otherwise modify the physiological effect of the test compounds. In fact, 3-NPA, which dissolves as readily in water as in 10% DMSO, is equally active at equivalent concentrations in the two solvents (Sutherland, 1982b) (Table 4).

This is the first report of nitro compounds from *Lotus pedunculatus*. They have previously been identified from foliage of several other legumes, notably crownvetch (*C. varia*) and *Astragalus* spp. (Gustine, 1979; Stermitz and Yost, 1978), of which crownvetch also is resistant to *C. zealandica* and apparently to other insects (Farrell, personal communication; Wheeler, 1974; Baluch et al., 1978). Byers et al. (1977) have described the toxicity to *Trichoplusia ni* of β-nitropropionic acid and the glucopyranoses karakin, coronillin, and cibarian when incorporated in a pinto bean rearing diet. However, as they point out, the compounds appear to have a feeding deterrent effect, and the

observed poor larval growth may have resulted, at least partially, from starvation rather than from direct toxicity. In our bioassay the former possibility was ruled out. However, as we have reported elsewhere (Sutherland et al., 1982b) the compounds do also deter feeding by *C. zealandica* larvae and may thus play a dual role in resistance of the plant to the insect.

Implication of the aliphatic nitro compounds in the resistance of *L. pedunculatus* to grass grub as either toxins or feeding deterrents seems certain, but how important their role is in comparison with that of the previously reported isoflavonoid feeding deterrents is not known (see Sutherland et al., 1975b; Russell et al., 1978). However, the presence of substantial levels of nitro compounds in the foliage as well as the root of *L. pedunculatus* (Sutherland et al., unpublished data) raises important considerations regarding the use of this pest-resistant species for forage as is contemplated in some sheep-grazing areas of New Zealand. Crownvetch foliage has previously been shown to be toxic to monogastric mammals and birds and the toxin identified as 3-nitropropionic acid (Gustine et al., 1974; Shenk et al., 1976). Gustine (1979) cautions against use of the plant in nonruminant feed formulations. The presence of free 3-NPA in *L. pedunculatus* was not investigated in this study.

The suitability of crownvetch foliage for ruminants is unclear. Peterson et al. (1974) have shown it to be very satisfactory, and Gustine (1979) approves its use for pasture and hay. However, Williams and James (1978) reported that some species of *Astragalus*, another nitro-bearing legume, are highly toxic to sheep and cattle. They correlate this toxicity with the presence of the aliphatic nitro compound miserotoxin (Stermitz and Yost, 1978) and note the lack of toxicity to ruminants of *Astragalus* species containing only the related toxins karakin and cibarian, which also occur in crownvetch. But Williams and James point out that although these compounds are less toxic than miserotoxin, they are synthesized in greater quantities, which can result in a roughly equal toxicity per gram of plant (Williams and James, 1978). Clearly the issue would have to be resolved before any pasture legume bred for an increased pest resistance (perhaps based on increased levels of such toxins) could be contemplated for use as forage for sheep or cattle. It may, of course, prove possible in the case of *L. pedunculatus* resistance to the subterranean grass grub to maintain or even increase resistance by modifying toxin levels in the root by selective plant breeding while decreasing, or at least not increasing, levels in the foliage.

Note Added in Proof—Williams (1983) has recently reported the presence of unidentified aliphatic nitro compounds in several species of *Lotus*, including *L. pedunculatus* and has found that foliage of the latter species is toxic to 1-week-old chicks.

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OCCURRENCE OF SEX ATTRACTANT PHEROMONE, 2,6-DICHLOROPHENOL, IN RELATION TO AGE AND FEEDING IN AMERICAN DOG TICK, *Dermacentor variabilis* (SAY) (ACARI:IXODIDAE)[1]

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Abstract—No detectable sex pheromone, 2,6-dichlorophenol (2,6-DCP), was found in *Dermacentor variabilis* engorged nymphs, teneral females, replete females, or unfed males. The amount of 2,6-DCP present in unfed females of different ages was extremely variable; no relationship between age and 2,6-DCP content in these unfed females was observed. Partially fed virgin females had less 2,6-DCP than unfed females, but there was no change in pheromone content during the course of feeding. Sex pheromone was also found in partially fed males.

Key Words—*Dermacentor variabilis*, Acari, Ixodidae, tick, American dog tick, sex pheromone, 2,6-dichlorophenol.

INTRODUCTION

The chlorinated phenol, 2,6-dichlorophenol (2,6-DCP), has been established as the sex pheromone of ixodid ticks of several different genera. This attractant excites sexually active male ticks, and enables them to orient to pheromone-emitting females. It has been found in at least eight species (Sonenshine et al., 1982; Silverstein, unpublished) and may be assumed to occur in many others. Although widespread, the occurrence of 2,6-dichlorophenol in metas-

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triate ticks is not universal. Wood et al. (1975) were unable to find it in four species of *Rhipicephalus* (Latr.), which responded to a mixture of phenol and p-cresol.

Little is known about the physiological events that affect the synthesis and secretions of 2,6-dichlorophenol. The pheromone is secreted by the foveal glands (Sonenshine et al., 1977) where it is stored and, presumably, where it also synthesized. Pheromone secretion is initiated by feeding, during which profound changes occur in the ultrastructure of the pheromone glands (Sonenshine et al., 1981). However, data on the time in the life cycle when pheromone biosynthesis begins or on changes in pheromone content with age, feeding, mating, and repletion are lacking. Nothing is known of the precise mechanisms by which sex attractant activity is regulated, although a neurohumoral pathway is suggested by the finding of neurosecretory activity in the foveal nerve (Sonenshine et al., 1981).

This study describes the changes in 2,6-dichlorophenol content during the life cycle of the American dog tick, *Dermacentor variabilis* (Say). Changes in the concentration of this pheromone may serve as indicators of important physiological events in the life cycle of these ticks. Measurements of sex pheromone content may provide a convenient way for evaluating the response of the pheromone system to pharmacologically active substances and, perhaps, provide further insight into natural regulatory mechanisms in these important disease vectors.

METHODS AND MATERIALS

Ticks. The American dog tick, *D. variabilis*, was colonized with collections obtained near Montpelier and Ashland in central Virginia. Immature ticks were allowed to feed on albino rats (*Rattus norvegicus*), adult ticks on laboratory rabbits (*Oryctalagus cuniculus*). Ticks and developing eggs were held in an Aminco-Aire Climate Lab® incubator (American Instrument Co., Silver Spring, Maryland) at 27°C ±1°C and 92% ± 2% relative humidity during the nonparasitic periods of their development. Weights of partially fed female ticks were determined with a Mettler semimicrobalance (Mettler Instrument Co., Cranbury, New Jersey).

Extraction of Pheromone. Specimens were selected with respect to life stage, sex, days post-molting, state of engorgement during feeding (i.e., number of days attached to hosts, virgin or mated, and part-fed or replete). A total of 17,730 specimens were used in this study. Partially fed virgin females attached 7 days which had been inoculated with 1% saline solutions were found to weigh approximately the same as uninoculated females ($t = 0.81$, 130 *df*, NS) and were included in the samples. Specimens selected in

accordance with these criteria were accumulated, and the samples were placed in cold, reagent-grade, double-distilled hexane (Omni-Solv®, Krackler Chemical Co., Albany, New York) in long-stem ampoules, flame-sealed, and held for chemical study. The sealed ampoules were subjected to repeated cycles of freezing in liquid nitrogen, thawing, and sonication with a Bransonic II ultrasonic probe (Branson Sonic Power Company, Danbury, Connecticut) (Sonenshine et al., 1976). The ampoules were opened, the hexane was decanted from the residue, and the procedure was repeated twice with redistilled pentane. The hexane–pentane solution was concentrated to about 5 ml by distillation through a packed column, and this solution was cooled and extracted three times with ice-cold 3% NaOH. The cold NaOH extract was acidified with ice-cold 4% HCl to pH 2, and the acidified solution was extracted three times with pentane and once with diethyl ether. The pentane and ether solutions were washed with water, dried over Na_2SO_4, and concentrated to about one ml distillation through a packed column.

Gas–Liquid Chromatography. Aliquots of the tick extracts were injected on a Varian model 2740 gas chromatograph equiped with an electron-capture detector (tritium–titanium foil) suitable for detection and quantitation of halogenated compounds. Originally, we used a 5.5-m × 4.0-mm ID glass column packed with 4% Carbowax 20 M on Chromosorb G. Nitrogen was used as the carrier gas at a flow rate of 54 ml/min. The detector temperature was 140° C while the injector port was 170°. Subsequently, the studies were continued with a 3.05-m × 1.7-mm ID glass column, 5% FFAP on Chromosorb G 80–100 mesh, 20 ml N_2/min, 180° C injector port, 190° C detector temperature, and 170° C oven temperature. Coinjection with an authentic 2,6-dichlorophenol (2,6-DCP) standard confirmed the identification and facilitated quantification of the tick pheromone.

Tests with known amounts of pure standards demonstrated that 40% of the original sample could be recovered with pentane, and all of the values given in the Results were corrected accordingly. Ether extraction gave higher recovery but resulted in interfering peaks.

RESULTS

Sex Pheromone in Different Life Stages. Sex attractant pheromone, 2,6-dichlorophenol (2,6-DCP), was found only in adult ticks, and only during specific physiological states. No pheromone was found in a sample of engorged nymphs collected 13 days postfeeding. In females, 2,6-DCP did not appear until the ticks were at least 12–18 days old (i.e., postemergence) (Table 1). The amount of 2,6-DCP in the unfed females was extremely variable, ranging from 0.05 to 1.50 ng/tick. The highest concentrations were found in

TABLE 1. CHANGES IN OCCURRENCE OF 2,6-DCP DURING LIFE CYCLE OF AMERICAN DOG TICK, *D. variabilis*

Life stage and physiological state	Days postfeed/molt	Individuals (No.)	Amount pheromone (ng DCP/tick)
Nymphs, engorged	13	150	0.0
Males, unfed	5–41	1816	0.0
Males, fed 9 days	10–30	312	0.35
Females, unfed	5–7	450	0.0
	6	300	0.0
	14–20	450	0.05
	12–18	4700	1.00
	22–28	4226	1.50
	24–25	280	1.50
	35	125	0.40
	35–38	1817	0.08
	38–40	222	0.35
	73	500	0.50
	105–120	236	0.06
	115–138	435	0.78
	158	277	1.00
	173	295	0.90
Females, virgin			
Fed 3 days	14–21	214	0.18
Fed 5 days	14–21	228	0.26
Fed 7 days	14–21	225	0.16
Fed 7 days[a]	14–21	176	0.20
Females, mated			
Fed 8 days (1 day postmate)	14–21	221	0.27
Females, mated			
Replete	14–21	75	0.00

[a] 1% saline prior to attachment.

females 22–28 days old, and in females 158–173 days old, the oldest populations examined.

Feeding resulted in a significant change in female tick pheromone concentrations. All samples of partially fed females exhibited greatly reduced concentrations of 2,6-DCP (0.16–0.26 ng/tick). Mating had no apparent effect on 2,6-DCP concentrations, at least not within the first 24 hr after copulation. However, replete females that had dropped from their hosts no longer had detectable 2,6-DCP.

No 2,6-DCP was found in unfed males. However, feeding males attached 7–9 days had substantial amounts of 2,6-DCP which were comparable to those found in feeding females.

Sex pheromone activity in *D. variabilis* reflects a cycle of chemical

synthesis, storage, release, and eventual depletion. Pheromone biosynthesis commences as the young females mature during the first few weeks following the nymphal molt. Subsequent events surrounding pheromone activity in the aging, starved females are unclear. The pheromone is stored, and none is released during this phase of the tick's life (Sonenshine et al., 1974). The extreme variability observed in the samples of aging, unfed females may be due to partial degradation, at unknown rates, of the stored compound. Whether additional biosynthesis occurs under these physiological conditions is unknown. Other explanations are possible. However, no other conclusions can be considered without additional study of sex pheromone content in these ticks.

Sexual activity commences in *D. variabilis* during feeding. This new physiological condition stimulates renewed sex pheromone activity. Pheromone is released by the feeding female, as demonstrated by bioassay (Sonenshine et al., 1974), while fresh 2,6-DCP synthesis also occurs at this time (Sonenshine et al., 1977). The amount of pheromone in the feeding, virgin females remains virtually unchanged; presumably, new pheromone production parallels its emission from the tick's body. Pheromone is still present in the females 1 day after mating, but all pheromone activity terminates when the female completes engorgement.

The finding of significant amounts of 2,6-dichlorophenol in fed males, but not in unfed males, should not be surprising. This phenol also occurs in males of *Amblyomma maculatum* Koch, often in amounts similar to that found in females of the same species (Kellum and Berger, 1977). Foveal glands (the sex pheromone glands) also occur in male *D. variabilis* (Sonenshine, unpubished), although they appear much smaller than in the females. Foveal glands similar in their ultrastructural characteristics to those of the unfed females were found in the males of *Hyalomma dromedarii* Koch (Sonenshine et al., 1983). Evidently, the glandular system needed to produce and store 2,6-dichlorophenol is present in males of at least several ixodid ticks. Whether the phenol occurs in the males or not appears to depend upon physiological events (e.g., hormonal induction of pheromone biosynthesis). However, no evidence of 2,6-dichlorophenol emission by male ticks has been described.

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AQUEOUS EXTRACTS FROM INDIGENOUS PLANTS AS OVIPOSITION DETERRENTS FOR *Heliothis virescens* (F.)[1,2]

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Abstract—In laboratory and field tests, water extracts from plant foliage deterred oviposition by *Heliothis virescens* (F.). A maximum reduction of 93% was attained in laboratory oviposition cages. When females were allowed free choice of treated or untreated tobacco plants in field cage and field tests, maximum reductions in oviposition were 71 and 83%, respectively.

Key Words—Insect ovipositional deterrents, plant odors, *Heliothis virescens.*

INTRODUCTION

For centuries man has observed that some plant species protect and/or promote the growth of other plant species. Utilizing various plant parts, organic gardeners have discovered many home remedies that are truly effective. Garlic, marigold, mints, and a number of other plants are known for their natural repellency to insects. Yepsen (1977) lists more than 30 common pests of gardens and orchards that are repelled by certain plants, which he lists.

Recently, scientists have become more interested in the utilization of plants or plant materials as insect oviposition and feeding deterrents in pest management systems (Boddé, 1982). Jermy (1966) reported that narrower host specialization is connected with greater sensitivity of insect chemo-

[1]Lepidoptera: Noctuidae.
[2]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

receptors to feeding deterrents. Reports include feeding deterrents from plant substances for the gypsy moth, *Lymantria dispar* (L.), (Trial and Dimond, 1979), and the armyworms, *Spodoptera exempta* (Hübner) and *S. littoralis* Boisd. (Kubo et al., 1980). Ladd et al. (1978) found that solvent extracts from seeds of the neem tree, *Azadirachta indica* A. Juss, successfully deterred the Japanese beetle, *Popillia japonica* Newman, from feeding on sassafras leaves, *Sassafras albidum* (Nutt.), and soybeans, *Glycine max* (L.). An extract from the foliage of western red cedar, *Thuja plicata* Donn, deterred feeding by the white pine weevil, *Pissodes strobi* Peck, and the alder flea beetle, *Altica ambiens* (LeConte), and served as an oviposition deterrent for the onion root maggot, *Hylema antigua* Meigen (Alfaro et al., 1981). Other oviposition deterrents reported from plant constituents include those for the cabbage looper, *Trichoplusia ni* (Hübner), (Renwick and Radke, 1981) and the leafhopper, *Amrasca devastans* (Distant) (Saxena and Basit, 1982). Chapman et al. (1981) found that trap catches of the aphid, *Cavariella aegopodii* (Scopoli), were reduced by the presence of an isolated plant odor. Altieri (1981) cited examples where tolerable levels of weeds in certain crops have reduced populations of some insect pests by either repelling the pests or providing alternate hosts for predators and parasites. Nettles (1980) found that a tachinid, *Eucelatoria* sp., is more strongly attracted to the odor of the food plant of its host than to the odor of its host, *Heliothis virescens* (F.). It is evident that host selection, plant or insect, is very complex.

There is little scientific information on which plants, or chemical constituents, might effectively inhibit oviposition by economically important insect pests. The study reported here was undertaken to determine the effect of water extracts of selected plants on oviposition by *H. virescens*, a serious pest of tobacco and cotton in the United States. Plants from which extracts were prepared were selected primarily from species growing in the area. The first phase of this study involved laboratory screening which was followed with field cage testing to select a few promising extracts for application to field plants grown under normal cultural practices for tobacco in north-central Florida.

Plant identifications were made by the senior author; botanical names conform first to "Gray's Manual of Botany" (Fernald, 1950), or alternatively to "Weeds of the Southern United States" (Anonymous, undated), "Florida Weeds—Part II" (Orsenigo, 1977), "Guide to the Poisonous and Irritant Plants of Florida" (Perkins, undated), and "Herbs and Things" (Rose, 1972) in descending order.

METHODS AND MATERIALS

Laboratory-reared *H. virescens* moths were confined in cages made from 3.8-liter Fonda® paper cans which had the tops replaced with white Viva®

paper towels and were held in laboratory environmental chambers. Each paper towel top was first cut into half and then taped together with ca. 0.6 cm of space between each towel half so that one side could be treated with an aqueous extract without bleeding of the treated half into the control (untreated) half.

The extracts were obtained primarily from plant leaves. In some tests stalks, stems, flower buds, blooms, or fruits were used. In one series of tests, we prepared extracts from the following commercially prepared Spice Islands products (Specialty Brands, Inc., San Francisco, California): sweet basil (basil, *Ocimum basilicum* L.); bay laurel (bay, *Laurus nobilis* L.); cayenne pepper (chili pepper, *Capsicum frutescens* L.); coriander, (*Coriandrum sativum* L.); dill weed (dill, *Anethum graveolens* L.); marjoram (sweet marjoram, *Origanum majorana* L.); rosemary (*Rosmarinus officinalis* L.); sage (*Salvia officinalis* L.); garden mint (spearmint, *Mentha spicata* L.); and thyme (common thyme, *Thymus vulgaris* L.). Each extract from fresh plant material was prepared by homogenizing 20 g of leaves or other plant parts in 100 ml of distilled water using a Waring® blender. The solution was then filtered through a white Viva paper towel. Extracts from the Spice Islands products were prepared in the same manner except that we used 200 ml of water because they were dehydrated.

Each test consisted of 6–15 ($\bar{X} = 10.7$) cages of *H. virescens* moths (5 pairs/cage), and each cage was considered a replication. One half of each paper towel top was treated with 5 ml of freshly prepared extract that was applied with a No. 10 camel hair brush. Preliminary tests were conducted using distilled water instead of the extract. No significant differences were noted in the percentage of eggs deposited on the water-treated side (55.2%) vs. the control side (44.8%) (16 replicates); therefore, the control side of the towel was not treated with water. The treatments were made in late afternoon and allowed to dry at room temperature (ca. 23.8° C) before they were placed in position on the cages. The treated cages were then held overnight in an environmental chamber maintained at ca. 26.8° C and 75% relative humidity with a 10-hr dark period. The following morning, the paper towel tops were removed from the cages, and the eggs were counted on the treated and untreated (control) surfaces.

Extracts selected from those that significantly altered egg deposition by *H. virescens* females in the laboratory cage tests were tested on potted Speight G-18 (cv.) tobacco plants that were in the prebutton (prebudding) stage of growth. Each test was conducted in a natural-colored Saran® field cage (round) with a 2.9-m diameter and a 1.2-m height with one vertical zipper. A test consisted of four treated plants that were placed alternatively with four untreated (control) plants in a circular pattern inside the cage (4 replications/night/cage). Each plant was rotated one position clockwise daily for 3–6 days. Preliminary tests using distilled water instead of the extract indicated no

significant differences in the percentages of eggs deposited on water-treated plants (49.8%) and the control plants (50.2%) (12 replicates); therefore, the control plants were not treated with water.

Applications of extract (75 ml/plant) were made to the tobacco plants outside the cage as a fine spray over the entire plant surface with a 350-ml Garden Favorite® trigger sprayer. The extracts were prepared identically to those used in the laboratory tests. After the plants were dry and then positioned, 20 pairs of 2- to 3-day-old *H. virescens* moths were released into the cage. Additional moths were introduced as necessary to maintain about the same number of pairs throughout the test period. Eggs deposited on the plants were counted and removed daily. Fresh tobacco plants were used in each test.

Some of the extracts that were effective in the laboratory and field cages for altering egg deposition on treated surfaces by *H. virescens* were tested on individual Speight G-18 tobacco plants in a field planted by a cooperating farmer using his usual cultural practices. In each test, 20 plants located at least 2 m apart were selected within an area of ca. 64 × 13 m that was located in a 4.8-hectare field. We carefully chose plants that were similar in growth and maturity (button to early bloom stages). Also, we only selected those plants that had some (<20) *H. virescens* eggs already deposited on them to ensure that the plants were indeed attractive to *H. virescens* females for egg-laying; these eggs were removed prior to treatment.

As in the cage studies, 75 ml of the prepared extract were sprayed on alternate plants. A total of 10 plants (two replications) were treated in each test; 10 plants were left untreated. All *H. virescens* eggs were counted and removed daily for 4–5 days.

In all studies, the number of eggs deposited by *H. virescens* in each replication was converted to percentages and the percent reduction (or increase) in oviposition was calculated as follows:

$$\frac{\%\text{ of eggs in control} - \%\text{ of eggs in treatment}}{\%\text{ of eggs in control}} \times 100$$

Data in each test were analyzed for significance between oviposition on treated and untreated surfaces using Student's *t* test.

RESULTS AND DISCUSSION

Reduction in oviposition by *H. virescens* was accomplished in the laboratory, field cage, and field tests (Table 1). Extracts from the leaves of seven plant species [*Sambucus* sp., elderberry; *Lonicera* sp., honeysuckle vine; *Zea mays* L., Pioneer X304C (cv.) corn; *Rubus* sp., blackberry; *Hydrangea* sp., hydrangea; *Solanum esculentum* Mill., Floriamerica (cv.) tomato; and

TABLE 1. EFFECTS OF WATER EXTRACTS FROM PLANT LEAVES ON OVIPOSITION BY *Heliothis virescens* IN LABORATORY, FIELD CAGE, AND FIELD TESTS, ALACHUA COUNTY, FLORIDA, 1982

Plant	Laboratory		Cage		Field	
	% reduction (Total No. eggs in test)	*t* value[a] (Replications)	% reduction (Total No. eggs in test)	*t* value[a] (Replications)	% reduction (Total No. eggs in test)	*t* Value[a] (Replications)
Caprifoliacea						
Sambucus sp.	93.3	22.276**	60.1	7.736**	82.2	11.511**
Elderberry	(420)	(8)	(5173)	(16)	(333)	(10)
Lonicera sp.	78.8	8.002**	66.3	11.651**	68.6	6.664**
Honeysuckle vine	(964)	(8)	(2588)	(16)	(474)	(10)
Gramineae						
Zea mays L.						
Corn, Pioneer X304C	85.8	17.305**	47.6	7.680**	79.7	16.753**
	(890)	(11)	(4333)	(12)	(365)	(10)
Corn, Silver Queen	70.1	9.293**				
	(786)	(11)				
Rosaceae						
Rubus sp.	88.4	23.991**	47.1	7.834**	83.2	19.307**
Blackberry	(421)	(7)	(3170)	(16)	(362)	(8)
Saxifragaceae						
Hydrangea sp.	75.6	11.250**	68.2	10.941**	80.5	7.741**
Hydrangea	(1091)	(12)	(3105)	(16)	(274)	(10)
Solanaceae						
Solanum esculentum Mill.	77.5	8.082**	56.1	10.343**	78.6	11.806**
Tomato, Floriamerica	(355)	(6)	(2435)	(16)	(386)	(10)
Physalis sp.	88.1	17.512**	66.8	9.752**	69.8	7.889**
Ground cherry	(1539)	(13)	(4840)	(24)	(276)	(10)

[a]Calculated by Student's *t* test from percent of eggs deposited in treatment and control: ** = significant at $P = 0.01$.

Physalis sp., ground cherry] in five families produced similar results in all three test environments. The percent reduction in the numbers of eggs deposited in tests with these extracts on the treated paper towels in the laboratory ranged from 75.6 to 93.3% ($\bar{X} = 83.9$) as compared to 47.1–66.8% ($\bar{X} = 58.9$) on the treated tobacco plants in the cage and 68.6–83.2% ($\bar{X} = 77.5$) on plants treated in the tobacco field.

The extract from elderberry leaves appeared to produce the highest reduction in the laboratory, whereas the extract from hydrangea leaves gave the highest reduction in the field cage. The extract from blackberry leaves appeared to be the most effective in the field, but was the least effective in the field cage. Extracts from hydrangea and honeysuckle vine produced the lowest reduction in the laboratory and field, respectively. However, we did not attempt to determine significant differences among the various extracts. Extracts from all seven plants significantly reduced egg deposition by *H. virescens* on the treated surfaces ($P = 0.01$, Student's t test).

Water extracts from the leaves of nine other plant species [*Helianthus* sp., Dalton 164 (cv.) sunflower; *Xanthium* sp., cocklebur; *Ipomoea guamoclit* L., cypress vine; *Brassica* sp., wild mustard; *Cassia obtusifolia* L., sicklepod; *Desmodium tortuosum* (Swartz) de Condolle, Florida beggarweed; *Trifolium repens* L., white clover; *Passiflora incarnata* L., passion flower; and *Nicotiana* sp., Speight G-28 tobacco] (five additional families) were tested in both the laboratory and field cage (Table 2). Each of the extracts significantly reduced egg deposition by *H. virescens* in the laboratory ($P = 0.01$, Student's t test). The extracts from Florida beggarweed and tobacco, which are common hosts of *H. virescens*, and the extract from cocklebur did not reduce oviposition by *H. virescens* on tobacco plants in the field cage.

Results obtained with an individual extract likely varied among the laboratory, field cage, and field tests because of such factors as insect and plant density, plant maturity, and various environmental conditions. The same extract was not tested in both the field cage and field on the same night; therefore, weather conditions were not always consistent. Since all the extracts were not tested simultaneously in the field, conditions were constantly changing due to plant growth and maturation, insecticide applications, and natural insect population fluctuations. However, even with the existing uncontrolled factors we were able to obtain consistent significant reductions in egg deposition in laboratory, field cage, and field tests using water extracts from leaves of several plant species.

In addition to the extracts evaluated in the field and/or laboratory and field cages, extracts from the leaves of 26 other plant species (three additional families) were tested in laboratory cages (Table 3). Extracts from the following five plants significantly reduced ($P = 0.01$, Student's t test) egg deposition (>76%) on the treated surfaces: *Chrysanthemum* sp., chrysanthemum; *Cnidoscolus stimulosus* (Michx.) Gray, bullnettle; *Secale creale* L.,

TABLE 2. EFFECTS OF WATER EXTRACTS FROM PLANT LEAVES ON OVIPOSITION BY *Heliothis virescens* IN LABORATORY AND FIELD CAGE TESTS, ALACHUA COUNTY, FLORIDA, 1982

Plant	Laboratory		Cage	
	% reduction (total No. eggs in test)	*t* value[a] (replications)	% reduction (total No. eggs in test)	*t* value[a] (replications)
Compositae				
Heliantus sp.				
Sunflower, Dalton 164	71.5	8.532**	63.2	9.110**
	(780)	(8)	(2195)	(16)
Mammoth Russian	78.9	7.556**		
	(538)	(8)		
Xanthium sp.	72.8	10.639**	11.9[b]	0.707 NS
Cocklebur	(1274)	(12)	(2931)	(8)
Convolulaceae				
Ipomoea quamoclit L.	80.1	14.634**	63.6	8.356**
Cypress vine	(2207)	(15)	(2374)	(12)
Cruciferae				
Brassicae sp.	84.6	11.886**	64.0	9.296**
Wild mustard	(1150)	(11)	(1852)	(12)
Leguminosae				
Cassia obtusifolia L.	82.1	17.724**	43.7	5.616**
Sicklepod	(642)	(12)	(2688)	(16)
Desmodium tortuosum (Swartz) de Condelle	80.1	14.823**	27.3[b]	1.699 NS
Florida beggarweed	(1361)	(12)	(2538)	(12)
Trifolium repens L.	87.5	8.557**	62.8	12.759**
White clover	(1826)	(12)	(5933)	(24)
Passifloracea				
Passiflora incarnata L.	82.6	16.534**	71.3	11.800**
Passion-flower	(831)	(11)	(2915)	(16)
Solanaceae				
Nicotiana sp.	74.7	10.681**	2.4[b]	0.163 NS
Tobacco, Speight G-28	(1154)	(13)	(1862)	(16)

[a]Calculated by Student's *t* test from percent of eggs deposited in treatment and control: NS = nonsignificant at $P = 0.05$; ** = significant at $P = 0.01$.
[b]Increase.

Wrens Abruzzi (cv.) rye; *Solanum psuedo-capsicum* L., Jerusalem cherry; and *Callicarpa americana* L., French mulberry. The most effective of these extracts for deterring egg deposition by *H. virescens* females was from rye (87.1%).

TABLE 3. EFFECTS OF WATER EXTRACTS FROM PLANT LEAVES ON OVIPOSITION BY *Heliothis virescens* IN THE LABORATORY

Plant	% reduction (total No. eggs in nest)	*t* value[a] (replications)
Araceae		
Diffenbachia sp.	66.7	7.673**
Diffenbachia	(837)	(10)
Compositae		
Chrysanthemum sp.	77.1	12.835**
Chrysanthemum	(1107)	(12)
Cirsium sp.	29.9	2.208*
Thistle	(1187)	(12)
Eupatorium capillifolium (Lam.)	74.1	6.985**
Dog fennel	(513)	(7)
Tagetes sp.	33.6	1.842 NS
Marigold	(1350)	(12)
Taraxacum officinale Weber	63.9	6.873**
Dandelion	(681)	(9)
Convolulaceae		
Ipomoea purpurea (L.)	67.9	6.728**
Morning-glory	(682)	(11)
Ericaceae		
Rhododendron sp.	66.1	3.913**
Azalea	(455)	(7)
Euphorbiaceae		
Cnidoscolus stimulosus (Michx.)	84.9	13.571**
Bullnettle	(486)	(8)
Geraniaceae		
Geraniaceae sp.	27.9	1.733 NS
Geranium	(1494)	(11)
Gramineae		
Secale creale L.	87.1	22.459**
Rye, Wrens Abruzzi	(1107)	(8)
Juglandaceae		
Carya illinoensis (Wang.) K. Koch	58.3	4.843**
Pecan (seedlings)	(1258)	(10)
Labiatae		
Nepeta cataria L.	49.4	2.090 NS
Catnip	(596)	(6)
Liliaceae		
Aloe sp.	57.7	5.077**
Aloe	(382)	(8)
Allium Schoenoprasum L.	68.9	7.965**
Chives	(341)	(6)
Magnoliaceae		
Magnolia sp.	13.2[b]	0.484 NS
Magnolia	(772)	(8)

TABLE 3. (Continued)

Plant	% reduction (total No. eggs in nest)	t value[a] (replications)
Malvaceae		
Hibiscus esculentus L.	43.3	3.680**
Okra, Clemson spineless	(1512)	(10)
Meliaceae		
Melia azedarach L.	66.7	6.270**
Chinaberry[c]	(959)	(10)
Phytolaccaceae		
Phytolacca americana L.	56.9	3.712**
Polkweed (young)	(332)	(7)
Polygalaceae		
Rumex crispus L.	47.6	4.702**
Curly dock	(267)	(6)
Solanaceae		
Solanum melongena L.	68.2	6.823**
Eggplant	(2003)	(11)
Solanum pseudo-capsicum L.	76.4	7.540**
Jerusalem cherry	(1626)	(8)
Solanum tuberosum L.	63.0	10.258**
Potato (red)	(848)	(10)
Umbelliferae		
Apium glaveolens L.	56.1	6.017**
Celery, Dandy®	(443)	(9)
Verbenaceae		
Callicarpa americana L.	76.5	14.980**
French mulberry	(1471)	(14)
Lantana sp.	63.8	12.697**
Lantana	(990)	(13)

[a] Calculated by Student's t test from percent of eggs deposited in treatment and control: NS = nonsignificant at $P = 0.05$; * = significant at $P = 0.05$; ** = significant at $P = 0.01$.
[b] Increase.
[c] Reduction with extract from mature drupes was 65.2%, t value = 12.539** (14 replications).

Aqueous extracts of the stems, blooms, and/or fruits were prepared from some of the plant species and evaluated in the laboratory. Although the extract from blackberry leaves reduced egg deposition in the laboratory by 88.4% (Table 1), no significant reduction (45.4%; t value = 2.488, six replications) was obtained with the extract from blackberry flowers. However, the reduction resulting from application of extract from white clover flowers was significant ($P = 0.01$) at 47.3% (t value = 4.217, nine replications), but it was less than the 87.5% reduction obtained in the laboratory using extract from the white clover leaves (Table 2). Similarly, an extract from chrysanthemum

flower petals produced a significant ($P = 0.01$) reduction of 54.7% (t value = 7.254, 10 replications), which was less than the significant reduction of 77.1% from the extract of chrysanthemum leaves (Table 3). The extract prepared from chive blooms gave a significant ($P = 0.05$) reduction of 75.3% (t value = 11.017, 10 replications) compared to the 68.9% significant reduction produced by the extract from chive leaves (Table 3).

When extracts from tobacco blooms and flower buds were each tested in the laboratory, a significant ($P = 0.01$) reduction of 53.1% (t value = 7.648, 16 replications) was attained with the bloom extract, but no significant ($P = 0.05$) reduction (4.9%; t value = 0.274, 14 replications) resulted from application of the flower-bud extract. A 74.7% significant reduction had been attained previously with extract from tobacco leaves (Table 1) collected from potted plants grown outdoors. We determined that the aqueous extract prepared from leaves taken from greenhouse tobacco plants produced similar results with a significant ($P = 0.01$) reduction of 72.6% (t value = 12.825, 10 replications) in the laboratory.

Extracts prepared from the okra flower buds and pods each were more effective than the extract from okra leaves in reducing egg deposition by *H. virescens* on treated paper towel cage tops. Significant ($P = 0.01$) reductions of 76.8% (t value = 11.131, eight replications) and 62.6% (t value = 9.430, 12 replications) resulted using extracts from okra buds and pods, respectively, whereas a significant reduction of 43.3% was attained with the extract from okra leaves (Table 3). Another extract which was made from a combination of Florida beggarweed buds, blooms, and seeds gave a significant ($P = 0.01$) reduction of 65.0% (t value = 5.264, eight replications) as compared to the 80.1% reduction in oviposition obtained with the extract from Florida beggarweed leaves (Table 1).

A greater reduction in egg deposition was attained with an extract from ground cherry leaves (88.1% reduction, Table 1) than with extracts from ground cherry fruit or stalks and stems, which gave significant ($P = 0.01$) reductions of 65.9% (t value = 8.767, nine replications) and 51.2% (t value = 5.457, 11 replications), respectively. When an extract was prepared from the stalk of celery and tested in the laboratory, it gave a significant ($P = 0.01$) reduction of 43.7% (t value = 4.348, eight replications) as compared to a 56.1% reduction using the extract from celery leaves (Table 3).

No significant reduction (6.9%; t value = 1.230, 12 replications) was obtained in the laboratory cage test with an extract prepared from unripe tomato fruit. But significant reductions in oviposition were obtained with the extract from tomato leaves in the laboratory (77.5% reduction), field cage (56.1% reduction), and field (78.6% reduction) (Table 1).

Corn and sunflower were the only plant species for which we tested more than one variety. The extract from corn leaves of Pioneer X304C, which is known to exhibit resistance to the fall armyworm, *Spodoptera frugiperda*

(J.E. Smith) (Gross et al., 1982) gave an 85.8% reduction in egg deposition by *H. virescens* females in the laboratory; the leaf extract from Silver Queen (cv.) significantly reduced egg deposition by 70.1% (Table 1). Extract prepared from sunflower leaves of commercially grown Dalton 164 (cv.) produced a 71.5% reduction as compared to the reduction of 78.9% by the extract from Mammoth Russian (cv.) sunflower leaves (Table 2).

When water extracts were prepared from the Spice Islands products, seven of the 10 extracts evaluated in the laboratory significantly reduced egg deposition at $P = 0.01$, Student's t test (Table 4). The most effective of these

TABLE 4. EFFECTS OF WATER EXTRACTS FROM SPICE ISLANDS PRODUCTS ON OVIPOSITION BY *Heliothis virescens* IN THE LABORATORY

Plant[a]	% reduction (total No. eggs in test)	t value[b] (replications)
Labiatae		
Origanum majorana L.	75.8	14.321**
Sweet marjoram (marjoram)	(1241)	(10)
Mentha spicata L.	55.9	4.668**
Spearmint (garden mint)	(801)	(10)
Ocimum basilicum L.	80.1	11.611**
Basil (sweet basil)	(361)	(7)
Rosmarinus officinalis L.	28.5	2.010 NS
Rosemary	(744)	(10)
Salvia officinalis L.	39.5	2.480*
Sage	(466)	(8)
Thymus vulgaris L.	43.3	7.691**
Common thyme (thyme)	(856)	(14)
Lauraceae		
Laurus nobilis L.	75.1	14.340**
Bay (bay laurel)	(997)	(14)
Solanaceae		
Capsicum frutescens	81.7[c]	3.472**
Chili pepper (cayenne pepper)	(1271)	(9)
Umbelliferae		
Anethum graveolens L.	71.1	6.771**
Dill (dill weed)	(610)	(7)
Coriandrum sativum L.	2.8	0.178 NS
Coriander[d]	(740)	(12)

[a] Manufacturer's name for plant product shown in parenthesis.
[b] Calculated by Student's t test from percent of eggs deposited in treatment and control: NS = nonsignificant at $P = 0.05$; * = significant at $P = 0.05$; ** = significant at $P = 0.01$.
[c] Increase.
[d] Reduction with extract from leaves taken from greenhouse plants was 68.2%, t value = 5.604** (12 replications).

was the extract from sweet basil which gave a reduction of 80.1%, followed by extracts from marjoram and bay laurel producing reductions of 75.8% and 75.1%, respectively. In contrast, the extract from cayenne pepper significantly increased egg deposition on the treated surface by 81.7%. No significant differences resulted from treatments using extracts prepared from either rosemary or coriander.

The cayenne pepper extract was the only extract from the Spice Islands products that was tested in either the field cage or field. Although this aqueous extract increased egg deposition by 81.7% in the laboratory (Table 4) and by a significant ($P = 0.01$) 74.0% (t value = 3.713, 20 replications) in the field cage, a contrasting 76.2% reduction (t value = 8.792, 10 replications) occurred in the field. This was the only instance where an extract evaluated in the field did not yield similar results to those obtained in the laboratory and field cage tests.

Overall, the mean number of *H. virescens* eggs deposited per replication was 109 (range = 25–676) in the laboratory tests, 195 (range = 22–782) in the field cage, and 47 (range = 7–331) on plants in the field. A total of ca. 140,000 *H. virescens* eggs were counted in this study. We made no comparisons to determine if the extract from any particular plant species actually decreased (or increased) the total number of eggs deposited per individual female moth. Neither did we attempt to determine how long each extract was effective after its application to the tobacco plants; however, results usually were consistent throughout each test period in either the field cage or field environment.

It is not surprising that most of the extracts tested demonstrated some deterrent properties to oviposition by *H. virescens* inasmuch as we selected plant species that were either reported to repel certain insects (Wallace and Mansell, 1976; Yepsen, 1977) or those that we observed to show little or no insect damage in the field. Additional studies are underway to determine the biological mechanisms involved and to isolate and identify the plant constituents responsible for altering oviposition behavior in *H. virescens*. These naturally occurring deterrent compounds could become important components in future pest management systems.

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BERBERINE: A NATURALLY OCCURRING PHOTOTOXIC ALKALOID

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Abstract—The isoquinoline alkaloid berberine, present in nine different plant families was found to be phototoxic to mosquito larvae. In the presence of near UV the LC_{50} for acute 24-hr toxicity was 8.8 ppm compared to 250 ppm for dark controls. Mosquito larvae that were treated with 10 ppm berberine plus near UV for 24 hr and then transferred to berberine-free water showed decreased larval survival and resulted in a smaller cumulative number of pupae and adults as compared to controls, during a subsequent 4-week development period. Berberine was found to be a singlet O_2 generator in experiments with the chemical trap 2,5-dimethyl furan. A slight increase in chromosome aberrations in Chinese hamster cells was also observed with berberine plus near UV treatment. The significance of the phototoxicity of berberine is discussed in relation to plant-insect relations.

Key Words—Berberine, phototoxicity, *Aedes atropalpus*, Diptera, Culicidae, singlet oxygen, UV, alkaloid, secondary plant substance.

INTRODUCTION

In a recent symposium, we reported on the phototoxicity of a range of plant secondary metabolites to insects (Arnason et al. 1983). The photosensitizing properties of polyacetylenes and furanocoumarins are well known. A search for other phototoxic metabolites that might act as protective agents in plants was undertaken. Berberine was one of the substances identified in the survey and forms the basis of the present detailed study.

One of the important isoquinoline alkaloids, berberine (Figure 1) is

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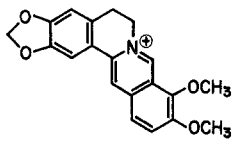
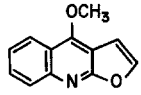


Berberine



Dictamnine

FIG. 1. The molecular structure of berberine and dictamine.

known to occur in at least nine botanical families: Annoncaceae, Berberidaceae, Juglandaceae, Magnoliaceae, Menispermaceae, Papaveraceae, Ranunculaceae, Rubiaceae, and Rutaceae (Jeffs, 1967; Manske, 1968, 1975; Raffauf, 1970; Santavy, 1970). It can be found in any part of the plant and concentrations of up to 10% have been reported (Manske, 1950). To date studies of the compound have been primarily concerned with phytochemical and pharmacological aspects.

Berberine has been shown to possess fungicidal and antibacterial properties (Greathouse and Watkins, 1938; Yoshikaju, 1976; Nakamura, 1977). In a previous study, we found that berberine affected the development rate and survival of insects at levels as low as 0.3% in the diet (Devitt et al., 1980).

The fluorescent nature of berberine was a circumstantial piece of evidence that suggested its possible photodynamic activity, i.e., the production of toxic activated O_2 species such as singlet O_2 ($^1O_2$) in the photoreaction, (Spikes, 1977) and led us to investigate its phototoxic properties. In the current paper we report on its action against the mosquito *Aedes atropalpus* and offer possible explanations as to its mode of action.

METHODS AND MATERIALS

The rock hole breeding mosquito, *Aedes atropalpus* was used to test berberine toxicity. Rearing conditions were previously reported (Philogène and Labaky, 1982).

Berberine (99%, Sigma) was recrystalized twice in ethanol and its purity verified by 2D chromatography on silica gel, using MeOH–$CHCL_3$ (35:36) in the first direction and benzene–MeOH (55:45) in the second direction (Rama Rao and Tandon, 1978).

The following concentrations were tested: 0.1, 1.0, 10, and 100 ppm. Twenty-nine fourth instar larvae were treated in each trial for a period of 24 hr under three different light conditions: (1) complete darkness; (2) two Indorsun fluorescent lamps (Verd-A-Ray Corp.) (5 W/m^2 total and 0.4 W/m^2 UV); and (3) two black light blue lamps (Westinghouse 20 T 12) and two Indorsun lamps (20 W/m^2 total and 3.5 W/m^2 UV). The light intensity was measured with a YSI radiometer and near UV intensity was estimated by using a Kodak Wratten filter No. 2B with a near UV cut off at 400 nm.

Following exposure to the above-mentioned conditions, the larvae were washed several times with dechlorinated water, placed in clean jars, and fed their normal diet of milkbone biscuits. Observations on their development were made to the adult stage.

A special control test consisted of 4th instar *A. atropalpus* larvae treated with a currently used mosquito larvicide, chlorpyrifos (dursban) (diethyl trichloropyridil phosphorothioate). The mosquito and insecticide were subjected to the same light conditions as the berberine-treated larvae.

Singlet Oxygen Measurement. The method of $^1O_2$ measurement was adapted from Ito (1978). Berberine (4.44×10^{-4} M) and 0.1 μl/ml 2,5-dimethyl furan (DMF) were incubated in 1 cm quartz cuvettes under two black light blue tubes (as above). Singlet O_2 generation was observed as the decrease in absorbance of DMF at 220 nm which is consumed in the reaction with $^1O_2$. An alpha-terthienyl (α-T) standard at the same concentration was used. Controls included direct irradiation of DMF without the sensitizer.

Chromosome Aberration Test. Chinese hamster ovary cells (DMO) were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), antibiotics (streptomycin sulfate 29.6 μg/ml, penicillin "O" N.F. sodium 125μg/ml, kanamycin 100 μg/ml, fungizone 2.5 μg/ml), and 7.5% sodium bicarbonate (1 μg/ml).

The cells of stock cultures were grown in 240-ml plastic culture flasks (Falcon) at 37°C in water and resuspended in the fresh medium. For seeding, the suspension was diluted to an approximate density of 70,000 cells/ml. An aliquot (2 ml) of this dilution was seeded on each 22-m^2 coverslip in plastic dishes (Falcon 35 × 10 mm) and kept in MEM with 10% FCS at 37°C for 2 days (60–80% confluency).

Berberine was dissolved in 95% ethanol and diluted in MEM with 2.5% FCS. The ethanol concentration in the first dilution did not exceed 1%. Subsequent twofold dilutions were made and 1 ml of each was added to the Petri dishes after removing tissue culture medium. Tests were carried out in duplicate, one series being irradiated and the second being maintained in the dark.

Cultures were incubated in the dark for 30 min at 37°C and the series to be irradiated was placed under a bank of four black light blue UV lamps (max. 350 nm, 15 W/m^2) for 30 min.

After irradiation, test solutions were removed, the coverslips washed two

times with MEM, and fresh medium with 10% FCS was added to the Petri dishes. Samples were incubated for 16 hr. Four hours prior to the harvesting, the cells were pretreated with 0.2 ml of colchicine (0.01%) in 2.5% (MEM). Sodium citrate (1%) was used for 20 min during harvesting. Air-dried coverslips were stained with 2% acid orecin, mounted, and 100 metaphase plates were analyzed for chromosome breaks and exchanges.

RESULTS AND DISCUSSIONS

Insecticidal Activity. Larval, pupal, and adult survival of *A. atropalpus* was significantly affected following treatment with berberine (Figures 2–4). The toxicity of the alkaloid not only increased with concentration but was enhanced by the presence of near UV light (Figure 2) which was not the case in tests with chlorpyrifos, a nonphototoxic insecticide (data not shown). The LC_{50} for acute 24-hr toxicity of berberine with 3.5 W/m^2 near UV, was 8.8 ppm as opposed to 250 for dark exposure.

Larvae treated with 10 ppm berberine and 0.4 W/m^2 near UV for 24 hr and then transferred to a berberine-free medium exhibited chronic toxicity effects which resulted in significant cumulative mortality in the presence of near UV (Figure 3). At the pupal state there were further effects, the UV and berberine-exposed individuals metamorphosing into a lower cumulative number of pupae (Figure 4). The same situation could be observed at the adult stage (Figure 5). Because of the long depuration time of larvae in berberine-

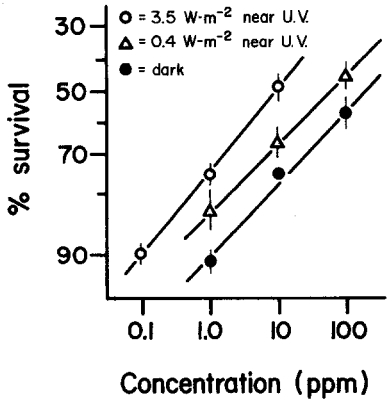


FIG. 2. Probit plots for the effect on 4th instar larvae of *Aedes atropalpus* of 24-hr berberine exposure in the dark (closed circles), exposure to berberine plus irradiation from 2 indoor sun lamps (triangles), or exposure to berberine plus irradiation from 2 indoor sun and 2 black light blue lamps (open circles). Bars represent standard errors.

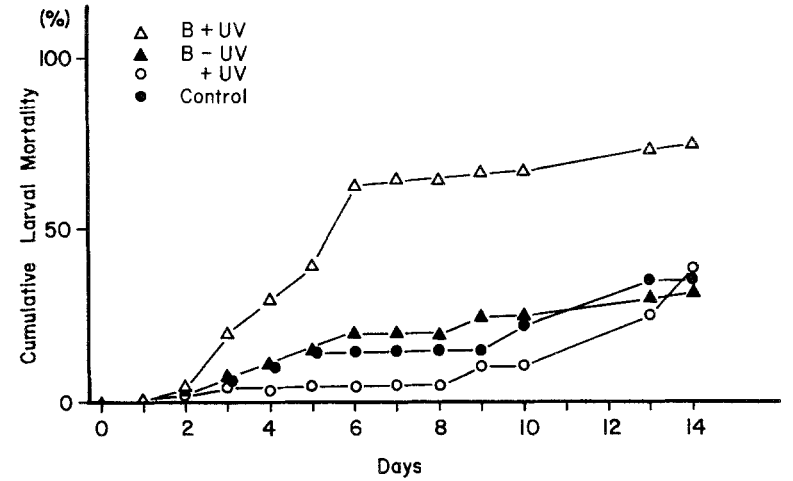


FIG. 3. Variation in cumulative larval mortality of *Aedes atropalpus* 4th instar larvae following 24-hr treatment with 10 ppm berberine plus exposure to near UV (B + UV) and subsequent transfer to berberine-free solution. Other treatments include 24 hr treatment with berberine but without UV exposure (B − UV), UV exposure without berberine treatment (UV), and a control with neither berberine nor UV exposure.

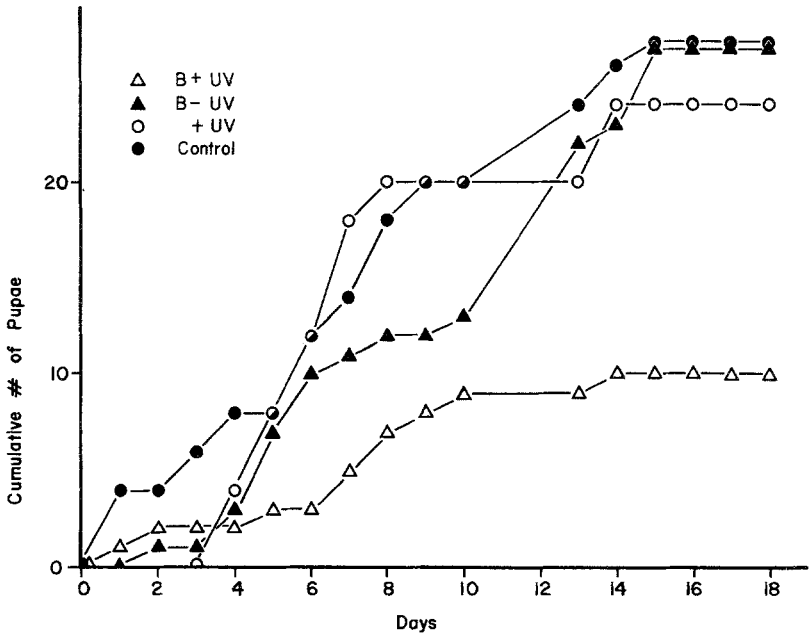


FIG. 4. Variation in the cumulative number of pupae produced from treatments described in Figure 3.

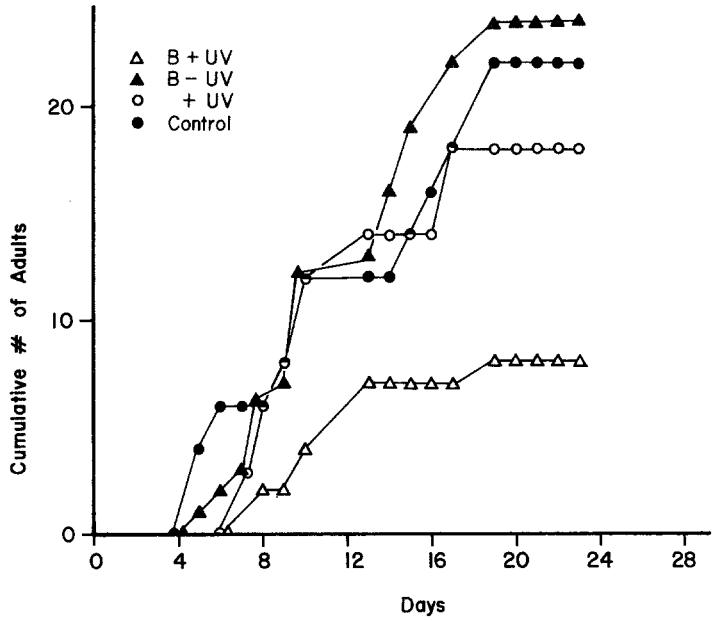


FIG. 5. Variation in the cumulative number of adults produced from treatments described in Figure 3.

free water, we believe there was very little toxic compound in contact with the mosquitoes beyond the larval stage. These results are suggestive of long-term carry-over effects following a brief initial exposure to berberine.

Mechanism of Action. The experiments with the singlet oxygen acceptor DMF demonstrated that berberine is a singlet oxygen generator. Initial rates of $^1O_2$ generation are linear with respect to DMF and a first-order rate constant was calculated (Figure 6). The value for berberine was $k = 2.1 \times 10^{-4}/S^1$, which is somewhat less than $k = 2.0 \times 10^{-2}/S^1$ determined for the potent phytodynamic sensitizer α-T (Arnason et al., 1981) at the same concentration. As the absorption band shapes and extinction coefficients are very similar, these values also reflect relative quantum yields for $^1O_2$ generation.

Slight cytogenetic damage was observed in the experiments in which berberine was used to photosensitize Chinese hamster cells. In the chromosome aberration test, near UV treatment slightly increased the number (from 0 to 0.05) of exchanges and breaks per metaphase plate and percent metaphase plates with chromosome aberrations (from 0 to 3.1) of a 50-ppm berberine treatment over the dark control. At higher concentrations berberine was toxic when irradiated, while at lower concentrations, no effect was observed (Table 1). This damage was considerably less than that created by dictamnine (Figure 1), a compound thought to form monofunctional adducts to DNA

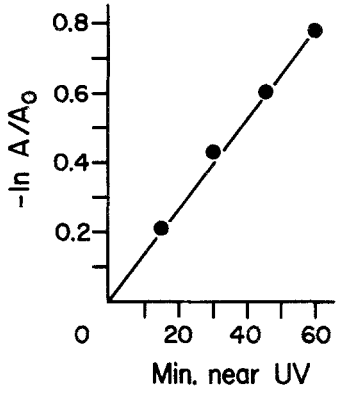


FIG. 6. Singlet O_2 generation by berberine irradiated with near UV as measured by the chemical trap DMF. The graph represents the first-order plot ($\ln A/A_0$) of the disappearance of DMF. Berberine concentration was 4.44×10^{-4} M and irradiation was provided by 2 black light blue tubes with a near UV intensity of 3.5 W/m^2.

(Pyffer et al., 1982) but greater than that observed with α-T (McCrae et al., 1981).

Other workers (Faddejeva et al., 1980; Maidi and Chauduri, 1981; Rungstitiyakorn et al., 1981) have recently shown that berberine intercalates DNA. This suggests that a possible mode of action for cytogenetic damage is the production of $^1O_2$ by berberine molecules bound to DNA, as is thought to be the case with the mutagen acridine orange (Ito, 1978). Further studies

TABLE 1. CHROMOSOME ABBERATION TEST WITH CHINESE HAMSTER CELLS TREATED WITH BERBERINE AND EXPOSED TO DARKNESS AND NEAR UV

		Exchange and breaks per metaphase plate		Percent metaphase plates with chromosome aberrations	
Compound	Conc. (ppm)	Near UV	Dark	Near UV	Dark
Berberine	100	T[a]	0.04	T[a]	2.30
	50	0.053	0	3.1	0
	25	0	0	0	0
Dictamnine	5	MI[b]	0	MI[b]	0
	2.5	2.7	0	82	0
	1.25	1.8	0	60	0

[a]toxic
[b]mitotic inhibition

are necessary, however, to ascertain the photodynamic and mutagenic properties of berberine.

Convergent Evolution. Secondary plant substances (SPS) have so far been studied primarily from the point of view of their phagostimulant or phagodeterrent effects on insects. Ecologists have been interested in the coevolution aspects (Gilbert, 1977; Zwölfer, 1978; Pesson, 1980) while physiologists have focused on the ability of insects to detect the compounds (Dethier, 1980). The current discovery of the phototoxicity of berberine, a widely distributed SPS, constitutes yet another example in the convergent evolution of the phototoxic protective mechanism in plants.

The biosynthetic routes leading to the synthesis of phototoxic substances are very diverse and include derivatization of lipids (polyacetylenes), phenolic biosynthesis from phenylalanine (hypericins, furanocoumarins), and transformation of several amino acids (isoquinoline, β-carboline, and furoquinoline alkaloids). In addition, these phototoxins are found in a very diverse group of plant families. The common phototoxicity of these metabolites would suggest a selective evolutionary advantage to this particular property. We submit that the absorption of light leads to a chemically excited state that is significantly more toxic (and hence protective) than interaction in the dark of secondary metabolites with targets in test species. Excited-state chemistry allows a variety of new processes to occur: covalent bond formation and electron and energy transfer processes. The ability of phytophagous insects to deal with the presence of such compounds will depend on the duration of their exposure to light, the opacity of their integument, and their ability to shield themselves from various wavelengths, particularly near UV.

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ATTRACTION OF MALE SPRUCE BUDWORM MOTHS, *Choristoneura fumiferana* (Clemens),[1] TO PHEROMONE-BAITED TRAPS IN SMALL-TREE THINNINGS[2]

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Abstract—Mean catches of spruce budworm, *Choristoneura fumiferana* (Clemens), moths were not significantly different among four small-tree thinning treatments of young spruce–fir–hemlock regeneration. Significant inverse relationships were found between trap catches and distances to nearby spruce–fir–hemlock overstory. Prevailing wind directions indicated that moths were attracted anemotactically to upwind pheromone sources. No definite trends were detected between catches and temperature or precipitation.

Key Words—Spruce budworm, *Choristoneura fumiferana*, Lepidoptera, Tortricidae, sex pheromone, small-tree thinnings, temperature, precipitation, wind, attraction distance.

INTRODUCTION

Pheromone traps have potential for detecting and monitoring low-level populations of the spruce budworm, *Choristoneura fumiferana* (Clemens). Before traps are used routinely for these purposes, numerous factors that may influence trap catches need to be investigated. Recent investigations showed the effects of trap age, trap saturation, and density or spacing of traps on catches of male spruce budworm moths in dense spruce–fir forests of Maine and Ontario (Houseweart et al., 1981). Previous studies dealt with identification of the spruce budworm sex pheromone (Weatherston et al., 1971; Sanders and

[1]Lepidoptera: Tortricidae.
[2]A contribution to the Canada/ United States (CANUSA) Spruce Budworms Program. Mention of proprietary products does not constitute an endorsement of those products by the USDA.

Weatherston, 1976) and with behavioral factors affecting pheromone communication and mating (Sanders, 1971, 1975; Sanders and Lucuik, 1972). Environmental factors that possibly influence pheromone trap catches of spruce budworm, such as small-tree thinnings and weather, had not been evaluated.

Holbrook et al. (1960) reported that captures of male gypsy moth, *Porthetria dispar* (L.) [= *Lymantria dispar* (L.)], were influenced by trap elevation above ground, type of surrounding vegetation, and weather. Saario et al. (1970) and Sharma et al. (1971) investigated the effects of surrounding vegetation on pheromone catches of field crop pests and concluded that dense crop foliage impeded dispersal of pheromone from traps. Dix et al. (1979) also indicated that heavy undergrowth restricted the dispersal of attractant for the male carpenterworm, *Prionoxystus robiniae* Peck.

Our study was undertaken to determine the possible effects of small-tree thinnings on pheromone catches of spruce budworm in Maine. Data were also obtained on temperature, precipitation, prevailing wind direction, and trap distance from nearby infested overstory, all factors that potentially influence trap catches.

METHODS AND MATERIALS

Thinning Treatments. The pheromone-trapping experiment was superimposed on an existing silvicultural thinning study located on the Penobscot Experimental Forest, near Bradley, Penobscot County, Maine. In 1976 four thinning treatments were made on a 13.4-hectare area of young spruce–fir–hemlock regeneration. Tree species were: spruce [both *Picea rubens* Sargent and *P. glauca* (Moench) Voss], balsam fir [*Abies balsamea* (L.) Miller], eastern white pine (*Pinus strobus* L.), eastern hemlock [*Tsuga canadensis* (L.) Carriere], paper birch (*Betula papyrifera* Marshall), red maple (*Acer rubrum* L.), and aspen (*Populus tremuloides* Michaux). Thinnings were accomplished with a power brush saw. Each treated area (plot) measured 24.4 m on a side (Figure 1). The four treatments were: (1) No release (control), no trees removed. (2) Row—no release, 1.5-m row was cleared, but no release work was done on the 0.9-m residual strip. (3) Row—release, 1.5-m row was cleared, and a potential crop tree was selected from the residual 0.9-m strip; then all other trees were removed. (4) 2.4 x 2.4-m spacing, one potential crop tree was selected from each 2.4- x 2.4-m area; all other trees were removed.

Each thinning treatment was replicated eight times, but not all available plots were used in the pheromone experiment.

Stand characteristics of nearby overstory were: basal area, 15.2 m^2/hectare and volume, 91.6 m^3/hectare. Species composition by percentage of basal area was: balsam fir (13%), spruce (35%), eastern hemlock (36%), and other species (16%).

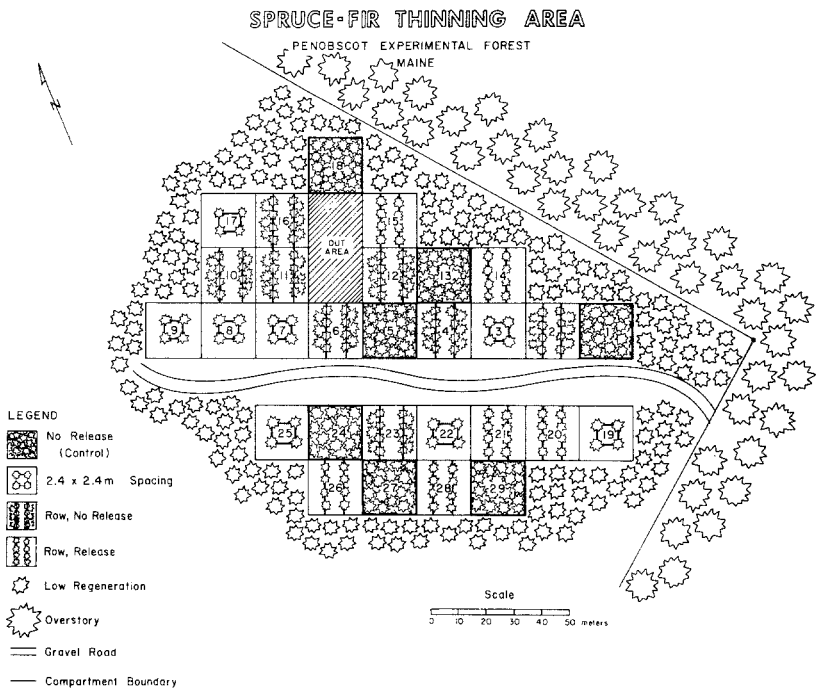

FIG. 1. Scaled map of spruce–fir–hemlock thinning area, Penobscot Experimental Forest, Maine, showing schematic plot layout, thinning treatments, and proximity to spruce–fir–hemlock–hardwood overstory.

Budworm Damage. Infestation by the spruce budworm was first noted in 1976 when upper crowns of nearby overstory spruce and fir were defoliated. However, little feeding damage was evident on small trees in the thinned area that year; only an occasional tree had one or two budworm damaged shoots. Less than 25% of the damage to small trees was attributable to spruce budworm in 1977 (Frank, personal communication). Spruce budworm damage estimates were not made in 1978; however, the trend of only lightly infested trees continued. Larval populations on small trees were not sufficient to warrant extensive branch sampling.

Field Attractancy Test. Pherocon 1CP® (Zoecon Corporation, 975 California Avenue, Palo Alto, California) traps were baited with a polyvinyl chloride (PVC) formulation containing 3% by weight of (*E*)- and (*Z*)-11-tetradecenal in a ratio of ca. 97 parts *E* to 3 parts *Z*. This synthetic formulation of both *E* and *Z* isomers is a potent lure for spruce budworm males (Sanders and Weatherston, 1976). Following Sanders (1978), each trap was baited with a PVC pellet, ca. 4 mm diam. x 10 mm long, and containing ca. 3.8 mg of attractant.

Four replicates of each thinning treatment were chosen randomly. One trap was deployed near each plot center. Traps were hung from mid- to upper-crown branches of small trees ca. 1.5–1.8 m above ground and near apexes of live branches. Trap openings faced ca. NE–SW.

Traps were deployed on June 26 and retrieved on July 21, 1978, for a 25-day trapping period. Traps were checked daily and trap bottoms were replaced with a new, fresh bottom each day. Retrieved bottoms were covered with Saran Wrap® for ease in transporting to the laboratory where captured moths were identified, sexed, and counted.

Temperature, Precipitation, Wind. Wind directional data were obtained from a recording anemometer located at Diamond International Company, Old Town, Maine, ca. 6.5 km north of the study plots. Temperature and precipitation data were obtained from the National Weather Service field station located at the University of Maine, Orono, ca. 5.8 km northwest of the study plots.

Distances to Overstory. Distances from each plot center to nearby spruce–fir–hemlock overstory were measured from a scaled map of the area and later ground-checked for accuracy.

Data Analysis. Moth catches were subjected to ANOVA tests to compare differences in trap catches among thinning treatments. Tree densities were subjected to ANOVA tests to compare differences among thinning treatments. Stepwise multiple regressions were used to test the relationships between trap catches and stand parameters (stems/hectare; tree height); simple linear regressions were used to test for correlations between trap catches and distances to nearby spruce–fir–hemlock overstory.

RESULTS

Thinning Treatments. Mean stems/hectare after thinning were: no release (38,727); row—no release (17,212); row-release (1622); and 2.4 x 2.4 (1708). Significantly more stems were present on the no release (control) plots than plots of the other treatments. The 2.4- x 2.4-m and row—release treatments did not differ significantly in total stems/hectare, indicating these thinnings had similar tree densities. Red and white spruces and balsam fir were the numerically dominant softwoods; paper birch, red maple, and aspen were the dominant hardwoods. For all species, mean dbh ranged from 2.1 to 2.4 cm, and mean height ranged from 2.3 to ca. 3.0 m.

Field Attractancy Test. Mean catch per plot over all days was not significantly different, regardless of thinning treatment ($F = 1.29$; $P = 0.05$). And, mean catches were not significantly different among thinning treatments for the 25-day trapping period (Table 1). Although total catches ranged from 978 to 1638 moths/plot, mean catches for individual plots within treatments did not vary significantly. Treatment–day interactions were also nonsignifi-

TABLE 1. PHEROCON 1CP TRAP CATCHES OF MALE *C. fumiferana* BY THINNING TREATMENT, FOUR REPLICATES, PENOBSCOT EXPERIMENTAL FOREST, MAINE, JUNE 26–JULY 21, 1978

Thinning treatment	Σ	$\bar{X}$/trap	SE	*F* value[a]
No release (control)	5003	50.0	3.3	0.59 NS
Row—no release	5529	55.3	3.0	
Row—release	5488	54.9	3.0	
2.4 x 2.4 m	5410	54.1	3.2	

[a]ANOVA; $P = 0.05$.

cant over all days, but effects due to treatments by individual day were highly significant ($F = 46.6$; $P = 0.01$) as expected, due to variable numbers of moths available during the flight period. Fewer moths were caught in the denser, no-release (control) plots, but these catches were not significantly lower than the other treatment means.

Peak moth activity was observed on July 11, 1978, when mean catches/trap ranged from 119.2 to 126.5 spruce budworm moths for the four treatments (Figure 2). After initial deployment, mean catches seldom dropped below 35–40 moths/trap until July 17.

Stand Parameters. No significant relationships were found between pheromone trap catches and stand parameters. Stepwise multiple regressions comparing mean trap catches with stems/hectare of each tree species indicated that balsam fir was the best entering variable, but the coefficient of multiple determination was very low ($R^2 = 0.13$). Moth catches were not significantly correlated to tree density of any or all species.

Distance to Overstory. Significant inverse relationships were found between mean trap catches and distances to the nearest spruce–fir–hemlock overstory (Figure 3) and to the northeast overstory (Figure 4). In both situations, fewer moths were caught as distance from overstory increased. No doubt the spruce–fir–hemlock overstory served as a reservoir and source of moths immigrating into the thinned area.

Temperature, Precipitation, and Wind. Mean daily temperatures generally exceeded 20°C during the 1978 flight period, and moths were caught during both periods of rainfall and periods of no precipitation (Figure 2). However, no definite trends were detected between catches and temperature or precipitation.

Prevailing winds for the 25-day trapping period were generally opposite to the directions of nearby overstory, i.e., prevailing winds (21 days) were from the SW and NW. Our data in Figures 3 and 4 indicate that moths were responding anemotactically to pheromone sources in the thinned area *upwind* from nearby overstory trees.

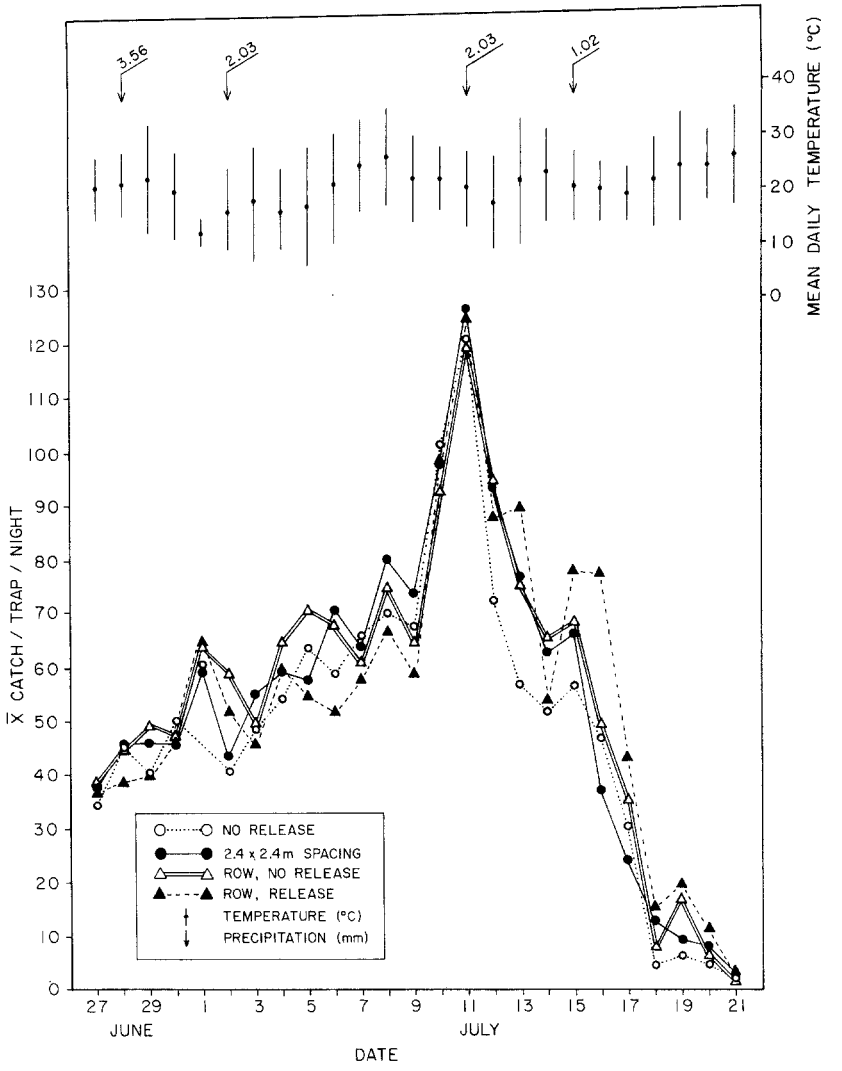

FIG. 2. Mean daily Pherocon 1CP trap catches of male spruce budworm moths by tree thinning treatments, Penobscot Experimental Forest, Maine, 1978.

DISCUSSION

We found no evidence that thinning treatment significantly influenced pheromone trap catches of male spruce budworm moths in young spruce-fir-hemlock regeneration. Because vegetation may restrict air movement and hinder the flow of pheromone molecules (Shorey et al., 1968; Hirano, 1976), we had expected to find fewer moths caught in traps where tree foliage was

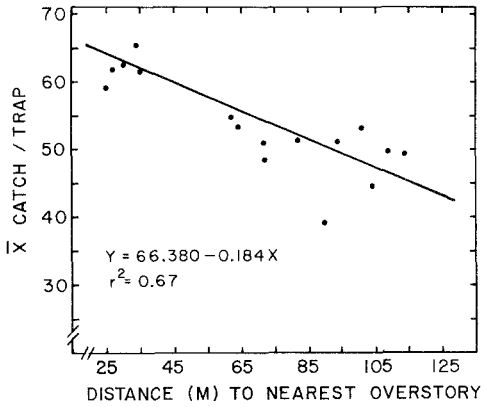

FIG. 3. Relationship of mean catch/trap in thinning area and distance in meters to nearest spruce-fir-hemlock overstory, Penobscot Experimental Forest, Maine, 1978.

most dense. Although most catches were lower in the more dense plots, which had the least budworm damage, catches were not significantly lower.

Numerous factors affect pheromone trap catches, including trap spacing, trap density, and pheromone lure strength. Houseweart et al. (1981) noted less interference in traps spaced 20 and 40 m apart than those spaced 5 and 10 m apart in a dense, mature spruce-fir forest of northern Maine. Similar data are not available for young spruce-fir-hemlock stands, open or dense. Because of existing plot layout, the minimum distance between traps in the small-tree thinning area was only 24.4 m; hence, some traps may have competed for flying moths. However, no consistent pattern of high-low catches for adjacent plots was evident.

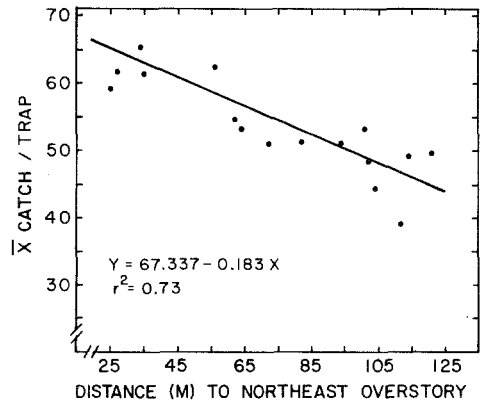

FIG. 4. Relationship of mean catch/trap in thinning area and distance in meters to northeast spruce-fir-hemlock overstory, Penobscot Experimental Forest, Maine, 1978.

The Pherocon 1CP traps we used in the field attractancy test functionally saturate, and trapping efficiency diminishes in both high- and low-density populations after ca. 50 moths are caught (Houseweart et al., 1981). Because many of the observed catches in the thinning area exceeded this level, we analyzed the data again, excluding catches of >50 moths/trap. Again, we found no significant differences (182 observations) between mean moth catches and thinning treatment.

The effective range of attraction for the PVC lure formulation and concentration used in the field attractancy test is unknown. Sanders (1981) reported that these lures were more attractive than virgin female moths. Our 3.8-mg lures undoubtedly attracted moths from nearby overstory trees. Distances from plot center to nearest overstory ranged from 25 to 114 m, and from plot center to northeast overstory (downwind) from 25 to 121 m. These distances are within the range of distances tested by Miller and McDougall (1973) for males responding to virgin female spruce budworm moths.

Although no definite trends were noted between trap catches and temperature or precipitation, our data indicate that spruce budworm moths responded anemotactically to upwind pheromone sources. Such response differs from emigration and displacement flights, which are generally downwind (Greenbank et al., 1980). Male budworm moths generally fly upwind (positive anemotaxis) in response to an attractive pheromone source (Sanders and Lucuik, 1972; Shepherd, 1979); however, effective distances for orientation and attraction are seldom known.

Future monitoring efforts in similar small-tree thinnings should consider: (1) pheromone lure strength, (2) proximity of pheromone traps to nearby budworm-infested overstory trees, (3) direction of prevailing winds, and (4) density or spacing between traps.

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NEW SEX ATTRACTANTS FOR 35 TORTRICID AND 4 OTHER LEPIDOPTEROUS SPECIES, FOUND BY SYSTEMATIC FIELD SCREENING IN THE NETHERLANDS

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Abstract—Most of the known sex attractants and pheromones found for Tortricidae attract species of the subfamily Tortricinae. In order to find more sex attractants for species of the subfamily Olethreutinae, about 60 one- and two-component lures were screened for attractancy in different biotopes in the Netherlands. Most of the chemicals tested were straight-chain dodecen-1-ols and their acetates. The species captured belonged to the following families and subfamilies: Tortricinae (5), Olethreutinae (30), Noctuidae (2), Gelechiidae (1), Gracillariidae (1). Some of the species captured are recorded as pest species in forestry: *Epinotia tedella, Gypsonoma aceriana, Cydia strobilella, C. zebeana, Petrova resinella, Blastesthia turionella*, and *B. posticana.*

Key Words—Sex attractants, field screening, Lepidoptera, Tortricidae, Olethreutinae, dodecen-1-ol, dodecen-1-ol acetate.

INTRODUCTION

To discover lepidopterous sex attractants, two different strategies are possible. One way is to isolate and identify substances produced in female pheromone glands. Another way is field screening of synthetic chemicals, singly or in blends, that have the potential to attract males of different moth species. In the former case the research is focused on a particular species, and one is quite sure that the chemical structures to be identified are real constituents of the natural sex pheromone. In field screening tests, the chemicals are known at the outset, but one does not know which species will be attracted. Moreover, sex attractants found by field screening do not necessarily correspond with

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natural sex pheromones. The advantage of field screening over chemical identification, however, is that sex attractants for many species may be found for a small expenditure of money and time.

Recent field screening studies have revealed many new sex attractants for lepidopterous species occurring in Japan (Ando et al., 1977, 1978, 1981), Canada (Weatherston et al., 1978; Steck et al., 1977, 1979, 1982), the United States (Roelofs and Comeau, 1970) and France (Frérot et al., 1979). These studies have appreciably broadened our knowledge about sex attractants, especially with regard to species of the families Noctuidae and Tortricidae where most sex attractant compounds appear to be straight-chain alken-1-ols, aldehydes, and acetates with a carbon chain of 10–18 carbon atoms. When a sex attractant is composed of two or more components, these are usually structurally related, being geometrical or positional isomers, or differ only in chain length or functional group (Steck et al., 1982; Ando et al., 1981).

As already noticed by Roelofs and Comeau (1971), there usually is a close relationship between the taxonomic position of species and the chemical structures to which they are attracted. Although this relationship is most characteristic at the family level, it usually also occurs at the subfamily level, or even at the tribal level (Roelofs and Brown, 1982; Steck et al., 1982). Knowledge of this relationship may be a great help when planning screening programs.

It is well known that sex attractants for species of the subfamily Tortricinae usually are composed of tetradecen-1-ol acetates and related structures, whereas those for olethreutine species most frequently consist of dodecen-1-ol acetates and corresponding alcohols (Roelofs and Brown, 1982; Kydonieus and Beroza, 1982). Despite the larger size of the subfamily Oelthreutinae, many more sex attractants are known for tortricine species than for olethreutine species. Many tortricine sex attractants were found by extensive field screening of C-14 structures (e.g., Ando et al., 1981). Comparable studies with C-12 structures are lacking thus far. In this paper the results are given of a field screening study, especially directed to species of the subfamily Olethreutinae.

METHODS AND MATERIALS

Some of the compounds used in this experiment were obtained from commercial outlets. The others were synthesized in our institute. All were ultimately purified by argentation chromatography (Voerman, 1979). They were assayed by GLC (glass column, 5.5 m x 2 mm ID, packed with 15% OV-275 on Chromosorb W AW DMCS 100–120 mesh) and by HPLC (Houx and Voerman, 1976) to ensure freedom from geometrical isomers (<1%). No ozonolysis–GC was performed to check for the presence of positional isomers.

For each chemical or blend of chemicals to be tested, 1 mg, dissolved in

CH_2Cl_2 (5 mg/ml), was applied to red rubber sleeve stoppers, 5 x 9 mm (Arthur H. Thomas company, Philadelphia, Pennsylvania, Cat. No. 1780-B10).

The lures were fixed into the top or triangle traps with exchangable sticky bottoms (10 x 21 cm). Tangle-Trap was used as sticky material (Tanglefoot Co., Grand Rapids, Michigan). The traps and lures were renewed after 6–7 weeks. The sticky bottoms were replaced when necessary (depending on capture of moths, saturation with nontarget insects, dust, etc.). The traps were usually fixed to tree branches 1.5–2 m off the ground. Thery were inspected weekly and the captured moths were taken to the laboratory for identification or removed if their identity was certain.

For identification, standard taxonomic works on West European moths were used (Bentinch and Diakonoff, 1968; Bradley et al, 1973, 1979; Hanneman, 1961). In doubtful cases specialists were consulted, and most species were checked against material from the entomological collections in Wageningen and Leiden. Generic and species names conform with those given by Lempke (1976), except the name *Cydia* which we used instead of *Laspeyresia*.

In 1982 field tests were conducted at the following sites: (1) a 10-year-old plantation of spruce (*Picea abies*) mixed with young birches and undergrowth of blueberries (*Vaccinium myrtillus*); (2) a 50-year-old pine forest (*Pinus sylvestris*) with dense undergrowth of blueberry (*Vaccinium myrtillus*), bordering on a *Calluna* heath; (3) a poor 50-year-old oak forest, with some birches near the limit of the trapping area; (4) a peatbog fen area with abundant bog myrtle (*Myrica gale*) willows (*Salix aurita*, *S. cinerea*) and birch (*Betula pubescens*); (5) a marshy forest with *Alnus glutionosa*, *Betula pubescens*, and *Salix cinerea*; (6) an unfertilized moderately dry meadow on loamy soil, rich in herbs; (7) a poplar stand with *Populus canadensis* and *Populus alba*; and (8) a poplar stand with *Populus alba*.

The first three sites are situated in the Planken Wambuis natural reserve near Ede (P), which is a dry area on poor, sandy soil. Sites 4 and 5 are situated in the Korenburgerveen natural reserve near Winterswijk (K). Sites 6 and 7 are near Wageningen, site 8 near Arnhem.

In this paper the chemical formulations are represented as follows: *Z* and *E* indicate the configurations of the double bond, the number following the *Z* or *E* gives the position of the double bond, the last number indicates chain length, : OH = alcohol, : Ac = acetate. Thus *Z*-8-dodecen-1-ol acetate, *E*10-14 : OH = *E*-10-tetradecen-1-ol, and so forth.

The main series of chemicals screened for their attractancy to Tortricidae (series T) consisted of 48 blends of C-12 and C-14 structures.

Blend Series T. *Z*7-12:Ac, *Z*8-12:Ac, *Z*9-12:Ac, *Z*10-12:Ac, *Z*8-12:OH, *Z*9-12:OH, *E*7-12:Ac, *E*8-12:Ac, *E*9-12:Ac, *E*10-12:Ac, *EE*8,10-12:Ac (one component per trap), *Z*8-2:Ac + *E*8-12:Ac (9:1, 1:1, 1:9), *Z*9-12:Ac + *E*9-12:Ac (9:1, 1:1, 1:9),*Z*10-12:Ac + *E*10-12:Ac (9:1, 1:1, 1:9), *Z*8-12:Ac + *Z*8-12:OH (1:1), *Z*9-12:Ac + *Z*9-12:OH (1:1), *E*10-

12:Ac + *E*10–12:OH (1:1), *EE*8,10–12:Ac + *EE*8,10–12:OH (1:1), *Z*7–12:Ac + *Z*9–12:Ac (1:1), *E*7–12:Ac + *E*9–12:Ac (1:1), *Z*8–12:Ac + *Z*9–12:Ac (1:1), *E*8–12:Ac + *E*9–12:Ac (1:1), *E*8–12:Ac + *E*10–12:Ac (1:1), *Z*11–14:Ac + *E*11–14:Ac (1:0, 9:1, 1:1, 1:9, 0:1), *Z*11–14:Ac + *Z*11–14:OH (9:1, 1:1, 1:9, 0:1), *Z*11–14:Ac + *Z*9–14:Ac (9:1, 1:1, 1:9), *Z*10–14:Ac + *E*10–14:Ac (1:0, 3:1, 1:3, 0:1).

At each of the sites 1–5 the full series of C-12 structures given above was tested. Blends with *Z*11–14:Ac were only tested in the coniferous habitats at sites 1 and 2, those with *Z*10–14:Ac or *E*10–14:Ac only at sites 4 and 5. The trap series at sites 1–5 operated from the middle of June to the end of August. At site 6 a selection of 29 blends of the T series was tested during July and August, including both C-12 and C-14 structures.

Blend Series G. At sites 7 and 8 a series of 15 mixtures was tested in July in order to find a sex attractant for *Gypsonoma acerina*, the larvae of which may cause damage in young poplar stands by boring into young shoots. *E*9–12:Ac + *E*9–12:OH (1:0, 3:1, 1:1, 1:3, 0:1), *E*10–12:Ac + *E*10–12:OH (1:0, 3:1, 1:1, 1:3, 0:1), *E*9–12:Ac + *Z*9–12:Ac (9:1, 3:1, 1:1), *E*9–12:Ac + *E*10–12:Ac (3:1, 1:3). The choice of blends was based on incidental capture of *Gypsonoma* species in the past.

RESULTS

Screening a wide array of one- and two-component lures in some contrasting habitats (dry–wet, coniferous–deciduous) appeared to be an effective and convenient way of finding new sex attractants for tortricid moths.

Table 1 gives the catches of species on those blends that were found to be most attractive. About 12 other attracted species have not been included in this table because very few of them were caught or because good sex attractants for them are already known. In those cases where significant numbers of a species were attracted to only one trap, repeat experiments are desirable.

As expected, most of the new sex attractants were found for species of the subfamily Olethreutinae. This confirms the rule that many species of this subfamily are attracted to mono- or diunsaturated C-12 compounds (acetates or alcohols) with the double bond at position 8, 9, or 10. For most species the most attractive blend caught at least 3–4 times as many males as did any other blend. These species apparently respond specifically to particular lures. Some species, however, are attracted to several blends, which differ mostly in the ratio of compounds. These latter species will be discussed below in more detail.

Dichrorampha plumbana was captured most effectively with *E*9–12:Ac + *Z*9–12:Ac (9:1), whereas only one specimen was caught on *E*9–12:Ac alone. However, when *E*7–12:Ac or *E*9–12:OH are added to *E*9–

12:Ac in a ratio of 1:1, attraction was greatly improved. This suggests that these compounds may also be present in the natural pheromone.

An interesting problem arose in the species *Blasthesthia turionella* and *B. posticana*, which are closely related, occur in the same habitats, and fly at the same time of the year. Both species were strongly attracted to three different lures: *E*9–12:Ac alone, *E*9–12:Ac + *E*8–12:Ac (1:1), and *E*9–12:Ac + *E*7–12:Ac (1:1). Neither of the species was attracted to *E*8–12:Ac or *E*7–12:Ac alone. Adding *E*8–12:Ac to *E*9–12:Ac did not significantly affect catches of either species, suggesting that neither species is sensitive to *E*8–12:Ac. Adding *E*7–12:Ac to *E*9–12:Ac reduced capture of *B. posticana by* about 30%, but did not reduce the capture of *B. turionella* males. This effect, however, may be due to trap position. Experiments with more replicates and compound ratios are necessary to test inhibitory or synergistic effects of *E*7–12:Ac on captures of the *Blastesthia* species. If the natural pheromone of both species consists of only *E*9–12:Ac, other mechanisms, like differences in daily calling times or different optimum release rates, should prevent interference between the species. It is also possible that other secondary compounds are involved.

Pammene inquilina belongs to a group of *Pammene* species frequently attracted to sex traps of the harmful species *Grapholitha molesta* and *Grapholitha funebrana* (Chambon and d'Aguilar, 1974; Sziraki, 1978). These traps are usually baited with*Z*8–12:Ac + *E*8–12:Ac (98:2–95:5). We found that adding 10% *E*8–12:Ac to pure *Z*8–12:Ac reduced catches of *P. inquilina* by approximately 25%. This suggests that *E*8–12:Ac may have a negative effect on that species. Two other *Pammene* species, *P. obscurana* and *P. populana* were attracted most to *Z*8–12:Ac + *Z*8–12:OH (1:1) and pure *Z*8–12:OH, respectively.

Another problem was encountered with two species of the genus *Gypsonoma*: *G. dealbana* and *G. aceriana*. Whereas the larvae of the former species feed on various deciduous trees, the latter species is confined to poplar trees. Both species can be found together in poplar stands. In the Korenburgerveen area, large numbers of *G. dealbana* were attracted to traps baited with *E*10–12:Ac (ca. 140 males/trap) and much less frequently to traps baited with *E*9–12:Ac + *Z*9–12:Ac (9:1) (ca. 20 males/trap). In the poplar stands near Arnhem and Wageningen, however, the same blends caught 63 and 55 males, respectively, suggesting that these blends are equally attractive. Further study is needed to resolve this discrepancy. *Gypsonoma aceriana* was also attracted to *E*10–12:Ac. Adding 25% of the corresponding alcohol (*E*10–12:OH) improved catches greatly. We do not know whether the latter blend is also attractive to *G. dealbana*, because that species had already vanished when this blend was tested.

Of the species captured, several are known as local pest species in forestry (Schwenke, 1978). Of these *Epinotia tedella* and *Cydia strobilella* may cause

TABLE 1. SEX ATTRACTANTS FOR TORTRICID AND OTHER MOTHS FOUND BY FIELD SCREENING IN THE NETHERLANDS IN 1982

Species caught	Area	Attractant	Males captured in (n) traps		Flight period
Tortricinae					
Archips oporana (L.)	P	Z11-14:Ac + E11-14:Ac (9:1)	92	(2)	6/15-8/5
Aphelia paleana (Hubner)[c]	W	Z11-14:Ac + E11-14:Ac (9:1)	47	(1)	7/12-8/9
Cnephasia interjectana (Haworth)	K	E10-12:Ac	195	(2)	6/15-7/27
Cnephasia longana (Haworth)	P	Z 9-12:Ac + Z 9-12:OH (1:1)	33	(2)	7/15-8/5
Epagoge grotiana (Fabricius)	K	Z10-14:Ac	52	(2)	7/6-7/27
Eulia ministrana (L.)	P	Z11-14:Ac + Z 9-14:Ac (9:1)			
Olethreutinae					
Ancylis apicella (Den. & Schiff.)	K	Z 8-12:Ac + Z 9-12:Ac (1:1)	84	(2)	5/19-8/10
Ancylis geminana (Donovan)	K	Z 9-12:OH	95	(2)	5/19-8/10
Ancylis uncella (Den. & Schiff.)	P	Z 9-12:OH	210	(3)	5/17-8/12
Apotomis betuletana (Haworth)	K, W	Z10-14:Ac	123	(3)	7/6-8/24
Bactra lancealana (Hubner)	K	E10-14:Ac	17	(2)	6/3-8/10
Blastesthia posticana (Zetterstedt)	P	E 9-12:Ac[a]	400	(3)	5/17-6/1
Blastesthiua turionella (L.)	P	E 9-12:Ac[a]	130	(6)	5/17-6/1
Cydia coniferana (Ratzeburg)	P	E 8-12:Ac + E10-12:Ac (1:1)	30	(2)	5/25-7/1
Cydia cosmophorana (Treitschke)	P	E 8-12:Ac + E10-12:Ac (1:1)	23	(2)	5/17-6/15
Cydia pactolana (Zeller)	P	Z 8-12:OH	289	(1)	5/25-7/1
Cydia strobilella (L.)	P, K	Z 8-12:OH	47	(4)	5/17-5/25
Cydia zebeana (Ratzeburg)	P	E 8-12:Ac + E10-12:Ac (1:1)	14	(1)	6/15-8/25
Dichrorampha plumbana (Scopoli)	K	E 9-12:Ac + Z 9-12:Ac (9:1)	32	(2)	5/19-6/17
Endothenia quadrimaculana (Haworth)	K	E10-14:Ac + Z10-14:Ac (3:1)	10	(1)	6/17-8/23
Epinotia nanana (Treitschke)	P	E 8-12:Ac + E10-12:Ac (1:1)	25	(1)	6/15-7/23
Epinotia rubiginosana (Herr.-Schaffer)	P	Z 9-12:Ac + Z 7-12:Ac (1:1)	47	(2)	5/25-6/25
Epinotia tedella (Clerck)	P	E 9-12:Ac + Z 9-12:Ac (9:1)	409	(1)	5/17-7/8
Eucosmomorpha albersana (Hübner)	K	E 9-12:Ac + Z 9-12:Ac (9:1)	60	(1)	5/19-6/17
Griselda myrtillana (Hump. & Westw.)	P	Z11-14:OH [+Z11-14:Ac (9:1)]	29	(3)	6/1-8/12
Gypsonoma aceriana (Duponchel)	R	E10-12:Ac + E10-12:OH (3:1)	53	(2)	7/6-8/3

Gypsonoma dealbana (Frölich)	K	*E*10-12:Ac	283	(2)	6/17-8/10
Gypsonoma sociana (Haworth)	K	*E*10-12:Ac	16	(1)	6/3-6/30
Metendothenia atropunctata (Zett.)	K, P	*Z*10-12:Ac	3733	(5)	5/17-8/25
Notocelia rosaecolana (Doubleday)	K	*EE*8,10-12:Ac	14	(2)	6/17-7/13
Olethreutes siderana (Treitschke)	[b]	*Z* 8-12:Ac	222	(1)	6/23-7/6
Pammene obscurana (Stephens)	P, K	*Z* 8-12:Ac + *Z* 8-12:OH (1:1)	42	(3)	5/17-6/15
Pammene inquilina (Fletcher)	P	*Z* 8-12:Ac	103	(2)	5/17-5/25
Pammene populana (Fabricius)	K	*Z* 8-12:OH	226	(2)	8/3-8/25
Petrova resinella (L.)	P, K	*Z* 9-12:Ac + 9-12:Ac (1:1)	524	(4)	5/17-6/15
Rhopobota ustomaculana (Curtis)	P	*Z* 9-12:OH	24	(2)	6/25-8/12
Strophedra weirana (Fabricius)	P	*E* 8-12:Ac + *E* 8-12:OH (1:1)	32	(1)	6/1-6/25
Other families					
Xestia xanthographa (Den. & Schiff.)[d]	W	*Z* 9-14:Ac + Z11-14:Ac (9:1)	15	(1)	7/19-7/26
Macdonnoughia confusa (Stephens)[d]	K	*Z* 7-12:Ac	55	(2)	5/19-8/23
Hypatima rhomboidella (L.)[e]	K	*E* 7-12:Ac	196	(1)	7/27-8/23
Phyllonorycter kleemanella (Fabricius)[f]	K	*Z*10-14:Ac + *E*10-14:Ac(1:0-1:3)	2055	(4)	5/19-8/23

[a]Or *E*9-12:Ac + *E*8-12:A (1:1) or *E*9-12:Ac + *E*7-12:Ac (1:1).
[b]Local screening in Oosterbeek with *Z*8-12:Ac + *Z*8-12:OH (1:0, 1:1, 0:1).
[c]Improved attractant compared with data given in literature.
[d]Noctuidae.
[e]Gelechiidae.
[f]Gracillariidae.

damage in spruce plantations; *Gypsonoma* aceriana in popular stands; *Petrova resinella*, *Blastesthia turionella*, and *Blastesthia posticana* in pine forests; and *Cydia zebeana* in larch stands. *Cydia zebeana* was captured for the first time in the Netherlands. It originates from eastern Europe. This species, of which the larvae are barkborers, is often confused with *Cydia millenniana*, another pest species on larch. The larvae of the latter species are gall formers.

DISCUSSION

The sex attractants of taxonomically related species often consist of similar chemical compounds. Knowledge about this relation between taxonomy and sex attractants greatly improves the predictability and success of field screening. The more known about a particular taxonomic group, the easier it will be to find new sex attractants for species in that group.

In the present study we could focus our field screening on species of the tortricid subfamily Olethreutinae, knowing that most of the sex attractants known from this group were dodecen-1-ols and dodecen-1-ol acetates. The results of our study strongly confirm this, although it also became clear that C-12 structures are not attractive solely to Olethreutinae and that some species of this group use other structures.

The chemical structures most frequently used as sex attractants in the Olethreutinae are dodecen-1-ols and dodecen-1-ol acetates that are unsaturated at positions 8 or 9 (*Z*8–12:Ac, *E*8–12:Ac, *Z*8–12:OH, *E*8–12:OH, *Z*9–12:Ac, *E*9–12:Ac, *Z*9–12:OH, *E*9–12:OH). Much less frequently used are *Z*7–12:Ac, *Z*10–12:Ac, *E*10–12:Ac, *E*10–12:OH, and *EE*8,10–12:Ac. It is remarkable that dococen-1-ols and their acetates are also frequently used by *Cnephasia* species belonging to the subfamily Tortricinae (see also Hrdý et al., 1979).

Of the tetradecen-1-ol acetates, *Z*10–14:Ac and *E*10–14:Ac are occasionally found to be sex attractants for Olethreutinae species. The favorite compounds for chemical communication in the Tortricinae, *Z*11–14:Ac and *E*11–14:Ac, did not attract any Olethreutinae species in our study. Another group of chemical structures that has been identified as sex pheromones in Tortricinae is that of the aldehydes. As far as we know, aldehydes have not been reported as pheromones or sex attractants in Olethreutinae. Unfortunately dodecenyl aldehydes could not be included in our field screening, but it would be interesting to incorporate these chemicals in future field tests.

With regard to the specificity of sex attractants, it is remarkable that particular blends often attract males of several species. In Tortricinae this is a well-known phenomenon; for example, several species are attracted by each of the single compounds *Z*11–14:Ac, *Z*11–14:OH, *E*11–14:Ald, and *Z*11–14:Ald (see for examples, Ando et al., 1981).

In our study Z8-12:OH, Z9-12:OH, E9-12:Ac + Z9-12:Ac (9:1), E10-12:Ac, E8-12:Ac + E10-12:Ac (1:1) all attracted three different species. In some cases the attracted species occurred in different habitats (e.g., *Ancylis geninana*, wet; *Ancylis uncella*, dry) or flew at different times of the year (e.g., *Cydia strobilella* and *Cydia pactolana*). In other cases, however, species can be found together in one trap at the same time, for example, *Ancylis uncella* and *Rhopobota ustomaculana*, or *Cydia coniferana*, *C. cosmophorana*, and *C. zebeana*.

Although it is likely that the attractancy and specificity of several sex attractants could be improved by further screening, it remains difficult to find blends that are very specific for certain species. For example, various blends have been tested for the oriental fruit moth, *Grapholitha molesta*, but none is really selective. The simple blends Z8-12:Ac + E8-12:Ac (98:2-95:5) that are very attractive to *G. molesta* attract as many as 20 other species mainly belonging to the genera *Pammene*, *Grapholitha*, *Epiblema*, and *Chephasia* (Chambon d'Aguilar, 1974; Sziraki, 1978; Alford, 1978).

Another problem is that males of some species are attracted to several different blends. This is not surprising if these blends differ only slightly, for example in their E-Z ratio. In other cases, however, one species may be attracted to quite different blends. In our study, for example, *Gypsonoma dealbana* was attracted strongly to E10-12:Ac and to E9-12:Ac (9:1). Although it is possible that our E10-12:Ac contained a little E9-12:Ac or vice versa, it seems more likely that two different attractants are involved. One could attribute this phenomenon to parapheromones (sex attractants that have features strongly in common with the natural pheromone even though they are not chemically the same) or to genetic polymorphism with genotypes producing (females) or responding to (males) different compounds. Such a polymorphism has been found in the grey larch bud moth, Zeiraphera diniana (Baltensweiler et al., 1978). Finally one may consider the possible existence of sibling species which have not been recognized as separate species thus far.

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STRUCTURE-ACTIVITY RELATIONSHIP OF UNSATURATED FATTY ACIDS AS MOSQUITO[1] OVIPOSITIONAL REPELLENTS

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Abstract—Various straight-chain unsaturated fatty acids from C_{14} to C_{24} were evaluated for their ovipositional repellency against gravid females of the southern house mosquito *Culex quinquefasciatus* Say, and the relationship between the structures of the fatty acids and their ovipositional repellency was determined. A double bond with *Z* configuration was prerequisite for an unsaturated fatty acid to be highly repellent; *E* isomers were less active or even inactive. No relationship was found between the repellency and the number of double bonds in the unsaturated fatty acids. In C_{18} monounsaturated fatty acids, (*Z*)-9 acid was more active than (*Z*)-11 and (*Z*)-6 acids, indicating that a double bond at the 9 position rendered an acid highly repellent. Among (*Z*)-9-alkenoic acids of different chain lengths, the most repellent was C_{18} acid which was also more active than (*Z*)-11-C_{20}, (*Z*)-13-C_{22}, and (*Z*)-15-C_{24} acids. Oleic[(*Z*)-9-octadecenoic] acid, which met all these criteria, was the most ovipositionally repellent among the unsaturated fatty acids tested.

Key Words—Unsaturated fatty acids, mosquito ovipositional repellents, *Culex quinquefasciatus*, Diptera, Culicidae, structure–activity relationship, oleic acid, stereoisomerism.

INTRODUCTION

Microbial fermentation of organic matter sometimes produces breakdown products which exhibit ovipositional attractancy or repellency toward mosquitoes (Kramer and Mulla, 1979). Chemical studies on organic infusions resulted in isolating and identifying acetic, propionic, isobutyric, butyric, isovaleric, and caproic acids as oviposition repellents for *Culex quinque-*

[1]Diptera: Culicidae.

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fasciatus Say (Hwang et al., 1980). The repellency of these lower aliphatic carboxylic acids was quantitatively studied against *Culex*, *Culiseta*, *Aedes*, and *Anopheles* mosquitoes (Kramer et al., 1980). Systematic studies on the ovipositional repellency of higher straight-chain aliphatic carboxylic acids revealed that octanoic, nonanoic, and decanoic acids were as active as, or more active than, their lower homologs against *C. quinquefasciatus*, *C. tarsalis* Coquillett, and *Aedes aegypti* L. (Hwang et al., 1982). Based on this information, octanoic and nonanoic acids were evaluated for their efficacy in repelling ovipositing *Culex* mosquitoes under semifield and field conditions (Schultz et al., 1982). Octanoic acid at 15 ppm was found to be effective in preventing mosquitoes from oviposition under semifield conditions; nonanoic acid produced complete ovipositional repellency for 1 day at 25 ppm, 2 days at 50 ppm, 5 days at 75 ppm, and 6–7 days at 150 ppm under field conditions.

During the course of our studies on oviposition-modifying substances produced by microbial fermentation of an infusion of chicken manure, we isolated and identified a series of homologous saturated and unsaturated fatty acids from C_9 to C_{18} by combined gas chromatography–mass spectrometry. Preliminary evaluation in laboratory olfactometers showed that saturated fatty acids were inactive, whereas some straight-chain unsaturated fatty acids were highly repellent to ovipositing mosquitoes. Further studies on higher saturated and unsaturated fatty acids were therefore carried out to correlate the structures of the acids and their ovipositional repellency. Here we report the structure–activity relationship of straight-chain unsaturated fatty acids from C_{14} to C_{24} as ovipositional repellents for mosquitoes.

METHODS AND MATERIALS

All chemicals (Table 1) were of 99% purity and obtained from Supelco, Inc. (Bellefonte, Pennsylvania). They were used without further purification; however, extreme care was taken to avoid unnecessary exposure of unsaturated fatty acids in the air above 10° C before bioassay tests. In describing stereoisomerism about a double bond in unsaturated fatty acids, the descriptors *Z* and *E* (Blackwood et al., 1968) are used in this paper; however, the traditional configurational descriptors *cis* and *trans* are also used to denote *Z* and *E*, respectively, for convenience.

C. quinquefasciatus was used in bioassay tests. Maintenance and handling of this mosquito were described by Kramer and Mulla (1979).

The bioassay procedure reported by Kramer and Mulla (1979) was followed. Briefly, each olfactometer unit consisted of a 1-liter polystyrene food cup and two glass Stender dishes. An aqueous suspension (4 ml) of a fatty acid, which was prepared by adding an acetone solution of an acid in

TABLE 1. UNSATURATED FATTY ACIDS BIOASSAYED FOR OVIPOSITIONAL REPELLENCY AGAINST *C. quinquefasciatus*

No.	Acid	Carbon atoms	Position and configuration of double bond
1	Myristoleic[(*Z*)-9-tetradecenoic]	14	9-*cis*
2	Palmitoleic[(*Z*)-9-hexadecenoic]	16	9-*cis*
3	Palmitelaidic[(*E*)-9-hexadecenoic]	16	9-*trans*
4	Oleic[(*Z*)]-9-octadecenoic]	18	9-*cis*
5	Elaidic[(*E*)-9-octadecenoic]	18	9-*trans*
6	Petroselinic[(*Z*)-6-octadecenoic]	18	6-*cis*
7	*cis*-Vaccenic[(*Z*)-11-octadecenoic]	18	11-*cis*
8	*trans*-Vaccenic[(*E*)-11-octadecenoic]	18	11-*trans*
9	Linoleic[(*Z,Z*)-9,12-octadienoic]	18	9-*cis*,12-*cis*
10	Linolelaidic[(*E,E*)-9-12-octadecadienoic	18	9-*trans*,12-*trans*
11	Linolenic[(*Z,Z,Z*)-9,12,15-octadecatrienoic]	18	9-*cis*,12-*cis*,15-*cis*
12	γ-Linolenic[*Z,Z,Z*)-6,9,12-octadecatrienoic]	18	6-*cis*,9-*cis*,12-*cis*
13	(*Z*)-11-Eicosenoic	20	11-*cis*
14	Homo-γ-linolenic[(*Z,Z,Z*)-8,11,14-eicosatrienoic]	20	8-*cis*,11-*cis*,14-*cis*
15	Arachidonic[(*Z,Z,Z,Z*)-5,8,11,14-eicosatetraenoic	20	5-*cis*,8-*cis*,11-*cis* 14-*cis*
16	Erucic[(*Z*)-13-docosenoic]	22	13-*cis*
17	(*Z,Z,Z,Z,Z,Z*)-4,7,10,13,16,19-docosahexaenoic	22	4-*cis*, 7-*cis*, 10-*cis* 13-*cis*, 16-*cis*, 19-*cis*
18	Nervonic[(*Z*)-15-tetracosenoic]	24	15-*cis*

water, was placed in a dish, and distilled water (4 ml) was placed in the other as control. The dishes were set side by side, 3.5 cm apart, and the food cup was inverted over them. The olfactometer was ventilated by applying vacuum (50 ml/min) from a glass tubing attached to the top of the unit. Five gravid mosquitoes were introduced into the olfactometer, and the results of oviposition were recorded 24 hr later. All tests were replicated at least eight times. The ovipositional activity is expressed as ovipositional activity index (*OAI*) and calculated according to the following formula:

$$OAI = \frac{N_T - N_S}{N_T = N_S}$$

wherein N_T represents the mean number of egg rafts in a treated sample, and N_S represents that in the control. All index values fall withing the range of +1 to −1. A negative *OAI* value indicates that more ovipositions took place in the

control than in the treated dish, thus signifying the latter to be repellent. The results were analyzed by the chi-square analysis to determine the significance of all indices.

RESULTS AND DISCUSSION

In discussing the relationship between the repellency and the structures of the unsaturated fatty acids, the following criteria were taken into consideration: (1) the geometric isomerism about the double bond, (2) the number of double bonds, (3) the position of the double bond, and (4) the chain length of the fatty acids.

The *OAI* values of the unsaturated fatty acids obtained in these studies are listed in Table 2. The importance of double-bond stereoisomerism in influencing the repellency of the fatty acids was best demonstrated by oleic acid (**4**) and its *trans* isomer, elaidic acid (**5**). Acid **4** with *cis* configuration was one of the most repellent among those evaluated, showing significant repellency even at 10^{-5} M, whereas acid **5** was not active at all concentrations used. A similar relationship was found between palmitoleic (**2**) and palmitelaidic (**3**) acids, between *cis*-vaccenic (**7**) and *trans*-vaccenic (**8**) acids, and between linoleic (**9**) and linolelaidic (**10**) acids. All *cis* acids were highly repellent, but all *trans* acids were inactive or weakly repellent. Of the saturated fatty acids (C_{15}, C_{16}, C_{17}, C_{18}, C_{19}, C_{20}, C_{21}, and C_{22}) tested, none showed any ovipositional repellency against gravid females at the 1×10^{-3} through 1×10^{-6} M concentrations. Furthermore, 9-octadecynoic acid, a C_{18} acid with a triple bond at the 9 position, did not show any repellency at the same concentrations. It is thus apparent that a double bond with *cis* configuration is required for an unsaturated fatty acid to be highly ovipositionally repellent against *C. quinquefasciatus*.

Oleic (**4**), linoleic (**9**), and linolenic (**11**) acids are all C_{18} acids and have 1, 2, and 3 double bonds, respectively; for the latter two acids, the double bonds are nonconjugated. An increase in the number of double bonds did not increase the repellency; oleic acid (**4**) was significantly repellent at 1×10^{-3}, 1×10^{-4}, and 1×10^{-5} M, and linoleic acid (**9**) and linolenic acid (**11**) showed significant repellency only at 1×10^{-3} and 1×10^{-4} M. γ-Linolenic acid (**12**), an isomer of linolenic acid having three double bonds at the 6, 9, and 12 positions instead of at the 9, 12, and 15 positions, however, was almost as repellent as oleic acid (**4**). (*Z*)-11-Eicosenoic (**13**), homo-γ-linolenic (**14**), and arachidonic (**15**) acids are C_{20} acids and possess 1, 3, and 4 double bonds, respectively. They all showed repellency at the 1×10^{-3} and 1×10^{-4} M concentrations; however, homo-γ-linolenic acid (**14**) was less active than acids **13** and **15**. The C_{22} unsaturated fatty acids, erucic (**16**) and (*Z*,*Z*,*Z*,*Z*,*Z*,*Z*)-4,7,10,13,16,19-docosahexaenoic (**17**) acids were both repellent at the 1×10^{-3}

TABLE 2. *OAI* OF UNSATURATED FATTY ACIDS AGAINST *C. quinquefasciatus*

No.	Acid	*OAI* ± SE[a] Concentration (M) 1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}
1	Myristoleic	−0.92 ± 0.08**	−0.03 ± 0.18	−0.03 ± 0.18	−0.16 ± 0.20
2	Palmitoleic	−0.87 ± 0.16**	−0.65 ± 0.08**	+0.22 ± 0.24	+0.18 ± 0.15
3	Palmitelaidic	−0.31 ± 0.19	−0.38 ± 0.14*	+0.04 ± 0.16	−0.06 ± 0.15
4	Oleic	−0.87 ± 0.06**	−0.91 ± 0.07**	−0.34 ± 0.09**	+0.02 ± 0.15
5	Elaidic	+0.07 ± 0.15	−0.08 ± 0.22	+0.29 ± 0.16	+0.09 ± 0.29
6	Petroselinic	−0.71 ± 0.13**	−0.29 ± 0.13	−0.14 ± 0.15	+0.29 ± 0.11
7	*cis*-Vaccenic	−0.87 ± 0.10**	−0.88 ± 0.06**	−0.12 ± 0.18	0 ± 0.16
8	*trans*-Vaccenic	−0.09 ± 0.17	+0.03 ± 0.18	−0.24 ± 0.12	0 ± 0.24
9	Linoleic	−0.86 ± 0.11**	−0.77 ± 0.10**	+0.16 ± 0.18	−0.29 ± 0.13
10	Linolelaidic	−0.04 ± 0.14	−0.50 ± 0.18*	−0.08 ± 0.30	−0.05 ± 0.22
11	Linolenic	−0.93 ± 0.02**	−0.81 ± 0.12**	+0.11 ± 0.15	+0.07 ± 0.18
12	γ-Linolenic	−1.00 ± 0**	−0.85 ± 0.12**	−0.35 ± 0.15*	−0.09 ± 0.07
13	(*Z*)-11-Eicosenic	−0.87 ± 0.08**	−0.94 ± 0.08**	−0.19 ± 0.15	+0.13 ± 0.18
14	*Homo*-γ-linolenic	−0.92 ± 0.08**	−0.57 ± 0.16**	−0.21 ± 0.24	+0.03 ± 0.24
15	Arachidonic	−0.83 ± 0.12**	−1.00 ± 0**	−0.13 ± 0.26	+0.06 ± 0.10
16	Erucic	−0.50 ± 0.16**	−0.47 ± 0.15*	−0.18 ± 0.28	+0.05 ± 0.24
17	(*Z*,*Z*,*Z*,*Z*,*Z*,*Z*)-4,7,10,13,16,19-Docosahexaenoic	−0.92 ± 0.06**	−0.62 ± 0.16**	−0.21 ± 0.14	+0.39 ± 0.15
18	Nervonic	−0.10 ± 0.19	+0.19 ± 0.20	+0.15 ± 0.13	+0.19 ± 0.20

[a]Positive *OAI* values indicate attractancy, and negative *OAI* values indicate repellency. *Significant from control at $P < 0.05$; **significant from control at $P < 0.01$.

and 1×10^{-4} M concentrations despite the former being monounsaturated and the latter being polyunsaturated. These comparisons indicated that there was no correlation between the repellency and the number of double bonds in the acids. The monounsaturated fatty acids were as active as, or more active than, the polyunsaturated fatty acids.

Of the three (*Z*)-octadecenoic acids, oleic acid (**4**, 9-*cis*) was more repellent than *cis*-vaccenic acid (**7**, 11-*cis*) which, in turn, was more repellent that petroselinic acid (**6**, 6-*cis*). Although no other data concerning the positions of double bonds were available, it seemed that a *cis* double bond at C_9 was essential in attaining a high ovipositional repellency in C_{18} fatty acids. Linoleic acid (**9**) has two 9-*cis* and 12-*cis* double bonds, and linolenic acid (**11**) has an additional 15-*cis* double bond. The additional 15-*cis* double bond did not render linolenic acid (**11**)more active than linoleic acid (**9**). However, γ-linolenic acid (**12**), which has a 6-*cis* double bond in addition to two 9-*cis* and 12-*cis* double bonds, was more repellent than linolenic acid (**11**) and as repellent as oleic acid (**4**). Therefore, the 6-*cis* double bond may contribute to repellency in C_{18} fatty acid.

(*Z*)-9-Alkenoic acids were typical examples in depicting the effect of chain length on the repellency. Myristoleic acid (**1**, 9-*cis*-C_{14}) was active only at 1×10^{-3} M, and palmitoleic acid (**2**, 9-*cis*-C_{16}) was active at 1×10^{-3} and 1×10^{-4} M. However, their higher homolog, oleic acid (**4**, 9-*cis*-C_{18}) was active even at 1×10^{-5} M. Thus, when the double bond was fixed at the 9 position and the carbon chain elongated from the ω end, the oviposition repellency of the unsaturated fatty acids would increase as the chain length increased with C_{18} acid being the most repellent. When the carbon chain elongated from the α end and the double bond shifted toward the ω end, the repellency would gradually decrease. Oleic acid (**4**, 9-*cis*-C_{18}) was highly repellent, but (*Z*)-11-eicosenoic acid (**13**, 11-*cis*-C_{20}) was less active. The repellency decreased further in erucic acid (**16**, 13-*cis*-C_{22}) and disappeared in nervonic acid (**18**, 15-*cis*-C_{24}).

Among the unsaturated fatty acids available to us, oleic acid, which seemed to meet all the criteria discussed above, was the most ovipositionally repellent to gravid females. On the basis of molar concentration, the repellency of oleic acid against *C. quinquefasciatus* even surpassed that of nonanoic acid previously reported (Hwang et al., 1982). The evaluation of oleic acid as an ovipositional repellent for *Culex* mosquitoes under field conditions is currently underway and will be reported elsewhere.

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SEX PHEROMONE OF A CONIFER-FEEDING BUDWORM, *Choristoneura retiniana*, Walsingham[1]

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Abstract—Sex pheromone components collected from female *Choristoneura retiniana* included 11-tetradecenyl acetates and alcohols. The major component was *E*-11-tetradecenyl acetate (E11-14:Ac) with a lesser amount of *Z* isomer necessary to induce male response. A 92:8 ratio of *E,Z*11-14:Ac appeared optimal. The alcohol component was present at about 10% of the total pheromone mixture, and traps baited with acetates plus alcohol surpassed unmated females in their degree of attractiveness. Chemical analysis indicated a 9:1 ratio of the *E*-*Z* isomers of 11-tetradecenyl alcohol (11-14:ol) pheromone components, although bait formulations containing a predominance of either the *E* or *Z* isomers were equally successful in field bioassays. Based on male response to traps, *E*- and *Z*11-14:Ac (92:8 *E*-*Z*) are essential pheromone components for long-range sex attraction. The 11-14:ol enhanced attraction when added at 10% of the total pheromone blend.

Key Words—*Choristoneura retiniana*, Lepidoptera, Tortricidae, sex pheromone, sex attractant, forset insects, Tortricidae, insect behavior, *E*- and *Z*-11-tetradecenyl acetates, *E*- and *Z*-11-tetradecenol.

INTRODUCTION

Found primarily in the Cascade Range of southern Oregon and the Warner Mountains and Sierras of California, *Choristoneura retiniana* Walsing-

[1]This paper reports the results of research only. Mention of a commercial product does not constitute a recommendation by the U.S. Department of Agriculture.

ham (= *C. viridis* Freeman) feeds on *Abies*, being principally associated with *A. concolor* (Gord. & Glend.) Lindl. ex Hildebr. (Powell, 1980). Freeman (1967) described this defoliator as a new species, but in a review of the nomenclature of conifer-feeding *Christoneura*, Powell (1980) established priority for *C. retiniana* based on a specimen described in 1871 by Walsingham (1879). The adults of this species vary considerably in color patterns (Freeman, 1967; Powell, 1980), and some specimens can be confused with other *Choristoneura*. For this reason, the field aspects of our study were conducted in an area containing only one other *Choristoneura*, the pine-feeding *C. lambertiana subretiniana* Obraztsov, a species which can be visually separated from *C. retiniana*.

Sanders et al. (1974) reported *E*-11-tetradecenyl acetate (*E*11-14:Ac) as a sex attractant for males of this species. In recent 1979 and 1980 field tests, however, the *E*11-14:Ac was generally ineffective and live-female baits were at least 10 times more attractive than the synthetic preparations. This study was undertaken to clarify the reasons for this apparent inconsistency and to more completely define the *C. retiniana* pheromone system.

METHODS AND MATERIALS

Fifth-generation laboratory-cultured *C. retiniana* females were subjected to pheromone collection procedures. The original insects were obtained from the Gearhart Mountain area about 18 km north of Bly, Oregon. Following analysis of pheromone collections, blends of synthetic components were compared for their relative attractiveness. Bioassays were conducted in the field based on the response of male moths to baited traps.

Pheromone Collections. Three methods were used for collecting pheromone. The first involved dipping or washing extruded abdominal tips in hexane. The specific techniques for this gland-washing method were reported by Sower et al. (1973). Prior to the hexane wash, the females were conditioned in a 16:8 light–dark regime during the pupal stage and for 2 days following adult emergence. Washings for pheromone collection took place on the second day 14–15 hr after the onset of illumination.

The second method incorporated the use of Porapak-Q® columns for collecting the pheromone emitted by females. Freshly emerged female moths in groups of 8–10 were placed in a 200-ml glass chamber. Compressed air was filtered through a Porapak-Q column, fed into the base of the chamber at a constant rate of 250 ml/min, and routed out the top through a second Porapak-Q column which trapped the pheromone. The amount of Porapak-Q used to absorb the pheromone was approximately 1 g. The moths were held in this collection system for 48 hours at a temperature of 21–2°C and a light regime of 16 hr light and 8 hr dark. The outflow Porapak-Q col-

umn was then eluted with about 2 ml of hexane. The glass chamber and columns were treated with silanizing reagent and thoroughly washed with methanol and hexane before use.

The third method of extraction was simply to soak excised female tips in hexane. All extracts were held in a freezer at −50°C until chemical analysis was performed.

Chemical Analyses. Female extracts were reduced in volume by evaporation at room temperature under a slow stream of nitrogen to a volume of ca. 50 μl and analyzed as follows.

Glass capillary column gas chromatography enabled clear separations of *E* and *Z* stereoisomers of pheromone constituents at levels as low as 0.5 ng/injection. A Hewlett-Packard 5880A gas chromatograph equipped with an open, tubular, wall-coated glass capillary column (60 m × 0.5 mm ID, J. & W. Scientific) with SP2340 liquid phase was used. The capillary injection system was operated in the splitless mode with inlet purging 30 sec after injection. Injector and detector temperatures were 200°C; the oven temperature was held at 90°C initially for 2 min, then temperature programed at a rate of 7°/min to a final value of 200°C. Carrier gas flow (helium) was 50 ml/min.

A Varian Aerograph 2700 gas chromatograph equipped with a glass column (2 m × 2 mm ID) packed with 1.5% OV-101 on 100- to 200-mesh Gas Chrom Q® was operated isothermally at 170°C. Injector and detector temperatures were 250°C. The instrument was interfaced with a Dupont 21-491B mass spectrometer equipped with a four-channel DuPont MSID accessory for selected ion monitoring. The following characteristic fragment ions were monitored: *m/e* 194 and *m/e* 166 (ions common to both 11-14:Ac and 11-14:ol). Unlike the capillary GC system, the *E* and *Z* isomers were not separable on the packed column.

Bioassay Procedures. Following identifications of possible pheromone components, synthetic blends were formulated into polyvinyl chloride (PVC) controlled-release bait pellets (Daterman, 1974). The relative attractiveness of these baits was evaluated in the field by comparing the numbers of male moths captured in traps baited with the different blends. Live females were also compared for attractiveness with selected blends of synthetics.

Traps used in these evaluations were triangular in shape, open at both ends, lined with an adhesive, and had a capture capacity of at least 65 *C. retiniana* males. Trap placement was randomized with individual traps spaced at least 25 m from any other trap. All traps were hung on the ends of host tree branches 2–2.5 m above the ground surface and left in the field for only 24–48 hr in order to minimize possible trap saturation effects. Synthetic baits were all 5-mm long × 3-mm diameter and contained either 0.01 or 0.1% pheromone components by weight. These baits were suspended from the interior center of the traps on insect pins. Based on the findings of Cory

et al. (1982), we estimated the 0.01% bait pellets would approximate the pheromone release rate of live females. Female moths used as bait were 12–48 hr old when placed in the field and were confined within small cylinders (25 × 75 mm) of fiberglass screen, which were positioned in the center of the traps on cardboard platforms.

The test area was located in Klamath County, Oregon, about 35 km north of Klamath Falls. Host trees made up about 50% of the stand composition, and population density of the insect was low with little or no obvious evidence of defoliation.

RESULTS AND DISCUSSION

Chemical Analyses. Analyses of the pheromone isolates obtained from *C. retiniana* females showed the presence of *E*- and *Z*-11-tetradecenyl acetates in a ratio of about 92 *E* to 8 *Z*. Also observed were the *E* and *Z* isomers of 11-tetradecenyl alcohol (11–14:ol). The alcohols occur at about 10% of the amount of the corresponding acetates, although the amounts detected in different samples varied from 0 to 20%.

The *E*:*Z* ratio of the acetate isomers was relatively consistent among samples (Table 1), and we consider the 92:8 *E*–*Z* proportions s representative. The ratio of *E* and *Z* stereoisomers of the alcohol was difficult to determine since the concentrations in extracts were often near the detection limit. The available data (Table 1) suggest that the *E*–*Z* isomer ratio of the alcohol was about 90:10; i.e., probably identical to the ratio of the acetate isomers.

None of our pheromone collections showed any evidence of 11-tetradecenyl aldehydes (11-14:Al), although *E*- or *Z*11-14:Al are known as

TABLE 1. SEX PHEROMONE COMPONENTS IDENTIFIED FROM *C. retiniana* FEMALES

		11-14:Ac		11-14:ol	
Collection method	No. females	*E*:*Z*	ng/♀	*E*:*Z*	ng/♀
Hexane soak	114	92:8	11	[a]	2
	33	93:7	19	[a]	1
Hexane wash	30	93:7	20	90:10	2
	33	92:8	21	90:10	2
	17	88:12	[a]	[b]	
Air collection	24	92:8	11	[b]	
	24	92:8	11	[b]	

[a]Not determined.
[b]Not detected.

major pheromone components of other conifer-feeding *Choristoneura* spp. (Cory et al., 1982; Silk et al., 1982; Weatherston et al., 1971; Sanders et al., 1974; Werner and Weatherston, 1980). The European fir budworm, *C. murinana* (Hb.), uses *Z*9–12:Ac in its pheromone blend (Bogenschütz, 1979; Priesner et al., 1980) but we found no evidence of C_{12} or C_{14} compounds with unsaturation at the 9–10 position in *C. retiniana*. The pheromone of the oblique-banded leafroller moth, *C. rosaceana* (Harris), is somewhat similar to that of *C. retiniana* in that both are composed of major 11–14:Ac and minor 11–14:ol components (Hill and Roelofs, 1979).

The first field bioassay emphasized evaluation of the attractiveness of the optimal blend indicated by chemical analysis and of the previously reported attractant, *E*11–14:Ac. The bait treatments and test results are listed in Table 2. This test confirmed that a combination of 11–14:Ac and 11–14:ol, with the *E* isomer of the acetate the principal component (Table 2), provided a very potent attractant for male *C. retiniana*. Although a previous study reported *E*11–14:Ac alone as a sex attractant for *C. retiniana* (Sanders et al., 1974), our nearly pure bait preparation of that component alone or in combination with 11–14:ol proved to be unsuccessful (Table 2). We suspect that the prior successful trapping (Sanders et al., 1974) was probably due to small proportions of other pheromone components present as contaminants in their supply of *E*11–14:Ac.

Because the results of the chemical analysis (Table 1) showed variation in the *E*–*Z* isomer ratio of *E*11–14:Ac, a trapping test was designed to evaluate the attractiveness of five different blends of the *E*–*Z* isomers of the acetate component. The results of this test (Table 3) demonstrated two points. First, that the 92:8 *E*–*Z* ratio caught significantly higher numbers than all other preparations with the exception of the 88:12 *E*–*Z* mixture (Table 3), which tends to verify the 92:8 *E*–*Z* ratio as being very close to the natural

TABLE 2. *C. retiniana* MALES CAPTURED BY TRAPS BAITED WITH LIVE FEMALES OR SYNTHETIC BLENDS FORMULATED IN PVC at 0.1% ACTIVE INGREDIENT

Bait[a]	Average moths/trap[b]	Range
90% 11-14: Ac (92:8 *E*-*Z*) + 10% 11-14:ol (9:1 *E*-*Z*)	43.0a	34–48
Live female	23.6b	7–44
95% *E*11-14:Ac + 5% 11-14:ol (9:1 *E*-*Z*)	1.7c	0–8
100% *E*11-14:Ac	0.7c	0–3
Unbaited check	0.0c	0

[a]Contains less than 0.5% opposite isomers as contaminants; 10 trap replications per bait treatment.

[b]Means followed by same letters not significantly different at 0.05% level of probability [analysis of variance and Tukey's test for significant differences among means (Snedecor, 1956)].

TABLE 3. *C. retiniana* MALES CAPTURED BY TRAPS BAITED WITH VARYING BLENDS OF *E* AND *Z*11-14:Ac FORMULATED IN PVC AT 0.1% ACTIVE INGREDIENT

Bait[a]	Average males/trap [b]	Range
90% 11-14:Ac (92:8 *E-Z*) + 10% 11-14:ol (9:1 *E-Z*)	13.3a	7-22
90% 11-14:Ac (88:12 *E-Z*) + 10% 11-14:ol (9:1 *E-Z*)	8.4ab	1-17
90% 11-14:Ac (84:16 *E-Z*) + 10% 11-14:ol (9:1 *E-Z*)	7.4b	1-15
90% 11-14:Ac (98:2 *E-Z*) + 10% 11-14:ol (9:1 *E-Z*)	7.3b	3-12
90% 11-14:Ac (80:20 *E-Z*) + 10% 11-14:ol (9:1 *E-Z*)	3.6b	0-8

[a]Ten trap replications per bait treatment.
[b]Means followed by same letters are not significantly different from one another at the same level of probability (Snedecor, 1956).

pheromone. Secondly, even the mixture containing only 2% *Z* isomer attracted substantial numbers of males, a fact which lends further credence to the notion that earlier data (Sanders et al., 1974) showing *E*11-14:Ac alone as a potent sex attractant, probably occurred because of the presence of a small amount of *Z*11-14:Ac as a contaminant.

The third bioassay emphasized comparison of female attraction to synthetic baits containing different alcohol components and a total pheromone concentration (.01%) which would approximate the release rate of females. Even at the lowered concentration, the synthetic blend of 90% 11-14:Ac (92:8 *E-Z*) and 10% 11-14:ol was significantly more attractive than live females (Table 4). Further, the synthetic bait containing only acetates was

TABLE 4. *C. retiniana* MALES CAPTURED BY TRAPS WITH SYNTHETIC BAITS CONTAINING DIFFERENT RATIOS OF 11-TETRADECENYL ACETATES, ALCOHOLS, AND THEIR *E, Z*-ISOMERS FORMULATED IN PVC AT 0.01% ACTIVE INGREDIENT

Bait[a]	Average males/trap[b]	Range
90% 11-14:Ac (92:8 *E-Z*) + 10% *Z*11-14:ol	16.7a	10-22
90% 11-14:Ac (92:8 *E-Z*) + 10% 11-14:ol (9:1 *E-Z*)	15.7a	10-21
90% 11-14:Ac (92:8 *E-Z*) + 10% *E*11-14:ol	10.6ab	2-18
94% 11-14:Ac (92:8 *E-Z*) + 6% 11-14:ol (9:1 *E-Z*)	9.7ab	4-16
100% 11-14:Ac (92:8 (*E-Z*)	7.4b	3-13
Unmated *C. retiniana* female	7.6b	2-20

[a]Pheromone blend components contain less than 0.5% opposite isomers as contaminants; seven trap replications per bait.
[b]Means followed by same letters are not significantly different from one another at the 5% level of probability [analysis of variance and Tukey's test for significant differences among means (Snedecor, 1956)]

TABLE 5. *C. retiniana* MALES CAPTURED BY TRAPS BAITED WITH SYNTHETIC BLENDS LACKING AN ALCOHOL COMPONENT OR CONTAINING 10% ALCOHOL WITH A PREDOMINANCE OF EITHER *E* OR *Z* ISOMER OF 11-14:ol

Bait[a]	Average males/trap[b]	Range
90% 11-14:Ac (92:8 *E-Z*) + 10% 11-14:ol (9:1 *E-Z*)	12.5a	7-17
90% 11-14:Ac (92:8 *E-Z*) + 10% 11-14:ol (1:9 *E-Z*)	10.6a	7-16
100% 11-14:Ac (92:8 *E-Z*) (no alcohol)	6.4b	3-11

[a]Eight trap replications per bait treatment.
[b]Means followed by same letters are not significantly different from one another at the 5% level of probability (Snedecor, 1956).

as effective as live females, and synthetic preparations containing alcohols trended toward higher capture numbers. The two most effective synthetic preparations contained a predominance of either the *E* or *Z* isomer of 11-14:ol (Table 4). We believe these data support the suggestion that alcohol as a minor component will enhance attraction. The lure preparation that contained a lower proportion of alcohol (6%), with the expectation that the higher release rate of the alcohol from PVC (Daterman, 1982) would more closely approximate the emission rate of females, was not more effective than the preparations containing 10% alcohol (Table 4).

A final bioassay to further evaluate the effects of the alcohol on attraction confirmed that the addition of a 10% 11-14:ol component did enhance attraction and optimize numbers of trapped moths (Table 5). Surprisingly, there was no significant difference in effectiveness between baits containing a predominance of either the *E* or *Z* isomers of 11-14:ol, and both were significantly more attractive than the preparation containing only the acetates.

SUMMARY

Chemical analyses indicated the sex pheromone of *C. retiniana* to be a blend of 11-14:Ac and 11-14:ol with *E-Z* isomer proportions of 92:8. The acetate was the principal component, comprising 90% of the total pheromone material. Field tests with baited traps confirmed that *E*11-14:Ac and *Z*11-14:Ac were necessary elements of the pheromone for male attraction, with the presence of the *Z* isomer required at about 8% of the total acetate. The addition of 10% 11-14:ol significantly improved attraction. This increase in effectiveness occurred when either the *E* or *Z* isomer of 11-14:ol predominated in the synthetic blend. Synthetic preparations formulated to approximate the release rate of females equalled or surpassed the numbers of males

captured in female-baited traps. We conclude that the 92:8 *E,Z*11–14:Ac is essential for long-range attraction of *C. retiniana* males and that the addition of 10% 11–14:ol, regardless of isomeric configuration, will further enhance attraction.

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INTERACTIONS OF TEMPERATURE AND FERULIC ACID STRESS ON GRAIN SORGHUM AND SOYBEANS

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Abstract—Experiments were conducted to test the hypothesis that allelopathic effects of ferulic acid (FA) may be altered by the temperature conditions of the growth environment. Growth of grain sorghum and soybean seedlings over a 10-day treatment period showed that a significant interaction effect occurred between environmental temperatures and FA treatments. Sorghum grown with an average day temperature of 37°C and soybeans grown at 34°C had greater dry weight reductions caused by FA than when the respective environments were 8°C and 11°C lower. The threshold concentration for inhibition of sorghum growth was 0.2 mM FA under the hot conditions and 0.4 mM FA with the cooler conditions. Soybeans were more sensitive than sorghum, and these inhibition thresholds for the hot and cool environments were 0.1 and 0.25 mM FA. These results demonstrate that temperature stress enhances allelochemical inhibition and indicate that interactions with the environment are an important consideration for understanding allelopathy.

Key Words—Ferulic acid, allelopathy, temperature stress, sorghum, soybean.

INTRODUCTION

Biochemical interference among plants (allelopathy) occurs in a variety of plant communities, and it is apparent that many plants can contribute toxins to the environment (Rice, 1974, 1979). The biological activity of allelochemicals depends on the presence of sensitive plant species, the degree of toxicity of the specific substances, the combination of allelochemicals present, and their residence time. Aspects of the environment are also important in allelopathy. Moisture conditions and soil type influence the availability

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and persistence of allelochemicals (Patrick et al., 1964; Wang et al., 1971; McCalla and Norstadt, 1974). The level of synthesis and accumulation of allelochemicals in donor plants may be raised by mineral deficiencies, temperature stress, ultraviolet light, and osmotic stress (Koeppe et al., 1969, 1970; Armstrong et al., 1970; del Moral, 1972; Lehman and Rice, 1972). However, many other environmental interactions have not been investigated.

Benzoic and cinnamic acids are frequently implicated in allelopathy. Vanillic, *p*-hydroxybenzoic, *p*-coumaric, caffeic, and ferulic acids are common ones identified in soil extractions where allelopathy is investigated. Quantities in the rhizosphere are extremely variable, with amounts reported for specific compounds ranging from less than 10 ppm to over 1000 ppm (Whitehead, 1964; Guenzi and McCalla, 1966; Wang et al., 1967; Lodhi, 1975, 1978; Turner and Rice, 1975; Chou and Patrick, 1976). The extent of soil binding and inactivation of phenolic acids also remains a complex unknown. Therefore, a persistent question in allelopathy has been whether the small amounts of allelochemicals that are often reported can be functional in regulation of germination and growth.

Part of the rationale supporting the regulatory role of small quantities of allelochemicals is that several different substances often act in cooperative ways (Rasmussen and Einhellig, 1977, 1979; Einhellig and Rasmussen, 1978; Einhellig et al., 1982; Williams and Hoagland, 1982). Allelopathic effects may also be viewed as a type of stress. Thus, it is plausible that additional environmental conditions may be important dimensions that modify growth interference from allelochemicals. Stowe and Osborn (1980) found that nitrogen and phosphorus availability influenced phytotoxicity of vanillic and *p*-coumaric acids. We have observed that under greenhouse conditions with adequate nutrients, there is considerable variation in response of bioassay plants to treatments with a known allelochemical.

The conjecture of these experiments was that when an allelochemical is near its threshold for inhibition of seedling growth, temperature of the growth environment would alter the allelopathic effect. High temperatures, yet growth temperatures still within the normal range of tolerance, create some stress on plant metabolism. Experiments were designed to determine if temperature stress would interact with and alter allelochemical effects from ferulic acid.

METHODS AND MATERIALS

Grain sorghum (*Sorghum bicolor* Moench., Dekalb Hybrid DK28) and soybeans [*Glycine max* (L.) Merr. cv. Corsoy] seedlings were used as the test plants in these experiments. Both are crop species which may be subjected to interference from allelochemicals released from weeds or decom-

position of crop residue (Colton and Einhellig, 1980; Schon and Einhellig, 1982; Einhellig and Schon, 1982), and they are sensitive to phenolic acids (Einhellig and Rasmussen, 1978, 1979). Seeds were germinated in vermiculite in the greenhouse, and after 6 days they were individually transplanted to opaque plastic vials containing nutrient solution. Containers for sorghum held 80 ml and those for soybeans held 120 ml. The nutrient solution consisted of 5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 0.9 mM $NH_4H_2PO_4$, 0.1 mM $(NH_4)_2HPO_4$, standard Hoagland's micronutrients (Hoagland and Arnon, 1950), and 72 μM iron supplied as sodium ferric diethylene-triamine pentaacetate (Sequestrene 330).

Seedlings were selected for uniformity after one day of acclimatization and treated with ferulic acid (Sigma Chemical Co.) dissolved in fresh nutrient solution. Ferulic acid (FA) was chosen as an allelochemical because of its common occurrence. Treatment levels for sorghum were 0, 0.1, 0.2, and 0.4 mM FA, and soybean treatments were 0, 0.1, and 0.25 mM FA. Each treatment utilized 24–30 seedlings which were divided into two subgroups with one subgroup placed in a higher temperature environment than the other.

Different greenhouses were utilized to obtain the two temperature environments for grain sorghum experiments. This allowed one environment to have a temperature stress while both retained full-sun growth conditions. The hot environment averaged 8°C above the cool from 1000 to 2000 hr, with 37°C (34–41) the mean (and range) for the hot environment, compared to 29°C (29–33) for the cooler environment. Night temperatures (2200–800 hr) averaged 20°C (18–23) and 18°C (16–19), respectively. These were the averages obtained by taking hourly temperatures during the 10-day treatment period for sorghum. The two environments also had some differences in humidity. The average day–night relative humidity for the hot environment was 24 and 56%, and it was 41 and 64% for the cooler environment. Soybeans were injured by the hot environment used for sorghum, and temperatures used with soybeans were lower. Soybean experiments were conducted in matched growth chambers using a 16:8 hr light–dark cycle with a light intensity of 300 $\mu E/sec/m^2$ (Lamba Instruments, L1-170 Quantum/Radiometer/Photometer). The accompanying temperature cycles during the light and dark were 34 and 29°C and 23 and 14°C, respectively, for the hot and cool environments. The chambers had no regulation of relative humidity and the averages were 40 and 53% and 63 and 73%.

The original treatment solution was replenished on the fifth day after treatment and abaxial leaf diffusive resistance was taken on this day using a Li-Cor model LI-1600 steady-state porometer. Abaxial resistances were obtained from the largest leaf of sorghum and from soybean unifoliates. Experiments were terminated after 10 days of treatment. At harvest, sorghum plants were measured for width of the largest leaf and base to tip shoot and

root lengths. Sorghum shoot and root dry weights were obtained after 48 hr at 104°C and shoot–root ratios were calculated. Soybean harvest consisted of taking the leaf area and dry weight for each seedling. Data of each experiment were analyzed by two-way analysis of variance (ANOVA). Within each environment a one-way ANOVA with Duncan's multiple-range test was applied. Each experiment was duplicated.

RESULTS

Sorghum seedlings treated with FA in the two temperature environments exhibited several differences in appearance and in dry weights by the end of the experiments. The overall comparison of temperature effects using the two-way ANOVA showed that plants grown under the hot conditions had significantly lower root weight and total plant weight, resulting in a higher shoot-root ratio (Table 1). The two-way ANOVA summation of FA effects across the two environments showed that the FA treatments caused significant alterations in dry matter accumulation in roots, shoots, and the total plant. Interaction effects between temperature and FA treatments also occurred. All sorghum weights and the shoot–root ratio showed a significant interaction of these two variables. Since the stress variables had a significant interaction on sorghum, further analysis of the effects of FA within each temperature was logical.

Ferulic acid inhibition of growth in each of the two temperature regimes was verified using a one-way ANOVA with Duncan's multiple-range test (Table 1). However, perhaps the most important observation concerning effects on dry weight is that seen by comparing FA inhibition in the hot regime to inhibitory effects under the cooler temperatures. This comparison demonstrates that the 0.2 mM FA treatment significantly reduced root, shoot, and total plant weights below the corresponding control with hot growth conditions, but the 0.2 mM FA treatment had no effect with cooler conditions. While 0.4 mM FA treatments inhibited seedling dry weights in both environments, these reductions were considerably greater with higher temperatures. Dry weights of sorghum grown in the hot regime with 0.4 mM FA were also significantly reduced below the inhibition caused by 0.2 mM FA. These comparisons were similar in the duplicate experiment (not shown).

Visual differences between sorghum seedlings grown in the two temperatures were evident. The shoots of seedlings grown with the hot conditions had a darker green color and the leaves were more narrow. This temperature effect on leaf width was verified by the significant probability obtained using the two-way ANOVA (Table 2). Plants in the hot environment also had a reduced root length and an increase in diffusive resistance when compared to those in the cool environment. A consideration of FA treatments irrespec-

TABLE 1. INTERACTION OF TEMPERATURE AND FERULIC ACID (FA) STRESS ON SORGHUM SEEDLINGS

Treatment	Dry wt[a] *in mg* ± SE (and % of control)			Ratio shoot–root
	Root	Shoot	Plant	
Hot[b] (37°C)				
Control	80.2 ± 4.5a	145.6 ± 8.3a	225.8 ± 12.1a	1.82 ± .06a
0.1 mM FA	81.9 ± 7.2a (102.1)	150.0 ± 16.9a (103.0)	231.9 ± 24.0a (102.7)	1.83 ± .05a
0.2 mM FA	66.4 ± 3.9b (82.8)	84.2 ± 5.5b (57.8)	150.6 ± 9.3b (66.7)	1.27 ± .02b
0.4 mM FA	26.0 ± 2.9c (32.4)	49.2 ± 4.1c (33.8)	75.2 ± 6.7c (33.3)	1.89 ± .14a
Cool (29°C)				
Control	102.7 ± 4.1a	123.5 ± 6.3a	226.2 ± 8.9a	1.20 ± .03a
0.1 mM FA	103.6 ± 5.4a (100.9)	116.6 ± 9.2a (94.4)	220.2 ± 14.5a (97.3)	1.12 ± .04ab
0.2 mM FA	109.0 ± 6.1a (106.1)	116.9 ± 7.3a (94.7)	225.9 ± 13.4a (99.9)	1.07 ± .03b
0.4 mM FA	72.6 ± 4.0b (70.7)	79.3 ± 4.8b (64.2)	151.9 ± 8.6b (67.2)	1.09 ± .02b
	Two-way ANOVA *F* value (and probability)			
Temperature	89.2 (0.0001)	0.1 (0.7621)	13.7 (0.0004)	224.9 (0.0001)
FA	33.8 (0.0001)	29.8 (0.0001)	31.5 (0.0001)	18.6 (0.0001)
Temp–FA	3.4 (0.0204)	8.2 (0.0001)	6.7 (0.0005)	13.9 (0.0001)

[a]Column means within a temperature regime that are not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.
[b]Day temperature mean. See text for details.

tive of temperature conditions demonstrated that FA affected diffusive resistance and each of the morphological parameters, as shown by the two-way ANOVA. Interaction effects between temperature and FA were significant for both root and shoot length.

Ferulic acid effects on seedling morphology occurred in both temperatures, but definite differences are apparent when FA treatments under the hot environment are compared to those under the cool (Table 2). These differences in morphology parallel effects found on dry weight. In the hot environment, 0.2 mM FA-treated seedlings had significantly reduced leaf width, root length, and shoot length below the corresponding control, but

TABLE 2. INTERACTION OF TEMPERATURE AND FERULIC ACID (FA) STRESS ON SORGHUM SEEDLING MORPHOLOGY AND DIFFUSIVE RESISTANCE[a]

Treatment	Day 5 abaxial resistance (sec/cm)	Leaf width (mm)	Root length (cm)	Shoot length (cm)
Hot (37°C)				
Control	2.1 ± 0.2a	13.9 ± 0.5a	18.9 ± 0.6a	24.0 ± 0.8a
0.1 mM FA	2.7 ± 0.3ab	13.3 ± 0.5a	17.7 ± 0.9a	24.4 ± 2.0a
0.2 mM FA	3.6 ± 0.4b	11.9 ± 0.6b	14.5 ± 1.0b	17.6 ± 1.1b
0.4 mM FA	3.7 ± 0.9b	8.6 ± 0.3c	7.6 ± 1.4c	8.9 ± 1.2c
Cool (29°C)				
Control	1.3 ± 0.2a	18.5 ± 0.5a	21.3 ± 0.9a	21.5 ± 0.8a
0.1 mM FA	2.5 ± 0.2b	18.2 ± 0.6a	20.0 ± 0.4a	20.0 ± 1.2a
0.2 mM FA	2.1 ± 0.1bc	17.1 ± 0.6a	21.0 ± 0.6a	21.0 ± 0.8a
0.4 mM FA	2.9 ± 0.2c	15.7 ± 0.7b	16.8 ± 0.4b	14.7 ± 0.7b
	Two-way ANOVA F value (and probability)			
Temperature	17.40 (0.0001)	188.62 (0.0001)	83.43 (0.0001)	0.80 (0.3767)
FA	11.91 (0.0001)	22.67 (0.0001)	37.70 (0.0001)	40.50 (0.0001)
Temp–FA	2.40 (0.0766)	2.45 (0.0671)	8.67 (0.0001)	9.72 (0.0001)

[a]Column means within a temperature regime that are not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.

this inhibition did not occur with the cooler temperature. In both temperatures, 0.4 mM FA reduced these morphological features, but effects were greater in the hot environment. In contrast, an increase in diffusive resistance was measured for all FA treatment levels under cool conditions, but only 0.2 and 0.4 mM FA caused an increase in resistance in the hot environment.

The general response of soybean seedlings to FA and temperature stresses used in these studies was similar to that found with sorghum. The two-way ANOVA probabilities demonstrated that both temperature and FA treatment had significant effects on leaf area and plant dry weight (Table 3). There was also a significant temperature–FA interaction on soybean leaf area and plant weight. Abaxial leaf resistance taken on day 5 was significantly higher under the hot regime, and a significant probability was also obtained from comparing all FA treatments without regard to the tempera-

TABLE 3. INTERACTION OF TEMPERATURE AND FERULIC ACID (FA) STRESS ON SOYBEAN SEEDLINGS[a]

Treatment	Day 5 abaxial resistance[b] (sec/cm)	Leaf area[b] (cm^2)	Dry weight[b] (mg)
Hot[c] (34°C)			
Control	3.5 ± 0.5a	70.9 ± 2.1a	410.0 ± 7.7a
0.1 mM FA	2.7 ± 0.2ab	71.0 ± 1.8a	349.0 ± 11.8b
		(100.2)	(85.1)
0.25 mM FA	2.1 ± 0.1b	40.2 ± 2.2b	259.4 ± 8.4c
		(56.7)	(63.3)
Cool (23°C)			
Control	1.3 ± 0.1a	52.6 ± 2.4a	393.5 ± 11.8a
0.1 mM FA	1.4 ± 0.1a	50.0 ± 1.8a	392.5 ± 12.7a
		(95.10)	(99.7)
0.25 mM FA	1.4 ± 0.1a	36.4 ± 2.6b	328.1 ± 14.8b
		(69.2)	(83.4)
	Two-way ANOVA F value (and probability)		
Temperature	59.6	63.4	11.7
	(0.0001)	(0.0001)	(0.0010)
FA	4.4	73.04	47.21
	(0.0175)	(0.0001)	(0.0001)
Temp–FA	6.6	8.85	7.3
	(0.0028)	(0.0004)	(0.0012)

[a]Values are the mean ± SE (and % of control).
[b]Column means within a temperature regime that are not followed by the same letter are significantly different, $P < 0.05$, ANOVA with Duncan's multiple-range test.
[c]Day temperature mean. See text for details.

ture. The interaction of the two stresses was further studied by comparisons of analyses within each temperature.

Soybeans grown at a day temperature of 34°C were significantly reduced in dry weight by 0.1 mM FA and plants grown with 0.25 mM FA had even lower weight (Table 3). In contrast, 0.1 mM FA did not alter the dry weight of soybeans at 23°C. Only the 0.25 mM FA treatment reduced growth in this temperature. At harvest, soybean leaf areas of the 0.25 mM FA groups were below the controls in both temperatures. Ferulic acid did not alter diffusive leaf resistance when soybeans were grown at 23°C, whereas at 34°C the 0.25 mM FA plants had a significant reduction in diffusive resistance. Data from the replicate experiment (not shown) were similar to effects shown in Table 3.

DISCUSSION

The data support the hypothesis that allelopathic effects of FA may be altered by environmental temperatures. The threshold for FA inhibition of seedling growth was significantly lower when plants were grown at temperatures near the higher end of their range of tolerance. Two previous reports have suggested an interaction of allelochemical inhibition with temperature. Glass (1976) noted that the effects of a mixture of phenolic acids on excised barley roots varied under several temperature regimes. Steinsiek et al. (1982) reported that the inhibitory action of extracts from wheat on weed-seed germination and growth was temperature-dependent, and several of the weeds they tested had the greatest inhibition at 35°C. Thus, allelopathic inhibition is closely tied with this aspect of the environment.

While FA-induced inhibition of both grain sorghum and soybean seedling growth was enhanced by temperature stress, there were some differences in response between the two test species. Inhibition of soybean seedlings was achieved by lower FA treatment levels than with sorghum, and soybeans were stressed by lower temperatures. In concert with temperature stress, 0.1 mM FA reduced soybean growth, while twice this concentration was required for inhibition of sorghum. These results illustrate that a combination of allelopathic and environmental conditions may alter growth of one species but not affect another. Such a differential in response to allelochemical action among species fosters allelopathic regulation in plant communities.

Grain sorghum seedlings treated with FA had an elevated diffusive resistance in all treatments except one. This stomatal effect was found at the lowest levels of FA treatment in the cool environment even though these treatments did not alter growth. Ferulic acid alteration of stomatal function reinforces previous work, suggesting one mechanism of allelopathic action may be an interference with plant water balance (Einhellig and Muth, 1980; Einhellig and Schon, 1982). However, the soybean plants in the hot environment that were inhibited in growth most strongly by FA had a decrease in diffusive resistance. This opposite response was unexpected, but it may have resulted from the interrelationship with heat stress. It is likely that both FA and temperature stress modify several different aspects of metabolism.

The fact that the action of an allelochemical such as FA is modified by another factor of the growth environment is extremely important for understanding allelopathy. Certainly several environmental variables influence plant growth, and it is seldom that all growth conditions are at their optimum. Other stresses, such as the temperature conditions, are a likely occurrence accompanying the presence of allelochemicals. The results of this study demonstrate that temperature stress enhances allelochemical inhibition, thus the magnitude of allelopathic action is directly related to the total conditions. A consideration of such interactions, along with the fact that

several different allelochemicals may be acting together, further clarifies the process of allelopathy under field conditions.

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DEMONSTRATION OF SEX PHEROMONES IN CADDISFLIES (TRICHOPTERA)

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Abstract—Field tests with live, caged females and whole-body extracts of females of the caddisfly *Gumaga griseola* (McLachlan) (Trichoptera: Sericostomatidae) demonstrate, for the first time, the existence of a chemically mediated sexual communication system in this insect order. Both live females and extracts are significantly more attractive to conspecific males than either empty control traps or traps baited with *Gumaga nigricula* (McLachlan) females. Anatomical structures suggest that semiochemicals are widespread in Trichoptera.

Key Words—Aquatic insects, attractant, caddisfly, *Gumaga*, odor, pheromone, scent, Sericostomatidae, sex pheromone, Trichoptera.

INTRODUCTION

Pheromones have been described for species in at least eight insect orders (Birch, 1974), but not the Trichoptera. Exocrine secretions have been reported for caddisflies, but they are thought to have defensive, not sexual, functions (Duffield et al., 1977; Duffield, 1981). Since sexual pheromones, elaborate scent organs (hair pencils, coremata), and well-developed mating behavior (female calling, male lek formation) are found throughout the closely related Lepidoptera (Willis and Birch 1982), we presumed that a similar chemically mediated sexual communication system should exist in Trichoptera.

There have been persistent reports of detectable odors among several caddisfly species (Kimmins, 1956; Benz, 1975), and Moretti and Bicchierai (1981) have described morphological features that they presumed were scent glands. Recently, Kelner-Pillault (1975) demonstrated experimentally that caged, wingless females of the terrestrial caddisfly *Enoicyla pusilla* Burm. (Limnephilidae) attracted males that were several meters away. However,

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neither Kelner-Pillaut nor, apparently, others have established that the nature of mating between caddisflies is chemically mediated. This paper presents the results of field experiments designed to determine if caged, virgin females of the sericostomatid caddisfly *Gumaga griseola* (McLachlan) attracted males, and if so, whether extractable chemicals were involved in this attraction.

METHODS AND MATERIALS

Field trials were conducted along Las Trampas Creek (Contra Costa County, California). Virgin *G. griseola* and *Gumaga nigricula* (McLachlan) adults were reared in the laboratory from pupae collected from Las Trampas Creek and Beasley Flat Spring (Mendocino County, California), respectively. Since only *G. griseola* occurs at the field site, trials with the congeneric *G. nigricula* females were used to test for interspecific attractivity.

For live-organism field trials, females from each species were placed separately into cylindrical screened cages (30 × 40 mm). One or two newly emerged, live, virgin females were placed into each cage; the cages were then placed into a commercial sticky trap (Pherocon 1C, manufactured by Zoecon Corporation, Palo Alto, California).

Extracts from whole, virgin *G. griseola* females were used in the chemical trials. For each extract (one per trap), five live females were placed into a glass vial with 2 ml of dichloromethane; after 1 hr, the bodies were removed and discarded. A rubber septum was then infused with the extract and stored at 0° C until used.

Each field trial consisted of both treatment traps and a control trap; the latter contained no females, or only the dichloromethane solvent. Since field tests were conducted as fresh specimens from rearings became available, the number of treatments and number of traps within a treatment varied among the five test nights (June 16–July 2, 1982). A total of 60 traps were used for the field trials.

The traps were placed in the field at dusk, not less than 10 m apart, under bridges or in vegetation along the stream course, and retrieved the following morning. The number and sex of all caddisflies captured were recorded at the end of each test. Data were transformed to $\sqrt{x + 0.5}$, and a t test, or analysis of variance and Dunnett's test (Dunnett, 1955), were used to compare the different treatment results with those of the control.

RESULTS

Five conclusions were apparent from the results of these field tests. First, caged *G. griseola* females attract conspecific males in significantly higher

TABLE 1. MEAN NUMBER OF *G. griseola* MALES CAPTURED/TRAP IN PHEROMONE FIELD TESTS CONDUCTED DURING 1982 AT LAS TRAMPAS CREEK (CONTRA COSTA COUNTY, CALIFORNIA)[a]

Treatment	Number of caged females/trap	16 June	17 June	20 June	23 June	2 July
Control	0	0(3)	0(3)	0.25(4)	0(1)	0(1)
G. griseola	1–2	7*(2)	10*(6)	12.2*(10)	35.5*(2)	NT
G. griseola extract	0	NT	NT	4.5*(2)	0.7*(3)	2.2*(5)
G. nigricula	1–2	NT	0.3(3)	0(5)	0(5)	NT

[a]Asterisks denote mean values of treatments significantly different from mean values of controls at $\alpha = 0.05$; NT = not tested. Number of traps is given in parentheses.

numbers than occurred in the control traps (Table 1). Second, in five traps tested with caged male *G. griseola*, neither males nor females were attracted. Third, caged *G. nigricula* females were no more attractive to *G. griseola* males than were the empty control traps (Table 1). Fourth, although males were more attracted to caged, live females than to whole-body extracts, these extracts attracted significantly more males than did the control traps (Table 1). Fifth, we observed that females were not attracted to either caged females or whole-body extracts.

DISCUSSION

This study is the first demonstration of a chemically mediated sexual communication system in the insect order Trichoptera. We have been unable to identify the specific glands in *Gumaga* associated with pheromone production, although like most caddisflies, *Gumaga* adults have well-defined exocrine glands on the fifth abdominal sternite. However, similar structures in the limnephilid caddisfly *Pycnopsyche scabripennis* (Rambur) were recently found to be defensive glands (Duffield et al., 1977). In addition to early reports of odors, Cummings (1914), Eltringham (1919), Mosely (1919, 1922, 1923), and Betten (1934) long ago described eversible organs on the head, thorax, or abdomen of caddisfly adults that are apparently similar to the coremata (air-filled tubes covered with hairs) found in Lepidoptera. Recent anatomical studies of caddisfly adults (Moretti and Bicchierai 1981, Roemhild 1980) also indicate that androconial organs, which are commonly referred to as scent or sex pheromone glands, are widely distributed throughout the order. If these structures are actually part of a pheromone system, their widespread presence would indicate that semiochemicals are used by many caddisfly species.

Since pheromones are common among Lepidoptera species, it is not sur-

prising that the closely related Trichoptera possess a similar chemical communication system. In fact, males of the philopotamid caddisfly *Dolophilodes novusamericana* (Ling) have been taken during two successive years in pheromone traps baited with two synthetic lepidopterous pheromones (94% 93:7/*E*:*Z*-11-tetradecenyl acetate and 6% 92:8/*E*:*Z*-11-tetradecenyl alcohol, and 100% 92:8/*E*:*Z*-11-tetradecenyl aldehyde), whereas no caddisflies were taken in control traps.

Studies of chemical communication systems can aid in our understanding of the reproductive biology of aquatic insects, but they may also increase our understanding of commensalistic (Esch and Coggins, 1974), predator-prey (Peckarsky, 1982), and other types of relationships. For example, systematic studies of *Gumaga* are now using pheromone response to compliment studies of larval morphology (Resh et al., 1981), egg-mass shape (Wood et al., 1982), and habitat occurrence for more complete species discrimination. Future studies of *Gumaga* pheromones will concentrate on characterizing the chemical compounds, site of production, and receptor organs used in eliciting this response.

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RESPONSE OF THE CLERID PREDATOR *Thanasimus dubius* (F.) TO BARK BEETLE PHEROMONES AND TREE VOLATILES[1] IN A WIND TUNNEL

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Abstract—Tree volatiles and pheromones produced by southern bark beetles were bioassayed for response by the clerid predator *Thanasimus dubius* (F.). Upwind flights in a laboratory olfactometer, modified from Visser (1976), were used to determine the attractiveness of compounds. Differences in response to a solvent control and pheromone treatment were tested for statistical significance using the Wilcoxon signed ranks test. Both sexes of *T. dubius* responded to frontalin, ipsdienol, and α-pinene in a dose-dependent manner with different but overlapping concentration ranges. Strong differences between the sexes were observed in response to *trans*-verbenol, verbenone, and *l*-β-pinene. Neither sex responded to ipsenol or *endo*-brevicomin.

Key Words—Coleoptera, Cleridae, *Thanasimus dubius*, beetle, predation, frontalin, ipsdienol, α-pinene, southern pine beetle, *Ips* spp., Scolytidae, ipsenol, *endo*-brevicomin, kairomone.

INTRODUCTION

Thanasimus dubius (F.) (Coleoptera: Cleridae) is a common predator of bark beetles infesting southern pines (Fronk, 1947; Vité et al., 1964; Thatcher and Pickard, 1966; Mignot and Anderson, 1969; Berisford, 1973). Adults and larvae of *T. dubius* feed on one or more life stages of southern bark beetles (Thatcher and Pickard, 1966; Mignot and Anderson, 1969).

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Vité and Williamson (1970) found that *T. dubius* responded in a balanced sex ratio to frontalin, an aggregating pheromone of the southern pine beetle (SPB) (Vité and Renwick, 1968). Simultaneous release of frontalin at rates of approximately 5 μl/hr and 50 μl/hr led to a 10-fold increase in trap catch for the higher concentration. No difference was found in response by *T. dubius* to frontalin alone or in combination with tree oleoresins. Response to frontalin was higher than to either *trans*-verbenol or verbenone at the concentrations tested. Verbenone elicited a low response which was predominately from males. Rose et al. (1981) reported trapping both sexes of *T. dubius* in response to a mixture of SPB and *Ips* spp. pheromones in east Texas.

Use of *Ips* spp. pheromones as kairomones was recorded in field tests for *T. formacarius* (L.) and *T. femoralis* (Zett.) in Europe by Bakke and Kvamme (1981). Bedard et al. (1980) reported that *Temnochila chlorodia* (Mannerheim), (Coleoptera: Trogositidae) used a kairomone to locate its prey, the western pine beetle, *D. brevicomis* LeConte, in the western United States.

While several bark beetle-produced chemicals have been tested in the field, (Vité et al., 1964, 1972; Vité and Renwick, 1968, 1971b; Kinzer et al., 1969; Vité and Williamson, 1970; Payne, 1970; Renwick and Vité, 1972; Werner, 1972a,b; Rudinsky, 1973; Renwick et al., 1976; Hedden et al., 1976; Payne et al., 1978; Dixon and Payne, 1980), no laboratory investigation of the response by *T. dubius* to single chemicals associated with SPB or *Ips* spp. has been reported. This study was initiated to investigate the response of *T. dubius* to compounds known to be produced during the attack and colonization of trees by SPB and *Ips* spp.

METHODS AND MATERIALS

Insects. Adult *T. dubius* collected in the field and/or reared as described by Nebeker et al. (1980) were maintained individually in the laboratory on a diet of SPB and *Ips* spp. They were held in a growth chamber at 25–28°C, 30–40% relative humidity and a 14:10 light–dark cycle. They were starved 24–72 hr prior to testing. Some insects were used repeatedly, but never exposed to treatments more frequently than once (5 min) in 24 hr.

Chemicals. The chemicals tested were known pheromones of SPB, *I. avulsus* Eichhoff, *I. calligraphus* Germar, and *I. grandicollis* Eichhoff or tree volatiles previously determined to be important in the behavior of *T. dubius* or its bark beetle hosts (Table 1). All chemicals were obtained from Chem. Samp. Co., Columbus, Ohio. Pentane was used as a solvent and served as the control. Dilutions were prepared serially to produced various concentrations of each chemical tested.

Bioassay. A low-speed, laminar-flow wind tunnel modified from Visser (1976) was used for the bioassay. It consisted of an all-glass test chamber $1.2 \times 0.5 \times 0.5$ m, through which purified room air of $27 \pm 2°$C was passed

TABLE 1. OPTICAL PURITY AND CONCENTRATIONS OF CHEMICALS BIOASSAYED FOR RESPONSE BY *Thanasimus dubius* (F.) IN LABORATORY OLFACTOMETER

Chemical	Chemical purity (%)[a]	Concentrations tested ($\log_{10}$) μg/ml
Frontalin	99	−5, −4, −3, −2, −1, 1, 3, 4, 5
Ipsdienol	94	−2, 1, 3, 4
Ipsenol	93	−2, 1, 4
trans-Verbenol	93	−2, 1, 4
Verbenone	93	−2, 1, 2, 3, 3.5, 4
α-Pinene	98	−2, 1, 3, 3.5, 4, 4.5
l-β-Pinene	98	1, 3, 3.5, 4, 4.5, 5
endo-Brevicomin	93	1, 4

[a]Purity determined by manufacturer's label, ChemSampCo, Columbus, Ohio.

at 5.4 m/sec. Spent air was exhausted from the building. Light from 6 F40D fluorescent bulbs located 28 cm above the test section was diffused by a plastic diffuser. Removable black panels were placed 50 cm from the sides of the test section to shield movements of the observer from the test insects. Tests were conducted in a darkened room between 1330 and 1700 hr. Tests conducted on days when the barometric pressure varied more than 0.3 mm Hg were repeated or deleted.

Insects were tested in groups of 10 individuals of the same sex. They were released from a platform placed at the center of the wind tunnel by allowing them to crawl from a 1-oz condiment cup in the center of the platform. Each replication consisted of observing the responses of 10 individuals during a 5-min exposure to solvent (pentane) followed (30 min later) by a second 5-min exposure to the compound at a particular test concentration. No more than 5 replications of each concentration were completed on any one day, and 10 replicates were tested per concentration. Test chemicals were released from plastic-covered cigarette filters (3 cm long) loaded with 1 ml of solution for each 5-min test. Tested compounds and concentrations were selected at random from a pool containing high, medium, and low concentrations. Subsequent concentrations were selected to bracket those showing activity after the first pool was completed.

Behavioral responses of three types were recorded: initial flights off the cardboard ring, including the number responding and the direction of the response (up-, down-, and crosswind); direction and numbers of flights after leaving the platform; and location of the *T. dubius* in the test section at the end of the test period. Location was scored as either fore (on or within 15 cm of the upwind screen of the arena) or aft (on or within 15 cm of the downwind screen). Differences between response to control and treatments were compared using the Wilcoxon signed ranks tests (one tailed, $P < 0.05$) as contained in the Statistical Package for the Social Sciences (Nie and Hull, 1977).

RESULTS

No pattern was observed in the occurrence of the three recorded behaviors—initial upwind flights (UF), total additional flights, and position in the arena (fore, aft)—of *T. dubius* at the end of the test, when these responses were considered in total. While some significant differences were determined statistically between control and treatment counts for the three types of responses, only UF provided a consistent measure of attraction to different concentrations of the compounds tested. Therefore, we used the frequency of UF to determine positive response (attraction) by *T. dubius*.

Southern Pine Beetle Compounds. UF by male *T. dubius* in response to frontalin were significantly different from the control ($P < 0.05$) for three concentrations: 10^{-4}, 10^{-3}, and 10^{-2} μg/ml, and to 10^{-1} μg/ml for females (Figure 1). The male response to frontalin changed with increasing stimulus concentration from 10^{-4} to 10^{-2} μg/ml with peak response to 10^{-3} μg/ml. UF in response to *trans*-verbenol were significant for males at 10^{-2} μg/ml and for female *T. dubius to* 10^{2} μg/ml and 10^{3} μg/ml (Figure 2). Males responded to verbenone with significant UF to 10^{1} μg/ml, while UF for females were significant in response to both 10 μg/ml ($P = 0.026$) and 10^{1} μg/ml (Figure 3). *endo-*

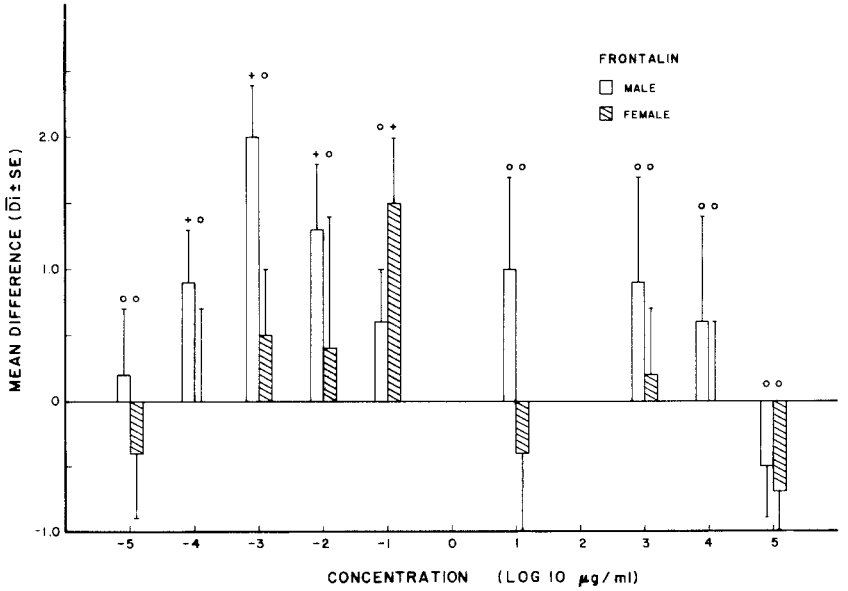

FIG. 1. Mean difference ($\bar{D}_i \pm$ SE = treatment − control) of initial upwind flights by male and female *Thanasimus dubius* (F.) in response to concentrations of frontalin. Significance levels [$P \leq 0.05$ = (+)] as determined by Wilcoxon signed ranks test.

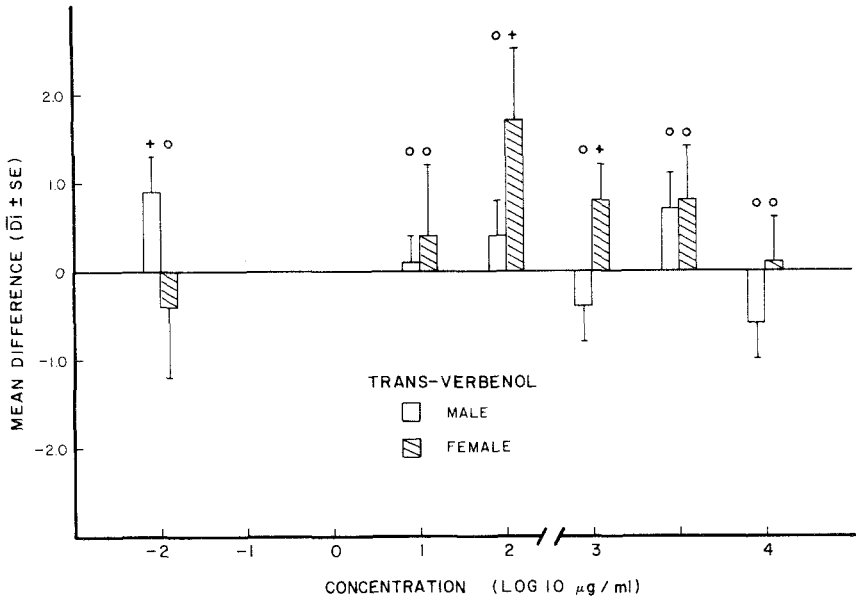

FIG. 2. Mean difference ($\bar{D}_i \pm$ SE = treatment − control) of initial upwind flights by male and female *Thanasimus dubius* (F.) in response to concentrations of *trans*-verbenol. Significance levels [$P \leq 0.05 = (+)$] as determined by the Wilcoxon signed ranks test.

Brevicomin was tested at two concentrations, but no significant UF by either sex of *T. dubius* were observed (Figure 4).

Ips spp. Compounds. Both sexes of *T. dubius* responded to concentrations of ipsdienol (Figure 5). Frequency of UF increased for males with increasing stimulus concentration from 10^1 to 10^4 μg/ml. Peak response was observed for males to 10^4 μg/ml. Females responded similarly to both 10^3 μg/ml and 10^4 μg/ml. Females did not respond to either the 10^{-2} or 10^1 μg/ml concentration. Both sexes of *T. dubius* responded in greater numbers to concentrations of ipsdienol than to concentrations of frontalin that elicited positive response. Three concentrations of ipsenol were tested, but no significant response ($P < 0.05$) in UF was observed (Figure 6).

Tree Volatiles. Response by *T. dubius* to α-pinene was similar for both sexes. UF were highly significant in response to $10^{3.5}$ μg/ml ($P = 0.011$), 10^4 μg/ml, and $10^{4.5}$ μg/ml (Figure 7). Frequency of UF for females peaked at $10^{3.5}$ μg/ml and decreased with increasing stimulus concentration. No significant response by either sex to three concentrations lower than $10^{3.5}$ μg/ml was observed.

Male and female *T. dubius* responded significantly to *l*-β-pinene (Figure

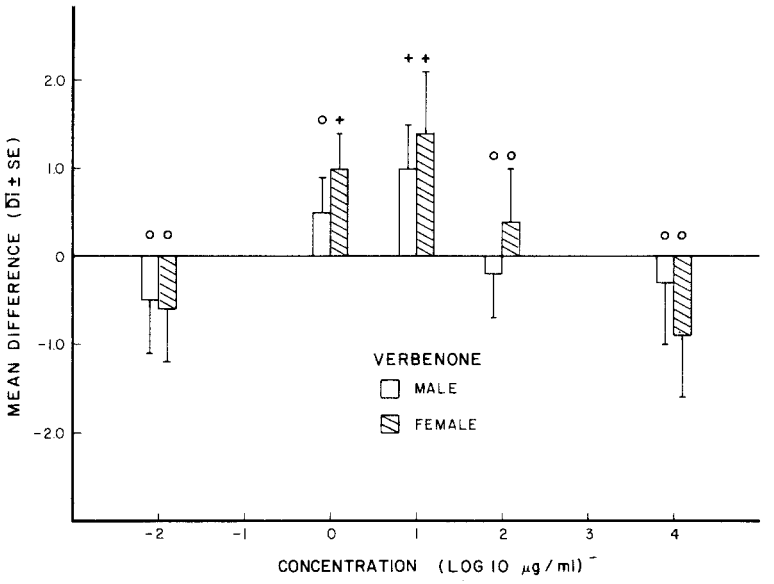

FIG. 3. Mean difference ($\bar{D}_i \pm$ SE = treatment − control) of initial upwind flights by male and female *Thanasimus dubius* (F.) in response to concentrations of verbenone. Significance levels [$P \leqslant 0.05 = (+)$] as determined by the Wilcoxon signed ranks test.

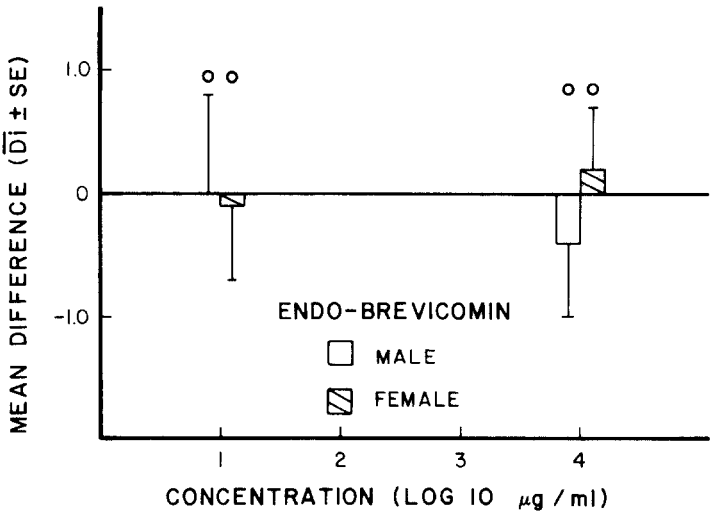

FIG. 4. Mean difference ($\bar{D}_i \pm$ SE = treatment − control) of initial upwind flights by male and female *Thanasimus dubius* (F.) in response to concentrations of *endo*-brevicomin. No significant response ($P < 0.05$) as determined by Wilcoxon signed ranks test.

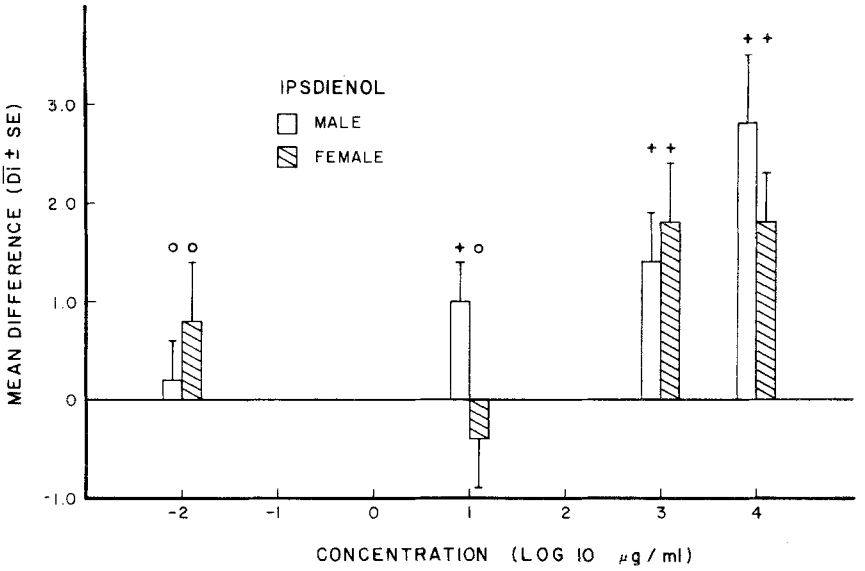

FIG. 5. Mean difference ($\bar{D}_i \pm$ SE = treatment − control) of initial upwind flights by male and female *Thanasimus dubius* (F.) in response to concentrations of ipsdienol. Significance levels [$P \leqslant 0.05$ = (+)] as determined by Wilcoxon signed ranks test.

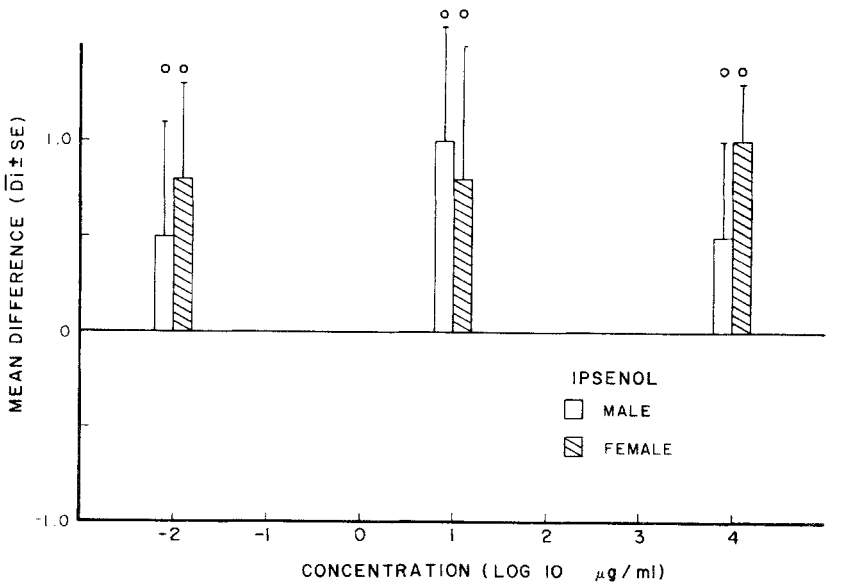

FIG. 6. Mean difference ($\bar{D}_i \pm$ SE = treatment − control) of initial upwind flights by male and female *Thanasimus dubius* (F.) in response to concentrations of ipsenol. No significant response ($P \leqslant 0.05$) as determined by Wilcoxon signed ranks test.

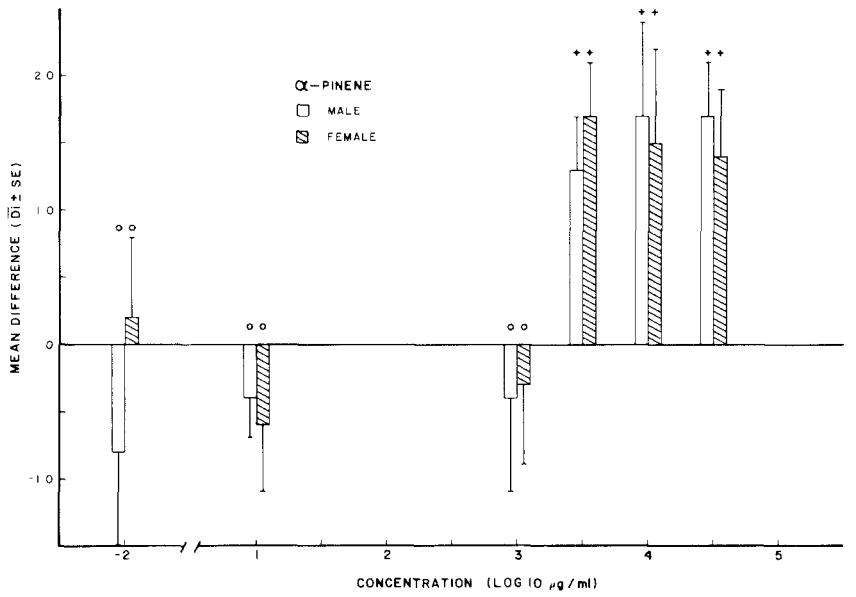

FIG. 7. Mean difference ($\bar{D}_i \pm$ SE = treatment − control) of initial upwind flights by male and female *Thanasimus dubius* (F.) in response to concentrations of α-pinene. Significance [$P \leqslant 0.05 = (+)$] as determined by Wilcoxon signed ranks test.

8). Male UF were highest to $10^{3.5}$ μg/ml and decreased with increasing stimulus concentration. The lack of female response to 10^4 μg/ml cannot be explained. UF by female *T. dubius* were significant to only one concentration: $10^{4.5}$ μg/ml ($P = 0.014$).

DISCUSSION

Results from this study agree with the response by *T. dubius* to pheromones in the field reported by Vité and Williamson (1970). We observed upwind response flights to several concentrations of frontalin, *trans*-verbenol, and verbenone by both sexes of *T. dubius*. While Vité and Williamson (1970) did not observe an increase in response to test pheromones when released together with tree volatiles, we observed response by *T. dubius* to both α- and *l*-β-pinene when tested individually. It appears that adult *T. dubius* can use a variety of compounds to find newly attacked host trees, choose their bark beetle prey, and perhaps respond to changes in prey availability mediated by bark beetle kairomones.

Ipsdienol is the primary attractant produced by *I. avulsus* and also serves as a component of the pheromone of *I. calligraphus* (Renwick and Vité, 1972;

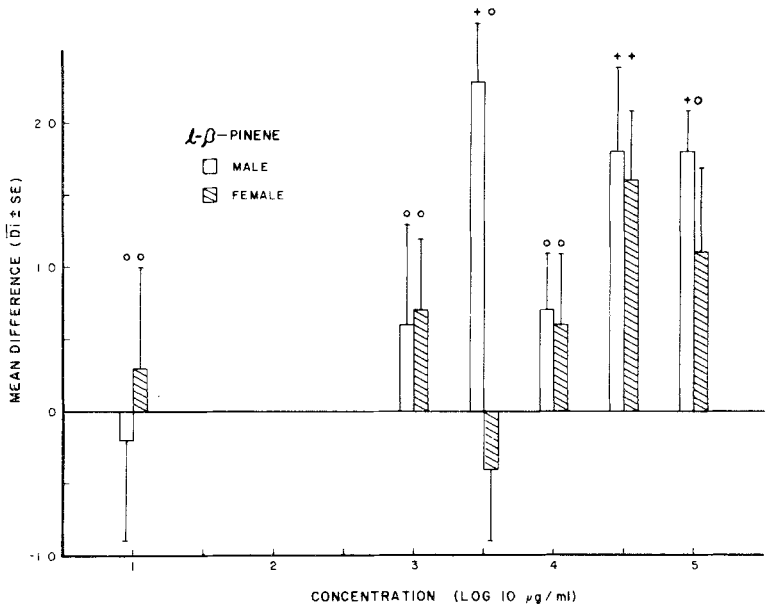

FIG. 8. Mean difference ($\bar{D}_i \pm$ SE = treatment − control) of initial upwind flights by male and female *Thanasimus dubius* (F.) in response to concentrations of *l*-β-pinene. Significance levels [$P \leq 0.05$ = (+)] as determined by the Wilcoxon signed ranks test.

Hedden et al., 1976). Ipsenol is the attractant pheromone of *I. grandicollis* (Vité and Renwick, 1971a). Positive response by *T. dubius* in this study is the first demonstration of response by *T. dubius* to single pheromones produced by *Ips* spp. These findings suggest that the predator can locate *Ips* spp. infestations in the field as well as SPB infestations. The authors have often observed *T. dubius* adults on trees infested only with *Ips* spp. in both Mississippi and Georgia.

Mason (1970) has shown that the attack pattern of *I. avulsus* is the most aggregated of the three *Ips* species, although less aggregated than the mass attack of SPB. Timely response by *T. dubius* to trees attacked by *I. avulsus* would therefore provide the predator with a greater food resource/unit time in comparison to the rate gained as a result of response to ipsenol produced by *I. grandicollis*.

While no response to ipsenol was observed, *T. dubius* could also prey upon *I. grandicollis* by responding to ipsdienol or frontalin. Birch et al. (1980) have shown that all the species of southern pine bark beetles often attack pines together and release their pheromones simultaneously. This occurrence would offer *T. dubius* a variety of compounds to use in locating host trees of its prey and also present the predator with a choice of different sized prey to capture. Mizell et al. (1981) have shown that *T. dubius* prefer the smallest

species, *I. avulsus*, in the laboratory when offered a choice of SPB, *I. avulsus*, and *Callosobruchus maculatus* (L.).

Kudon and Berisford (1980) and Berisford (1981) have presented experimental data and a conceptual model to explain the behavior of hymenopterous parasites with respect to preference for host and host kairomones and to changes in the relative density of SPB and *Ips* spp. in the southeastern United States. Their data suggest the preference of parasites for the kairomones of the bark beetle species from which they emerge (Kudon and Berisford, 1980). They further suggest that host preferences for the kairomones of the beetle host from which they emerge as adults is retained only if that host is abundant. Failing to find the beetle species from which it emerged, the parasite switches to the more abundant host present (*Ips* spp. → SPB, SPB → *Ips* spp., etc.) through its ability to respond to the kairomones of several host species. The next generation emerges with a preference, in the adult, for the kairomones of the host from which it emerged (Berisford, 1981).

While we did not determine the preferences of *T. dubius* for the kairomones of *Ips* spp. or SPB, our data suggest the ability of this predator to react in the system in a similar manner as do parasites. This response may be mediated by the hunger level of the predator (see Mizell, 1981). The ability to locate, feed, and oviposit upon a variety of prey species whose relative densities fluctuate greatly in space and time would be as advantageous to *T. dubius* as it is to the parasite.

There may have been some differences in the results if we had tested combinations of chemicals. This and the question of kairomone preference remain the subject of future experimentation.

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Book Review

Insect Pheromones. Studies in Biology No. 147. M.C. Birch and K.F. Haynes. Edward Arnold (Publishers) Limited, London and Baltimore, 1982. $8.95, 58 pp.

Birch and Haynes have written an excellent short treatise on insect pheromones. They have succeeded in meeting the expectations of their publishers to provide, for students and teachers of general biology, an authoritative and modern view of significant developments in biology, giving attention to methods, practical applications, and further reading. The authors have not limited their treatment to intraspecific communication only, i.e. pheromones, but have considered both physical and other biological stimuli that regulate these important behaviors. This approach not only allows for a more holistic or natural view of insect pheromones, but it safely avoids the considerable literature on other aspects of insect chemosensory behavior; for example, food plant and prey selection, and defense behavior. The extensive modern literature on any one of these subjects would easily justify another volume in this series. The subject is further limited to sex and aggregation pheromones. If the book has any shortcomings, perhaps the very brief introductory treatment of the behavior of bees, ants, and termites might be considered as one. Certainly, most students of biology could not fail to be fascinated by observing the human behaviors that we all know so well (i.e., nest, mate, and individual recognition; aggression; alarm; territoriality; etc.), expressed in the lowly insect societies largely through pheromonal communication. Also, the abundant and excellent work on the stored products beetles (Dermestidae) is excluded. These studies give important insight into the diversity and complexity of pheromone communication in another family of *Coleoptera.* But I suspect that parsimony and the abundance of literature, as well as personal research interests, motivated the authors to select their examples mainly from moths, butterflies, and bark beetles. At the same time, we are spared the long lists of compounds and insects from which they have been identified, as well as the distracting citations to the literature that accompany technical reviews of this subject.

The book is organized into seven chapters and, except for a short introductory chapter, all are about the same in length. In Chapter 1, the development of research on pheromones is traced from Fabre's pioneering late 19th century observations of male attraction to female moths, to

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Butenandt's identification of the silkworm moth pheromone in the late 1950's. Butenandt's achievement ushered in the era of "chemical ecology" of insects. Terminology and the types of interspecific and intraspecific behavior elicited are described. By comparing insectan and mammalian behavior, the student reader can easily identify insects with only a superficial knowledge of their biology and taxonomy. Examples of sex pheromones from eight families of moths, aggregation pheromones of the western pine beetle, courtship behavior of male queen butterflies, spacing (epideictic) pheromone of the female apple maggot fly, alarm and queen pheromone of the honeybee, recruitment pheromone of ants, and the maturation pheromone of the desert locust all are excellent illustrations of the ubiquity of pheromone communication in insects (Chapter 2).

The biosynthesis of insect pheromones remains a research frontier for biochemists, chemists, and physiologists. What little work exists is presented in Chapter 3; for example, *de novo* biosynthesis (cabbage looper moth) and conversion of host terpenes (California 5-spined ips), release (gypsy moth, California 5-spined ips), and perception (silkworm moth, silk moth, red-banded leaf-roller moth). The physiological and environmental variables (i.e., "context") that influence release and perception of pheromones are emphasized.

How flying insects orient to odors and how far downwind odors are perceived have fascinated insect behaviorists for the past 3–4 decades. Identification of the potent sex attractant pheromones of moths in the late 1960's and the 1970's provided a new impetus to this research. In Chapter 4 the authors give a clear description of the only experimentally proven mechanism (for male Indian meal moth) to guide flying insects to an odor source; that is, optomotor anemotaxis stimulated by odor stimuli. The intricate and extremely rapid (1.5 sec) sequence of courtship behavior of the Oriental fruit moth as seen through the video camera is described here and in Chapter 5.

The chemical and biological methods used to isolate and identify pheromones (Chapter 5) are illustrated from the authors' research on the artichoke plume moth and the pine engraver beetle. During these studies, the authors utilized an assay that permitted the full behavioral sequence to be expressed at each step of the isolation and identification procedures. The electroantennogram method used successfully to identify active pheromone components from many species of moths is also illustrated in this chapter. Unraveling the complex olfactory behavior of insects requires a strong interaction between chemical and biological methods. However, as the authors point out here, development of the appropriate assay of the behavior in question is the key element of this research. Further, the authors note that only through a clearer understanding of insect behavior will the potential for practical applications be realized. In Chapter 7 they describe the use of

pheromones in monitoring pest populations (largely moths), and controlling these populations by mass-trapping (western pine beetle, ambrosia beetles, smaller European elm bark beetle, European spruce beetle, codling moth, red-banded leaf roller) and mating disruption (pink bollworm, artichoke plume moth). We are cautioned about the possibility (likelihood?) of evolving resistance to pheromones and/or a shift to other behaviors.

The authors speculate in Chapter 6 that pheromone communication was probably a prerequisite for the evolution of multicellular organisms, as evidenced by the aggregation pheromones of slime molds, and were possibly the antecedents of hormones. Pheromones may have evolved from existing metabolites (cuticular waxes) and host odors. They discuss the importance of pheromones in maintaining reproductive isolation. Finally, as indicated by the burgeoning literature, there is good support for their conclusion that it is ". . . more interesting . . . to demonstrate the role of sex and aggregation pheromones in reproductive isolation [than] arguments about the adaptiveness of pheromone communication systems."

Most of the significant research findings (except for the social insects and dermestids) that have created the present day foundation of the exciting interdisciplinary science of "chemical ecology" have been used by Birch and Haynes to build their story for students and teachers of biology. The style of writing is enjoyable to read and complex concepts are lucidly described. This delightful book will perhaps recruit new students trained in both the biological as well as physical sciences to continue the exploration of this fascinating and important area of biology.

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BEHAVIORAL RESPONSES OF MALE *Heliothis zea*[1] MOTHS IN SUSTAINED-FLIGHT TUNNEL TO COMBINATIONS OF 4 COMPOUNDS IDENTIFIED FROM FEMALE SEX PHEROMONE GLAND

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Abstract—Each of the four compounds that have been identified from sex pheromone glands of *Heliothis zea* female moths was examined for its ability to elicit sexual responses from male moths in a flight tunnel. Males flew upwind to (*Z*)-11-hexadecenal alone, but greater levels of behavioral activity were evoked with the addition of (*Z*)-9-hexadecenal to the treatment. Addition of hexadecanal or (*Z*)-7-hexadecenal to the initial two components had no effect in raising the behavioral response of the males in the flight tunnel whether added singularly at both the normal gland-emission ratio or at varying ratios or in combination at the normal ratio. Live, calling females elicited levels of sexual activity from males not significantly different from that elicited by the mixture of (*Z*)-11- and (*Z*)-9-hexadecenal on cotton wicks.

Key Words—Corn earworm, *Heliothis zea*, Lepidoptera, Noctuidae, flight tunnel, sex pheromone, moth behavior.

INTRODUCTION

Klun et al. (1980) identified four compounds from the sex pheromone gland of the female corn earworm moth, *Heliothis zea* (Boddie): (*Z*)-11-hexadecenal (*Z*11–16:Ald), hexadecanal (16:Ald), (*Z*)-9-hexadecenal (*Z*9–16:Ald), and (*Z*)-7-hexadecenal (*Z*7–16:Ald) in approximately a 92:5:2:1 ratio. The combination of *Z*11–16:Ald and *Z*9–16:Ald captured as many males as did all four compounds when evaporated from cotton wicks in some field tests (Sparks et al., 1979; Hartstack et al., 1980; Klun et al., 1980) and in addition,

[1]Lepidoptera: Noctuidae.

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the two-compound mixture occasionally caught as many or more males than traps baited with live females (Hartstack et al., 1980; Klun et al., 1980). However, in other studies, traps baited with females caught significantly more males than did the best pheromone treatment (Sparks et al., 1979), although there was variability of catch dependent upon style of trap used. In addition, number of males captured was dependent upon the type of pheromone dispenser used, of which cotton dental wicks were the most attractive (Hartstack et al., 1980; Lopez et al., 1981). We undertook a flight tunnel study to complement the work that has been done in the field and to determine the role, if any, that each of the four compounds plays in sexual communication in *H. zea*.

METHODS AND MATERIALS

Rearing. H. zea larvae were raised on a modified pinto bean diet (Shorey and Hale, 1965) at the UC Riverside campus or on a casein–wheat germ diet at Agresearch, Inc. (Los Angeles, California). The two colonies were kept separate throughout the experiment to detect any differences that might exist. Pupae were segregated by sex; moths were segregated by age and the sexes kept in separate chambers after emergence. Larvae and adults were maintained at 24 ± 2° C on a 14:10 light–dark photoperiod. Adults always had access to an 8% sucrose solution.

Chemicals. All compounds were obtained from the Controlled Release Division of Albany International Corporation. Purity, as a percentage of total volatiles measurable by gas–liquid chromatography, was determined on a 30-m × 0.24-mm SP2330 column using a Varian 3700 GC run isothermally at 135° C. Purities for the compounds were as follows: *Z*11–16:Ald, *Z*9–16:Ald, 16:Ald (all >99%), and *Z*7–16:Ald (95%). Each compound contained no detectable quantities of the other three compounds. The hexane solutions used in the experiments were formulated prior to each series of tests and were stored at <−20° C. Emission rates were measured from the cotton wicks to be used in the behavioral tests in a device modified from Baker et al. (1981). The 15-mm-diam dental wicks (#3, Johnson & Johnson) were cut down to ca. 10-mm lengths and impregnated with 10 μl of solution containing varying amounts of *Z*11–16:Ald. The emission rate of 3000 ng of *Z*11–16:Ald, 0.73 ng/min, was approximately that emitted by a forcibly extruded sex pheromone gland of a *H. zea* female, 0.42 ng/min (Pope et al., 1983). In the first experiments (Tables 1–3), the other three compounds (*Z*9–16:Ald, 16:Ald, and *Z*7–16:Ald) were loaded in relation to *Z*11–16:Ald at ratios similar to those found in gland extracts (Klun et al., 1980): 30, 150, and 30 ng, respectively. In later experiments (Tables 4 and 5), a series of loadings of these three compounds were varied as binary mixtures of each compound to 3000 ng of *Z*11–16:Ald.

Compound Mixtures on Cotton Wicks. Male moths were tested during the 5th through 8th hr of their 4th, 5th, or 6th scotophase in a clear plastic flight tunnel (1 m wide at the floor, 0.9 m high, 3.65 m long) modified after Miller and Roelofs (1978). Moths were acclimated to flight tunnel conditions (0.5 m/sec wind velocity, 24 ± 3° C, 0.3 lux light level) ca. 30 min prior to testing and transferred to individual release cages ca. 15 min prior to testing.

Cotton wicks, in lots of 50, were cut down to 10-mm lengths, soaked in distilled acetone for 30 min, and oven-dried at 120° C overnight to remove potential contaminants. Wicks were loaded with 10 μl of pheromone solution in hexane 15–20 min before testing. Treatments within an experimental series were drawn in random order for each daily testing in a complete block design. At the beginning of a trial, the loaded wick was placed in the center of a 2-mm-thick metal ring washer (30 mm OD, 13 mm ID) which was then placed at the center of a 15 × 15-cm piece of sheet metal situated 15 cm above the tunnel floor on a sheet-metal platform. The ring washer prevented the males from knocking the wick off the platform. Each male was placed in the middle of the pheromone plume (predetermined by a $TiCl_4$ smoke source) ca. 3 m downwind of the wick and was given 1 min to take flight out of the cage. If found to be incapable of sustained flight (due to damage, etc) after attempting to fly, the male was not scored and then replaced with a new moth. Males that were capable of flight were observed until they left the pheromone plume and contacted the flight tunnel ceiling or flew out the back of the flight tunnel. Those males capable of sustained flight were scored for the following behaviors: UpW—Moth flies upwind in the pheromone plume from the release point; Pl—moth flies within 10 cm of platform edge; S—moth lands on source; Hp—after landing on the source, moth everts his hairpencils; and C—after landing on the source, moth exhibits the copulatory response (i.e., full hairpencil eversion, curling of the abdomen).

For each behavioral category, no male contributed more than 1 data point and the number reported for each category in each table is the percentage of flight-capable males that exhibited at least that level of behavior (i.e., a male that exhibited a Hp response added 1 positive response to each of the first four categories but not the last). Observations of hairpencil extrusions near the source were aided with a flashlight that had several layers of red cellophane pasted over the lens and whose light beam was shielded slightly by the observer's fingers. The light did not appear to affect the moths' behaviors.

All approaches by males were recorded, although most moths made only one approach and then flew up and out of the plume toward the top of the flight tunnel. Males were used once and then discarded from further testing; no more than 8 males were flown to one treatment per day. If moths from both colonies were used in the same series, equal numbers of flights for each colony were recorded for each treatment to eliminate potential bias. Each

trial (i.e., the amount of time the wick was in the flight tunnel airstream) lasted no more than 15 min.

All statistical analysis was performed using a χ^2 2×2 test of independence with Yates' correction at the 0.05 level of significance.

Female vs. Wick. Cotton wicks impregnated with the two-component mixture (3000 ng Z11–16:Ald–30 ng Z9–16:Ald) were tested against live, calling *H. zea* females that were in the 2nd through 6th hr of their 4th scotophase. Pretrial acclimation and handling are described previously (Vetter and Baker, 1983). Data were recorded from males that flew upwind in the plume for >0.25 m or hovered in the plume for >2 sec. If a male flew out of the release cage but did not fly upwind in the plume, no data were recorded since it was not known if he had stopped responding to the pheromone or if the female had stopped calling and no pheromone was present. The calling female was contained on the platform by an inverted release cage. When the wick was tested, it was pinned to the top on the inside of the cage (loaded end down) to mimic the female's calling position. Upon landing on the cage containing either the wick or female, the male was observed for 10 sec and removed. [This should not have biased the results since male *H. zea* exhibit their most intense response on the first approach (97%, $N = 406$).] This removal was necessary to prevent the male from disturbing the female, whereupon she might cease calling. No more than eight males were allowed to fly to a single female, and most trials with a female lasted 10 min, none more than 20 min.

RESULTS

One-Compound Treatments. When each compound was tested singularly, only Z11–16:Ald elicited significant levels of upwind flight, but these levels were significantly lower than the control, the four-compound mixture (Table 1). No significant differences were noted in subsequent behavioral levels among the four individual compounds; all elicited significantly fewer responses than the four-compound mixture, although Z11–16:Ald did evoke slight activity in each category.

Two-Compound Mixtures. All six possible combinations of binary mixtures from four compounds were tested. Those treatments containing Z11–16:Ald again elicited small percentages of upwind flight while those lacking it were inactive (Table 2). As in Table 1, the greatest attrition in behavior occurred between UpW and Pl levels of activity. Only one two-compound mixture, the combination of Z11–16:Ald and Z9–16:Ald, evoked response levels not significantly different from the four-compound mixture in all categories of behavior. Hereafter, this blend will be referred to as the two-component mixture.

Three-Compound Mixtures. To determine if either 16:Ald or Z7–16:

TABLE 1. RESPONSE OF MALES TO ONE-COMPOUND TREATMENTS

Compound	% behavioral response[a] (32 flights/treatment)				
	UpW[b]	Pl	S	Hp	C
Z11-16:Ald	25b	6b	6b	3b	3b
Z9-16:Ald	0c	0b	0b	0b	0b
16:Ald	0c	0b	0b	0b	0b
Z7-16:Ald	0c	0b	0b	0b	0b
Four-compound mixture	72a	44a	28a	28a	25a

[a]UpW—moth flies upwind in plume from release point; Pl—moth flies < 10 cm from platform edge; S—moth lands on source; Hp—after landing, moth everts hairpencils; and C—after landing, moth exhibits copulatory response.
[b]Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P < 0.05$).

Ald play a communicatory role in the sexual behavior of *H. zea*, each compound was admixed to the two-component mixture and then the response of males to these blends was compared to the two-component and four-compound mixtures. Addition of either 16:Ald or Z7-16:Ald to the two-component mixture caused no increase of response over that of the two-component mixture (Table 3). Also, the four-compound mixture did not significantly increase behavioral activity over that of the two components.

Varying Ratios of 16:Ald. Since the addition of 16:Ald (1:20 ratio to Z11-16:Ald) to the two-component blend evoked no significant increase of

TABLE 2. RESPONSE OF MALES TO TWO-COMPOUND MIXTURES

Treatments[a]				% behavioral response[b] (28 flights/treatment)				
Z11-16:Ald	Z9-16:Ald	16:Ald	Z7-16:Ald	UpW[c]	Pl	S	Hp	C
+	+			71a	36a	25a	25a	25a
+		+		11bc	0b	0b	0b	0b
+			+	29b	0b	0b	0b	0b
	+	+		0c	0b	0b	0b	0b
	+		+	0c	0b	0b	0b	0b
		+	+	0c	0b	0b	0b	0b
+	+	+	+	82a	29a	25a	25a	21a

[a]A "+" in the column indicates the presence of this compound in the treatment.
[b]Abbreviations for the behavioral responses as described in Table 1.
[c]Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P < 0.05$).

TABLE 3. RESPONSE OF MALES TO THREE-COMPOUND MIXTURES

Treatments	Additional compound	% behavioral response[a] (100 flights/treatment) UpW[b]	Pl	S	Hp	C
Z11-16:Ald + Z9-16:Ald	16:Ald	88a	43a	38a	35a	24a
	Z7-16:Ald	89a	34a	28a	28a	9b
	None	84a	40a	32a	30a	21a
	16:Ald + Z7-16:Ald	81a	43a	35a	28a	21a

[a]Abbreviations for the behavioral responses as described in Table 1.
[b]Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P < 0.05$).

TABLE 4. RESPONSE OF MALES TO 3-COMPOUND DOSAGE SERIES INVOLVING 16:ALD AND Z7-16:ALD AS VARIABLE COMPOUND

Amount (ng) loaded on wick (ratio to Z11-16:Ald)[a]	% behavioral response[b] (50 flights/treatment) UpW[c]	Pl	S	Hp	C
16:Ald					
0	76ab	46a	38ab	24a	12a
150 (1:20)[d]	82ab	54a	46ab	42a	20a
300 (1:10)	92a	48a	38ab	30a	16a
900 (1:3.3)	68b	36a	26b	22a	12a
3000 (1:1)	86ab	54a	48a	38a	22a
Z7-16:Ald					
0	78a	46a	38a	32a	28a
30 (1:100)[d]	84a	50a	48a	46a	36a
100 (1:30)	78a	48a	46a	44a	34a
1000 (1:3)	70a	40a	34a	32a	24a
3000 (1:1)	82a	46a	40a	34a	28a

[a]The amount (ratio) of Z11-16:Ald to Z9-16:Ald loaded onto the wick was 3000 ng and 30 ng, respectively (100:1).
[b]Abbreviations for the behavioral responses as described in Table 1.
[c]Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P < 0.05$).
[d]The natural loading in approximate ratio of compounds as found in gland extracts (Klun et al., 1980) and volatile collections (Pope et al., 1984).

the sexual response of *H. zea* males in comparison to the two-component mixture (Table 3), a series of treatments was run with a variable loading of 16:Ald to examine whether it might be active at some higher ratio. Addition of 16:Ald to the two-component mixture in ratios to Z11–16:Ald of 1:20 [the natural gland-emission ratio (Klun et al., 1980; Pope et al., 1984)], 1:10, 1:3.3, and 1:1 neither elevated nor depressed the behavior of the males compared to the two component mixture (Table 4).

Varying Ratios of Z7–16:Ald. Similar to the previous experiment, a wide range of ratios of Z7–16:Ald were added to the two-component blend to determine if it would affect behavioral responses. As with 16:Ald, addition of Z7–16:Ald to the two-component mixture in ratio to Z11–16:Ald of 1:100 (the natural gland-emission ratio), 1:30, 1:3, and 1:1 had no effect on any of the behaviors in comparison to the two-component mixture (Table 4).

Two-Component Dosages and Ratios. A series of two experiments was run using the two-component mixture; the first one varied the ratio of Z9–16:Ald while holding the amount of Z11–16:Ald constant and the second was a serial dilution of the two components held at a constant ratio to one another. Z9–16:Ald was admixed with Z11–16:Ald in ratios of 1:100 (the natural

TABLE 5. RESPONSE OF MALES TO 2-COMPONENT DOSAGE SERIES INVOLVING Z9–16:ALD AS VARIABLE COMPOUND (Z11–16:ALD LOADING HELD CONSTANT) AND RATIO OF Z11–16:ALD HELD CONSTANT IN A SERIAL DILUTION

Amount loaded on wick	% behavioral response[a] (50 flights/treatment)				
	UpW[b]	Pl	S	Hp	C
Z9–16:Ald (ratio to Z11–16:Ald)					
0 ng	24c	0c	0b	0b	0b
30 ng (1:100)[c]	86a	46a	42a	38a	34a
100 ng (1:30)	76ab	48a	40a	38a	32a
1000 ng (1:3)	68ab	32ab	28a	26a	22a
3000 ng (1:1)	62b	20b	10b	4b	2b
Z11–16:Ald (100:1 ration to Z9–16:Ald)					
0.3 μg	70a	28a	26a	24a	14b
3 μg[c]	84a	26a	24a	22a	18ab
30 μg	86a	42a	42a	36a	36a
300 μg	78a	40a	26a	24a	20ab
3000 μg	80a	38a	32a	28a	24ab

[a]Abbreviations for the behavioral responses as described in Table 1.
[b]Percentages in the same column having no letters in common are significantly different according to a χ^2 2 × 2 test of independence with Yates' correction ($P < 0.05$).
[c]The natural loading resulting in an emission rate similar to maximally emitting females (Pope et al., 1984).

TABLE 6. RESPONSES OF MALES TO 2-COMPONENT MIXTURE VS. LIVE, CALLING FEMALES

Treatments	% behavioral response[a] (50 flights/treatment)				
	UpW[b]	Pl	S	Hp	C
Calling female	98a	62a	60a	48a	22a
2-component mixture[c]	98a	54a	50a	42a	32a

[a]Abbreviations for the behavioral responses as described in Table 1.
[b]Percentages in the same column with no letters in common are significantly different according to a χ^2 2×2 test of independence with a Yates' correction ($P < 0.05$).
[c]Cotton wick loaded with 3000 ng Z11-16:Ald and 30 ng Z9-16:Ald.

gland-emission ratio), 1:30, 1:3, and 1:1 as well as a treatment consisting of Z11-16:Ald alone. There were no significant differences in the behavioral responses when the 1:30 and 1:3 ratio treatments were compared to the natural ratio treatment, although there was a slight decrease in response with increasing amounts of Z9-16:Ald (Table 5). At the 1:1 mixture, the responses decreased significantly from those to lower ratios, although 10% of the males still landed on the wick. Consistent with the previous experiments, Z11-16:Ald alone elicited upwind flight but addition of 1% Z9-16:Ald increased activity significantly.

In the serial dilution series, Z11-16:Ald + 1% Z9-16:Ald was diluted in tenfold steps and 10 μl were loaded in tenfold decrements from 3000 to 0.3 mg onto the cotton wicks. There were no significant differences among the five treatments, except the 0.3-μg loading which elicited fewer copulatory attempts than the 30-μg treatment (Table 5).

Wick vs. Live Female. There were no statistical differences between the responses of males to live, calling females and the two-component mixture on cotton wicks (Table 6). The overall percentages of males responding were higher than in earlier experiments since males that flew straight up and out of the plume from the release cage were discarded from the data whereas previously they were included.

DISCUSSION

In our flight tunnel, the initiation of upwind flight and subsequent close-range sexual behaviors in male *H. zea* were mediated by the two pheromone components, Z11-16:Ald and Z9-16:Ald. Although Z11-16:Ald alone was capable of eliciting upwind flight and, once, copulatory behavior (Tables 1, 2, and 5), all sexual behaviors were increased significantly with addition of 1% Z9-16:Ald (Tables 2 and 5).

Activity of Z11-16:Ald alone is consistent with earlier findings in activa-

tion bioassay chambers (Roelofs et al., 1974; Klun et al., 1980). All of the above was also consistent with the findings of Carpenter and Sparks (1982a) in a field-flight-tunnel study. However, they tested treatments simultaneously in a transverse array that allowed for the mixing of up to as many as five different pheromone plumes, thereby making the isolated effect of single treatments difficult to observe.

Although 16:Ald and *Z*7–16:Ald were also found in the sex pheromone glands of *H. zea* females (Klun et al., 1980), in our flight tunnel, they were inert in regard to *H. zea* sexual communication. These compounds have also been found to be emitted by *H. zea* females (Pope et al., 1984) in ratios similar to those reported by Klun et al. (1980). However, there was no effect of either compound on altering the response of males in comparison to the two components when admixed as singular additions to the two-component mixture in natural ratios (Table 3) or added together to form a four-compound mixture (Tables 2 and 3). These data are consistent with field trapping experiments (Sparks et al., 1979; Hartstack et al., 1980; Klun et al., 1980). Furthermore, in our study, when each compound was tested in a dosage series where the load varied from natural to 1:1 ratios (to *Z*11–16:Ald), the presence of either 16:Ald or *Z*7–16:Ald caused no significant changes in male response when compared to those elicited by the two-component mixture (Table 4). Despite the fact that both compounds are present in female gland and volatile emissions, we could find no evidence that 16:Ald or *Z*7–16:Ald plays any behavioral role in pheromone communication in this species.

In the flight tunnel, male *H. zea* responded to a wide range of ratios and loadings. First, although the ratio of emission of the two components is ca. 100:1, males responded equally well even when *Z*9–16:Ald accounted for 25% of the treatment (Table 5). A decrease in the response was observed only when the 1:1 ratio was tested. In field trapping, Klun et al. (1982) observed some decrease in trap catch of male *H. zea* when 93:7 *Z*11–16:Ald–*Z*9–16:Ald was tested (as compared to 96:4), significant decrease at 87:13, and almost no trap catch at 76:24. Flight-tunnel assays may be less discriminating than field trapping tests because they lack long-distance flight, but significant reduction of response was eventually observed at the highest level of *Z*9–16:Ald tested.

Responses to the two-component mixture and to live, calling females were not significantly different even at close range (Table 6). This was a somewhat surprising result because male *H. zea* use vision at close range (<12 cm) once they have been attracted from greater distances (Carpenter and Sparks, 1982b). However, in our study, all females were contained in an inverted wire release cage, and some may have been only partially visible as they called from the upwind portion of the cage. Also, although only females that could elicit upwind flight in males were used, there happened to be greater same-day variation among the male's response to calling females than to pheromone

evaporated from cotton wicks; some females were much better than wicks, some much worse. This may be the reason for some of the variation in field trapping (Sparks et al., 1979; Klun et al., 1980) when live females were compared with pheromone components on cotton wicks.

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URINE FRACTIONS THAT RELEASE FLEHMEN IN BLACK-TAILED DEER, *Odocoileus hemionus columbianus*

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Abstract—Flehmen (or "lipcurl") is a response of male mammals primarily to female urine, performed primarily during the reproductive season. To elucidate the components of female urine that release the Flehmen, urine of black-tailed deer (*Odocoileus hemionus columbianus*) was fractionated, and the fractions were presented to captive male conspecifics during three rutting seasons. The active principle (one or more compounds) is water-soluble, of low volatility, not readily extractable with organic solvents, and between 200 and 12,000 daltons in molecular weight. Several urine components were identified, but none of these showed activity.

Key Words—Black-tailed deer, *Odocoileus hemionus columbianus*, urine, Flehmen, deer.

INTRODUCTION

Flehmen (or lipcurl) is a response primarily by male mammals usually to female urine, but sometimes to male urine. It occurs in artiodactyls, perissodactyls, felids, viverrids, chiroptera, sirenia (Estes, 1972), swine (Martys, 1977), marsupials, (Gaughwin, 1979), lower primates (Evans, 1980), shrews, (Baxter, 1981), and proboscids (Rasmussen et al., 1982). Urine is first sniffed, then licked and taken into the mouth. Then the upper lip is curled, the jaws parted, and the head raised and turned from side to side or moved up and down, depending on the species; The animal breathes deeply. Earlier suggestions that urine is introduced into the vomeronasal organ during Flehmen

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(Knappe, 1964; Estes, 1972) have been confirmed by Ladewig and Hart (1980), who found dyed urine in the posterior portion of the vomeronasal organ of male goats only after Flehmen but not after mere sniffing and licking.

In black-tailed deer (*Odocoileus hemionus columbianus*), usually only males show Flehmen. We did not observe it in two castrates over three and six years, respectively. Three females, who were related to one another, were observed to perform Flehmen on rare occasions. Flehmen occurs only during the reproductive season, from late September to early January, and is most frequent in November (Müller-Schwarze, 1979; Henderson et al., 1980). The readiness of Flehmen changes with the season, but not with the kind of stimulus: urine from males, estrous and anestrous females, and fawns of both sexes release the same Flehmen responses if presented out of context. However, the intensity of the response to these stimuli varies seasonally with the level of reproductive behavior (Altieri and Müller-Schwarze, 1980). In males kept without females, male urine releases as much Flehmen as male urine does in mixed groups (Müller-Schwarze and Altieri, 1983).

No specific post-Flehmen behavior has been identified in black-tailed deer, most Flehmen being followed by routine activities, such as feeding. Likewise in goats, 95% of the Flehmen responses have no behavioral consequences related to reproduction (Shank, 1972). There is also no evidence that Flehmen is a display that would modulate behavior in conspecifics.

Flehman is thus a motor pattern in the service of sensory evaluation of urine stimuli that are potentially important for reproduction. The following behavioral steps form the sequence of such urine testing: (1) The male is attracted to the urinating female by visual, auditory, and/or olfactory stimuli. (2) The urine is sniffed. This is the first step when urine is encountered on the ground in the absence of a female. (3) The urine is tasted by licking or allowing the urine to run over the muzzle as it is voided. (4) The urine sample enters the vomeronasal organ during the Flehmen proper. (5) The accessory olfactory system evaluates the stimulus. (6) Characteristic post-Flehmen behavior may or may not be exhibited.

Urine from humans and domestic animals has been subjected to extensive chemical analysis to correlate characteristic components with normal and disease states. However, there have been only a few recent investigations of urine to determine the nature of constituents that comprise chemical signals (Sokolov et al., 1979; Beauchamp et al., 1980; Marchlewska-Koj, 1980; Novotny et al., 1980; Schilling et al., 1980; Wilson et al., 1980). The principal component of the characteristic odor of black-tailed deer urine has been identified as (*Z*)-4-hydroxydodec-6-enoic acid lactone (Brownlee et al., 1968), an effective stimulus for eliciting approach, sniffing, and licking in social encounters (Müller-Schwarze, 1969, 1971).

The purpose of the present study was to identify the components of female black-tailed urine that release the Flehmen response.

METHODS AND MATERIALS

Source of Urine. Urine was obtained during the breeding season from adult captive females that were not in estrus. The samples were collected from tame females with a beaker on a pole 1.5 m in length during "regular" urination (as opposed to "rub-urination" with hindlegs rubbed together, Müller-Schwarze, 1971).

Bioassay. Two to 12 adult black-tailed deer bucks were tested between September and late January during the 1979–1980 (average six males) and 1980–1981 (average three males) seasons, and again in October/November 1981 (average three males). To keep more than 10 adult males poses problems as they are incompatible, and each needs a separate pen. These males ranged in age from 2 to 6 years. Late in the breeding season fewer males responded; hence fewer were tested. Each male was tested only once a day. A test lasted 2 min or until the urine was consumed, whichever occured first. Sample volume was 2 ml urine, or its equivalent in the case of fractions, presented in a glass bowl at ambient temperature since Flehmen was independent of urine temperature. The glass bowl was attached to a pole 2 m in length and thus passed through the fence of the pen. The males approached the urine bowl on their own accord. For each fraction, the maximum possible number of males were tested. Since not all males were always interested in the sample, different numbers of males were tested. For each set of fractions, whole urine and distilled water served as control stimuli. If at least one male gave the Flehmen response, the sample was considered behaviorally active. Three measures of activity were used: (1) overall rate of Flehmen (total number of Flehmen responses divided by number of tests; Table 1 and Figure 1); (2) number of males that showed Flehmen; (3) mean Flehmen rate (average of individual rates; Tables 2 and 3); and (4) duration of individual Flehmen responses.

Each male was given each fraction at least twice. This allocation was determined by the number of males available, the need to avoid habituation to frequent stimulation, and the time available during the 3-month-long breeding season.

Analytical and Preparative Techniques. Proton magnetic resonance spectra were obtained on a Varian XL-100 instrument, and mass spectra on a Finnigan 4000 GC-MS instrument. A Varian 3700 gas chromatograph was used for analytical gas chromatography (GC), and a Varian 1700 gas chromatograph equipped with a Brownlee-Silverstein splitter and thermal gradient collector was used for preparative GC. The glass columns (1.74 m × 4 mm or 1.7 mm ID) used for gas chromatography were 3% OV-101 or 5% SE-30 on Chromosorb G AWDMCS 60/80. Methyl esters were prepared by treatment of the substrate with ethereal diazomethane. Ethyl esters were prepared by stirring the substrate in ethanol with anhydrous potassium carbonate and ethyl iodide for two days.

TABLE 1. FLEHMEN BY BLACK-TAILED DEER MALES TO URINE FROM DIFFERENT SPECIES OF MAMMALS

Urine type	No.of ♂♂ tested	No. of Flehmen	Fl/♂[a]	No. of ♂♂ that did Flehmen	% of ♂♂ responding
Black-tailed deer♀	106	137	1.29 (1.25)	27	60
White-tailed deer♀	5	2	0.40 (0.40)	2[c]	40
Immat. ♂ goat	6	4	0.50 (0.67)	2[c]	33
Elk♀	4	0	0.00 (0.00)	0	0
Dairy cow	6	1[b]	0.17 (0.00)	1	16
Dog ♂	7	3[b]	0.43 (0.00)	1	14
Human ♂	7	2[b]	0.29 (0.00)	1	14
Human♀	7	1[b]	0.00 (0.00)	1	14

[a]Flehmen rate for all males tested. In parenthesis are values excluding one particularly responsive male.
[b]These Flehmen responses to urine from species other that black-tailed deer were due to one particularly responsive individual.
[c]These two males were different ones from the one mentioned under *b*.

RESULTS

The following results are based on a total of 846 individual presentations, during which 358 Flehmen responses were recorded over three seasons.

Series Specificity. Conspecific urine released the strongest and most consistent Flehmen response in all males. In contrast, urine from several other mammal species released a weak Flehmen response or none (Table 1). Thus, the Flehmen response to urine is highly species specific, although some individuals are less selective. Since urine is sniffed from some distance, coding for specificity must lie in the volatiles of the urine.

Seasonal Changes of Responsiveness. The rut of black-tailed deer peaks in November. This is the time when bucks are most responsive to female urine. Little or no Flehmen occurs from February through August (Henderson et al., 1980; Altieri and Müller-Schwarze, 1980).

Figure 1 shows the seasonal variation of the overall rate of Flehmen (average number of Flehmen per male) in response to whole female urine (solid line and dots) for 1979–1980 (top) and 1980–1981 (bottom). The peak in responsiveness occurred in October or November but varied from year to year. The duration of Flehmen decreased from September to December in 1980–1981 (bottom; dotted line and squares), but not significantly in 1979–1980 (top graph). The Flehmen response rates to urine fractions (bottom; triangles) were generally lower than those to whole urine. Reconstituted urine and fractions always released responses considerably weaker than those to the original sample (Table 2, first line compared with last three).

Fractionation and Responses to Urine Fractions. Urine taken during

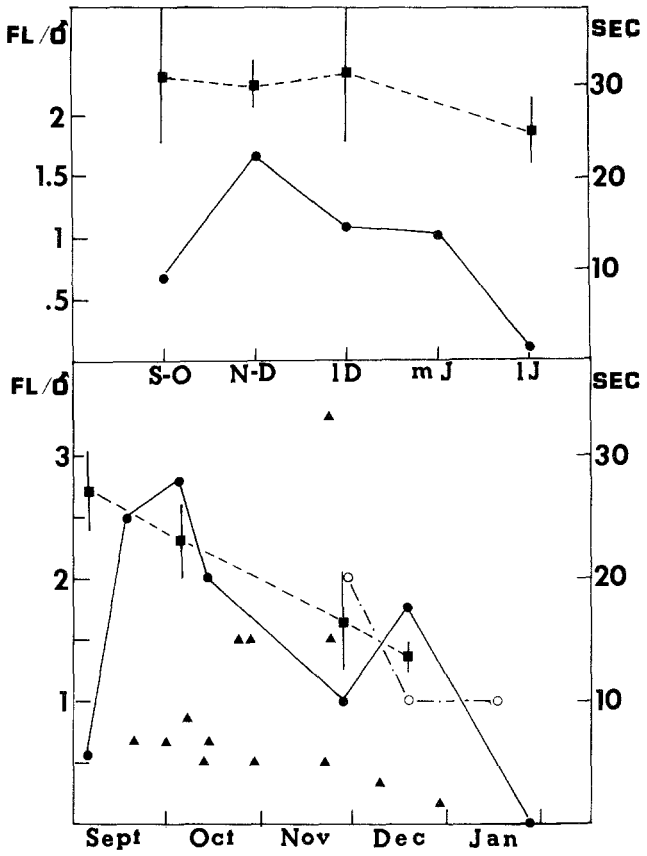


FIG. 1. Seasonal variation of Flehmen response to female black-tailed deer urine and its fractions. Top. Solid line represents rate of Flehmen to female urine during the 1979–1980 season. Left ordinate shows number of Flehmen divided by number of males tested. Broken line shows average duration (± SEM) of Flehmen in seconds (right ordinate). Abscissa: S-O, September/October; N-D, November/early December; D, late December; mJ, mid-January; J, late January. Bottom. Solid line, rate of Flehmen; broken line, duration of Flehmen, 1980–1981 season. Ordinates same as top. Triangles mark rates of Flehmen to various fractions, and circles mark rates of Flehmen to estrous urine.

the first rutting season was fractionated chemically as follows (Figure 2): The urine was acidified with H_2SO_4 to pH 4–5 at 0° C, saturated with NaCl, and extracted with ether leaving the extracted aqueous layer (F). The ether solution (A) was extracted with 2% KOH and with water, leaving an ether solution of neutrals and bases (C). The KOH solution (B) was acidified to pH 2 and extracted with ether to give an ether solution containing phenols and carboxylic acids (D). The extracted aqueous solution (E) was neutralized with

TABLE 2. RELATIVE ACTIVITIES OF VARIOUS URINE FRACTIONS DURING FIRST SEASON (SEPTEMBER–NOVEMBER 1979)

Urine type	No. of different individual ♂♂ tested	Total tests[a]	Males that Flehmened at least once	Flehmen rate[b] (FR) ($\bar{X} \pm$ SE)	FR/FR ♀ urine	Duration (sec) ($\bar{X} \pm$ SE)
Black-tailed deer ♀ urine	4 (7)[c]	40	3	1.98 ± 1.08	1.00	27.0 ± 2.0
Ether extract (A)	4 (7)	29	3	0.95 ± 0.38	0.49	22.6 ± 1.3
Aqueous phase (F)	3 (7)	12	2	0.33 ± 0.22	0.17	18.5 ± 10.3 NS[d]
Ether extr. of A (neutrals) (C)	3 (7)	9	2	1.89 ± 1.57	0.98	14.8 ± 6.7
Ether extr. of aqueous phase (phenols, acids, lactones) (D)	3 (6)	9	2	0.94 ± 0.47	0.49	18.0 ± 3.9
Aqueous layer after 3 ether extraction neutrals (G)	4 (6)	11	1	0.25 ± 0.25*	0.13	17.6 ± 4.7
Distilled water	4 (9)	9	1	0.13[b] ± 0.13*	0.07	26.0 + 0[e]
A, C, D, F, G recombined ("synthetic urine")	3 (6)	4	3	1.00 ± 0.00	0.52	21.5 ± 2.2
C, D, G recombined (reconstituted ether extract A)	4 (7)	10	3	0.33 ± 0.17	0.17	18.3 ± 3.7
A + F (reconstituted urine)	4	4	1	0.25 ± 0.25[f]	0.13	33.0 ± 0

[a] N = Cumulative number of males tested, including repeated testing of same males.
[b] Flehmen rate: For each male, total number of Flehmen responses divided by total number urine presentations. Rate listed is mean of individual rates.
[c] Data are based on the smaller number of males; in parenthesis, total number of males tested.
[d] NS = not significant.
[e] One particularly Flehmen-prone male showed Flehmen once to water.
[f] Difference from whole urine (line 1) significant at $p < 0.05$.

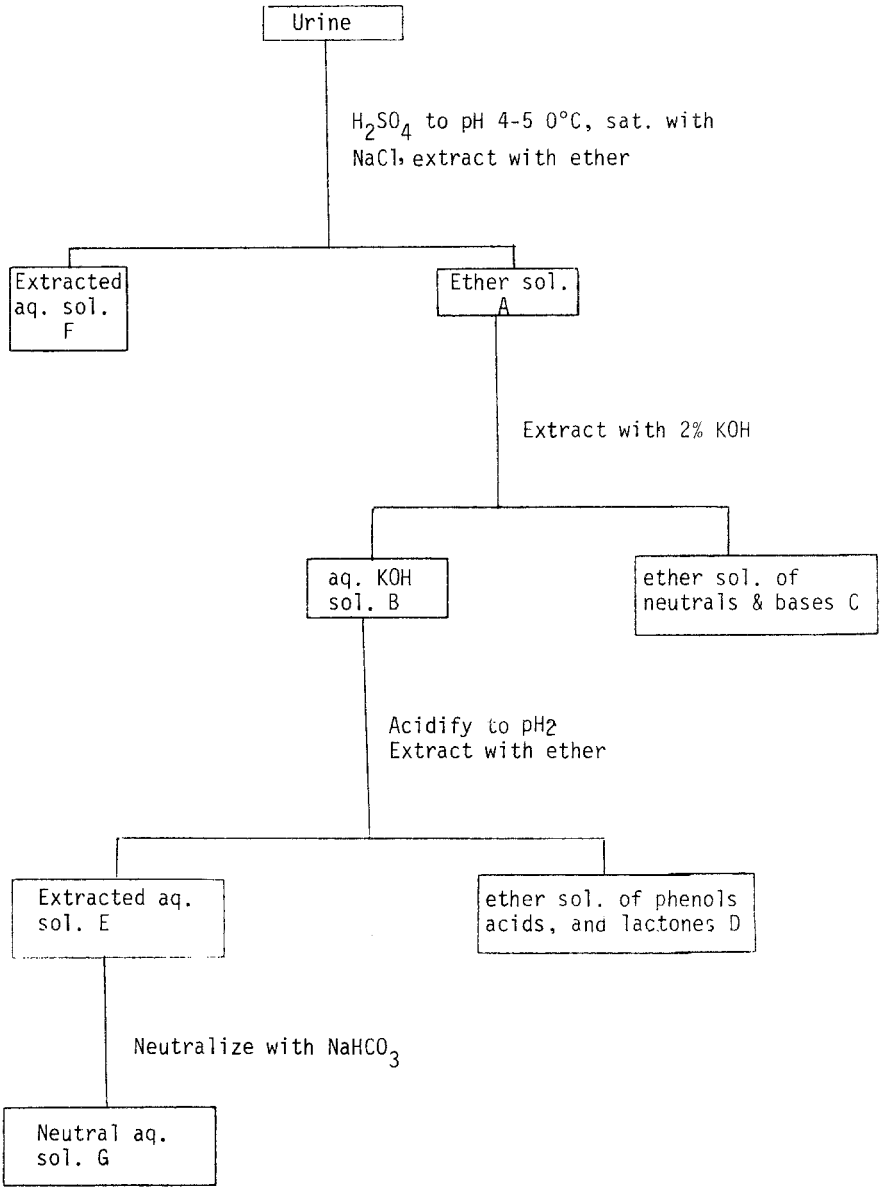
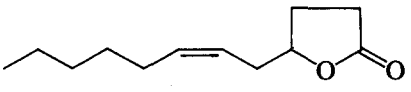


FIG. 2. Chemical fractionation of female black-tailed deer urine taken during the first rutting season.

$NaHCO_3$ (G). The whole urine sample gave a Flehmen rate 0.95. Dilution with distilled water to 50% gave the same Flehmen rate, but activity was lost on dilution to 25%. The activities of the fractions are shown in Table 2.

On the assumption that treatment with acid or base destroyed the activity, another urine sample was saturated with NaCl and exhaustively extracted with ether. The ether solution was concentrated and fractionated by thin-layer chromatography on silica gel. Although the ether solution gave a Flehmen rate of 0.45, neither the fractions nor a recombination were active.

During the second rutting season, urine from female black-tailed deer was saturated with salt and extracted with ether. The principal component of this extract was the deer lactone (**I**), identified by comparison with authentic material by GC-MS.



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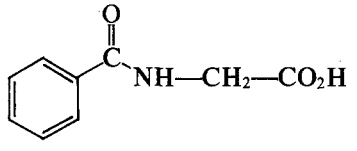

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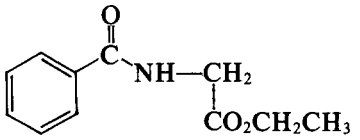

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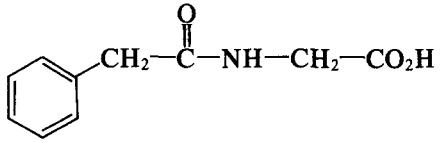

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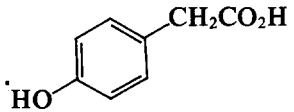


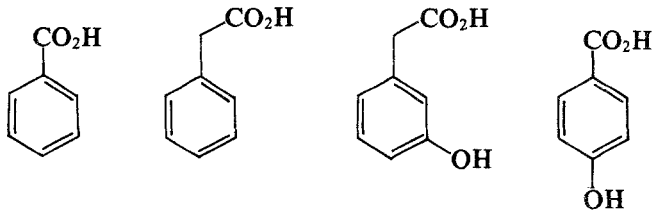
V

Acidification of the aqueous phase from the above extraction with dilute sulfuric acid followed by a further ether extraction gave an extract that contained the glycine derivatives (**II**), (**III**), and (**IV**), and *p*-hydroxyphenylacetic acid (**V**) as major components. Hippuric acid (**II**), the principal component, crystallized from the urine on standing in the refrigerator. These components were identified by comparison with authentic material by GC and GC-MS, and by proton NMR spectra. Both of these ether extracts were sampled by the deer, but no Flehmen occurred. The aqueous phases were rejected by the deer presumably because of the added salt.

Extraction of unsalted urine with ethyl acetate in a continuous liquid-liquid extractor gave an organic extract that did not give a Flehmen response, and an aqueous residue that elicited Flehmen. Removal of the water from whole urine by evaporation at 80–90° C (and 60° C) at water-pump vacuum on a rotary evaporator gave a residue which, on dilution to the original volume with distilled water, elicited a Flehmen response (Table 3, line 2). This residue contained none of the volatiles that were previously extracted with organic solvents. This was checked by extraction of the residue with ether as previously.

Urine was next subjected to dialysis against water through a cellulose bag with an exclusion limit of 12,000. The dialysate that passed out of the bag and the retentate were evaporated to dryness on a rotary evaporator at 80–90° C, then made up to the original volume of urine with distilled water. Only the material that passed through the bag gave a Flehmen response. Thus the molecular weight of the active material must be less than 12,000. The active material was fractionated on Biogel P-2 with water as the eluent. One of the four fractions collected (molecular weight 200) gave a positive Flehmen response, but subsequent fractionation of the active fraction on the same column gave only inactive fractions.

Exhaustive continuous diethyl ether extractions (five days) of the active fraction from Bio-Gel chromatography led to the isolation of a mixture of organic acid derivatives. The seven principal components **(II, III, IV, VI–IX)** were identified by GC-MS of their methylation products and by coinjection with authentic samples on GC. Isolation of the glycine derivatives by preparative GC allowed confirmation of their structure from the proton NMR spectra. The methyl esters were prepared from the acids by reaction with diazomethane, and, as this also alkylates the phenolic hydroxyl group, the acids were also alkylated with ethyl iodide to confirm that the natural compounds were free phenols and not methyl derivatives.



VI **VII** **VIII** **IX**

Continuous ethyl acetate extraction of the aqueous phase from the above diethyl ether extraction resulted in the isolation of urea which was identified from its MS and by TLC comparison with authentic material. The aqueous phase from this ethyl acetate extraction was desalted using Bio-Rex-mixed-

TABLE 3. RELATIVE ACTIVITIES OF VARIOUS URINE FRACTIONS DURING SECOND SEASON (1980–1981)

Urine type	No. of different individual ♂♂ tested	Total tests	Individuals which Flehmened at least once	Flehmen rate (FR) ($\bar{X}$)	FR sample/FR ♀ urine	$\bar{X}$ duration (sec)
Black-tailed deer ♀ urine, nonestrous	4	32	3	1.70 ± 0.76	1.00	21.5 ± 2.0
Diluted residue from urine evaporation	3	3	1	1.00 ± 1.00	0.59	11.0 ± 0*[a]
Estrous urine	3	5	3	2.17 ± 0.93	1.28	22.0 ± 3.1
50% Estrous urine + 50% distilled water	2	2	2	1.00 ± 0.00	0.59	10.0 ± 1.0 *
25% estrous urine + 25% distilled water	2	2	0	0	0	0
Ether extract	4	6	0	0	0	0
Aqueous phase, salted			0	0	0	0
Aqueous phase, unsalted	3	8	2	0.66 ± 0.51	0.39	9.0 ± 3.3**
MW < 12,000	3	15	2	0.67 ± 0.55	0.39	17.0 ± 2.0 *
MW > 12,000	3	5	1	0.17 ± 0.17*	0.10	2.0 ± 0 *
Steroids removed	3	3	2	3.33 ± 2.85	1.96	9.0 ± 5.0
Steroids	2	2	2	2.00 ± 0.00	1.18	12.0 ± 6.0
Biogel fractions of MW < 12,000:						
1	3	5	1	0.17 ± 0.17*	0.10	9.0 ± 0*
2	3	5	2	0.33 ± 0.17	0.19	10.0 ± 2.0*
3	2	8	1	0.13 ± 0.13	0.08	7.0 ± 0*
4	3	5	0	0	0	0
Distilled water	3	6	0	0	0	0
Hippuric acid	3	3	0	0	0	0

[a]Statistical significance of difference from whole urine: $*P < 0.05$; $**P < 0.01$. Significant paired comparisons are connected by brackets.

bed ion exchange resin. Concentration of the aqueous fractions from this treatment resulted in the isolation of further urea.

The overall rate of Flehmen (Fl/♂) to whole female urine varied from 0.5 to 5.0 during the 1980–1981 season. The average for the whole season (September–December) was 1.88 ± 0.38 (SE) Fl/♂ (95% confidence interval (ci): 1.02–2.74 Fl/♂). The number of males that showed Flehmen to whole female urine averaged 60% (95% ci: 46–74%; 99% ci: 41–79%). Water, on the other hand, never released Flehmen. The overall responses to fractions were intermediate between whole urine and water: the average rate was 0.94 Fl/♂, and 51.3% of the males responded on the average.

Fractions both with and without steroids released Flehmen (Table 3). Hippuric acid (**II**), a major urinary constituent in black-tailed deer, did not release Flehmen (Table 3).

The males varied individually in their response intensity; of two males, each responded up to 13 times with Flehmen to one urine sample, while others gave one Flehmen or none to the same stimulus. The more responsive males showed Flehmen most frequently to whole urine.

During the third season, the urine extracts, chromatographic fractions, and individual compounds that had been stored in the frozen state were tested alone and in combination. No clear-cut Flehmen responses occurred, although the test animals were fully responsive to whole urine that had been held frozen during the collecting season.

DISCUSSION

There is general agreement that, in most ungulates, Flehmen is released by nonvolatile compounds in the urine, as contact is a prerequisite for the response (Müller-Schwarze, 1979; Hart, 1980). Horses, however, show Flehmen after mere sniffing (Stahlbaum et al., 1983).

The search for the Flehmen-releasing principle in female urine is complicated by the fact that the intensity of the Flehmen response, as measured by the probability of its occurrence and its duration, varies with the physiological state of the receiving male. Only fractions tested during the response peak in October and November can be compared directly. Using responses to whole urine as control is designed to alleviate this problem. However, the deer may respond to different compounds in different ways at different phases of the rutting season. It is possible that at a certain time of the breeding season, a fraction is too weak to release a response while whole urine still does. Figure 1 demonstrates that the responses to fractions were almost always weaker than those to whole urine.

Our evidence indicates that the active principle, which may consist of several compounds, is water soluble, of low volatility, not codistillable with water, not readily extractable with organic solvents, heat stable, and between

200 and 12,000 in molecular weight. However, during the first season, some active material was extracted with ether from whole urine that was saturated with NaCl. It is possible that salting liberated an organic-soluble moiety from a water-soluble complex.

The loss of Flehmen-releasing activity with fractionation of the urine has also been described for the Asian elephant, *Elephas maximus* (Rasmussen et al., 1982). The two tested elephants showed 5.9 Flehmen-like responses to whole estrous urine, and 3.8 to solvent extracts. Our experiments and those of Rasmussen et al., however, are not directly comparable since we used distilled water as vehicle for the urine fractions, while Rasmussen et al. suspended the fractions in nonestrous urine. Of the four possible reasons they list for the loss of activity, two—interference by solvents and by the nonestrous urine—may not apply, as we observed a similar loss despite a different technique. This leaves incomplete extraction or loss of one or several crucial compounds during processing as possible causes of loss of activity.

We found considerable individual variation of Flehmen frequency. Only five individuals showed Flehmen, and only three did so consistently. Generally, the shyer the buck, the less likely he was to exhibit Flehmen.

The individual variation of selectivity and frequency of Flehmen, if it exists in free-ranging deer, may considerably influence intermale competition and, hence, breeding success ("inclusive fitness"). Thus, rather than being "statistical noise," it may be part of the breeding strategy in this (and possibly other) species.

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EFFECT OF FOLIAGE PROXIMITY ON ATTRACTION OF *Choristoneura occidentalis* AND *C. retiniana* (LEPIDOPTERA: TORTRICIDAE) TO PHEROMONE SOURCES

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Abstract—Pheromone-baited traps located close to both host and nonhost crowns were more attractive than traps located between crowns for both *C. occidentalis* Freeman and *C. retiniana* (Walsingham) at both 10 m and at 1.5 m above the ground. At 10 m height, traps located in host foliage were more attractive than those located in nonhost foliage, but at 1.5 m height there was no significant difference. These results were obtained for both dense and sparse populations of *C. occidentalis* and sparse populations of *C. retiniana*. We conclude that the tree species on which a virgin female is located is not an important factor restricting mating between closely related *Choristoneura* spp. Also, the tree species on which a trap is located may not be an important factor that must be standardized in developing pheromone monitoring systems for *C. occidentalis* and *C. retiniana*.

Key Words—Pheromone trap placement, western spruce budworm, modoc budworm, *Choristoneura occidentalis*, *Choristoneura retiniana*, Lepidoptera, Tortricidae, sex pheromone, sex attractant.

INTRODUCTION

Field tests (Sanders et al., 1974; Daterman et al., 1977) indicated that (*E*)-11-tetradecenal was a sex attractant for the male western spruce budworm, *C. occidentalis* Freeman, and (*E*)-11-tetradecenyl acetate was a sex attractant for the male Modoc budworm, *C. retiniana* (Walsingham) [= *C. viridis* Freeman (Powell, 1980)]. Subsequently, Cory et al. (1982) and Silk et al. (1982) identified the active components of the *C. occidentalis* sex pheromone as (*E*)- and (*Z*)-11-tetradecenal in the approximate ratio 92:8. Recent studies

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by Daterman et al. (1983) indicate that the active components of the *C. retiniana* pheromone are (*E*)- and (*Z*)-11-tetradecenyl acetate, and (*E*)- and (*Z*)-11-tetradecenol in a ratio of 84:7:8:1.

C. occidentalis occurs through much of the western coniferous forest and is associated principally with Douglas-fir, *Pseudotsuga menziesii* (Mirb). Franco. Through parts of its range, *C. occidentalis* occurs sympatrically with *C. retiniana* which feeds mainly on white fir, *Abies concolor* (Gord. & Glend.) Lindl. [Douglas-fir is a secondary host of *C. retiniana* (Volney et al., 1984)], and with *C. lambertiana* Busck which is associated with various pine species (Freeman, 1967; Stehr, 1967; Powell, 1980). It has been proposed that reproductive isolation between conifer-feeding *Choristoneura* spp. may be controlled by geographic separation, specific attractant pheromones, daily periods of sexual activity, seasonal periods of moth flight, and postmating incompatibility (Smith, 1953, 1954; Sanders et al., 1971; Sanders, 1977). In addition, it has been proposed by Smith (1953) that reproductive isolation may be affected by females and males selectively congregating to mate on the foliage of their own host tree species. We have observed in *C. occidentalis* and *C. retiniana*, as others have observed with *C. fumiferana* (Clem.) (Wellington, 1948; Greenbank et al., 1980), that virgin females are poor fliers and thus we expect that they usually mate on the tree on which they fed as larvae. In this study we attempted to determine if males orient to host foliage and/or differentiate between host and nonhost foliage when flying to a pheromone source.

Knowledge of this behavior is also important to the development of operational budworm monitoring methods using pheromone traps. Knowledge of environmental factors affecting trap catch is necessary for standardizing trapping procedures in order to obtain better correlations between pheromone trap catch and absolute insect density (Cardé, 1979).

METHODS AND MATERIALS

Traps and Lures. All traps used in this study were Pherocon 1CP traps (Zoecon Corp., Palo Alto, California). In experiments 1, 2, and 4 (see below) traps were baited with red rubber septa (#8753-D22, Arthur H. Thomas, Philadelphia, Pennsylvania) loaded with the appropriate pheromone components. Baits for *C. occidentalis* were loaded with 12.2 μg of (*E*)-11-tetradecenal and 1.1 μg (*Z*)-11-tetradecenal and will be referred to as TDAL. Baits for *C. retiniana* were loaded with 30.9 μg (*E*)-11-tetradecenyl acetate, 2.6 μg (*Z*)-11-tetradecenyl acetate, 1.16 μg (*E*)-11-tetradecenol, and 0.11 μg (*Z*)-11-tetradecenol and will be referred to as TDAC. Using Butler and McDonough's (1979, 1981) equations, these loads release an estimated 3 ng total attractant per hour which approximates the mean release rate of one *C. occidentalis* female (Cory et al., 1982; Silk et al., 1982). The TDAL bait was

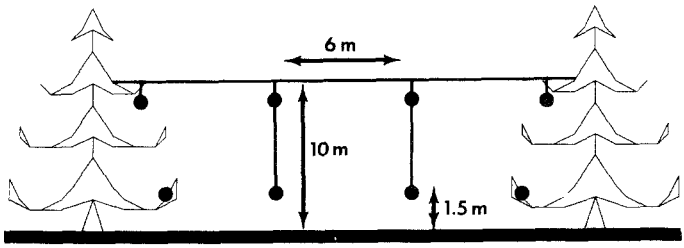

FIG. 1. Pheromone trap configuration used in experiment 1. Each dot represents one Pherocon 1CP trap.

calculated to release (*E*:*Z*)-11-tetradecenal at the 92:8 ratio that naturally emanates from female *C. occidentalis* (Cory et al., 1982; Silk et al., 1982). The TDAC lure was calculated to release (*E*:*Z*)-11-tetradecenyl acetate-(*E*:*Z*)-11-tetradecenol at the 84:7:8:1 ratio that naturally emanates from *C. retiniana* females (Daterman et al., 1983). In experiment 3, traps were baited with a 3-mm-diam. × 5-mm-long polyvinyl chloride (PVC) lure containing 0.01% 92:8 (*E*:*Z*)-11-tetradecenal (supplied by G.E. Daterman, United States Forest Service).

Experiment 1. This experiment was conducted in 1981 in a series of small meadows in San Antonio Canyon, Sandoval County, New Mexico. Light defoliation indicated that a moderately dense population of *C. occidentalis* existed in the area. Eleven sets of eight TDAL-baited traps were deployed as shown in Figure 1. The paired host trees in each case were open-grown Engelmann spruces, *Picea engelmannii* Parry, of similar height (ca. 15 m) and crown proportions (foliated to ground level). Traps were left up for 1 night, after which the trapped males were counted. A Wilcoxon signed-ranks test (Conover, 1971) was performed to determine if there were differences between trap catches in (1) host foliage at 10 m and midair at 10 m, (2) host foliage at

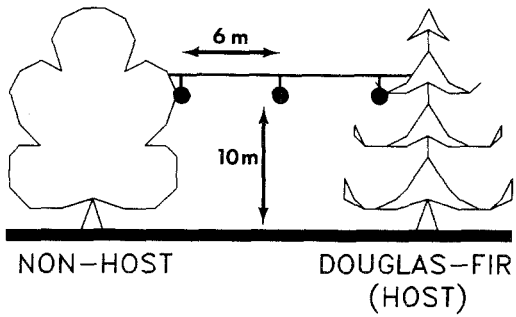

FIG. 2. Pheromone trap configuration used in experiment 2. Each dot represents one Pherocon 1CP trap.

1.5 m and midair at 1.5 m, (3) host foliage at 1.5 m and host foliage at 10 m, and (4) midair at 1.5 m and midair at 10 m.

Experiment 2. This experiment was conducted in 1981 in a series of small meadows in San Antonio Canyon and repeated in 1982 near Hyatt Lake, Jackson County, Oregon. Populations of both *C. occidentalis* and *C. retiniana* were sparse in the Hyatt Lake area. Traps were deployed in a manner depicted in Figure 2. The paired host and nonhost trees were open-grown trees of similar height and crown proportions. At the San Antonio Canyon site, 18 sets of three traps were deployed with ponderosa pine, *Pinus ponderosa* Laws., as the nonhost and 10 sets were deployed with quaking aspen, *Populus tremuloides* Michx., as the nonhost. In each case, the host tree species was Douglas-fir. All traps were baited with TDAL (*C. retiniana* was not known to occur in the area). Traps were left up for 1 night, after which the males trapped were counted. At the Hyatt Lake site, seven sets of three TDAL-baited traps were deployed, and seven sets of three TDAC-baited traps were deployed. The nonhost species was ponderosa pine and the host tree species was Douglas-fir in all Hyatt Lake tests. Traps were left for 3 nights, after which the trapped males were counted. A Wilcoxon signed-ranks test was used to determine if trap catch varied significantly between traps located in (1) host foliage and midair, (2) nonhost foliage and midair, and (3) host foliage and nonhost foliage.

Experiment 3. At each of 15 study sites in southern Oregon and northern California in 1979 (Figure 3 and Table 1), 10 traps baited with the PVC TDAL

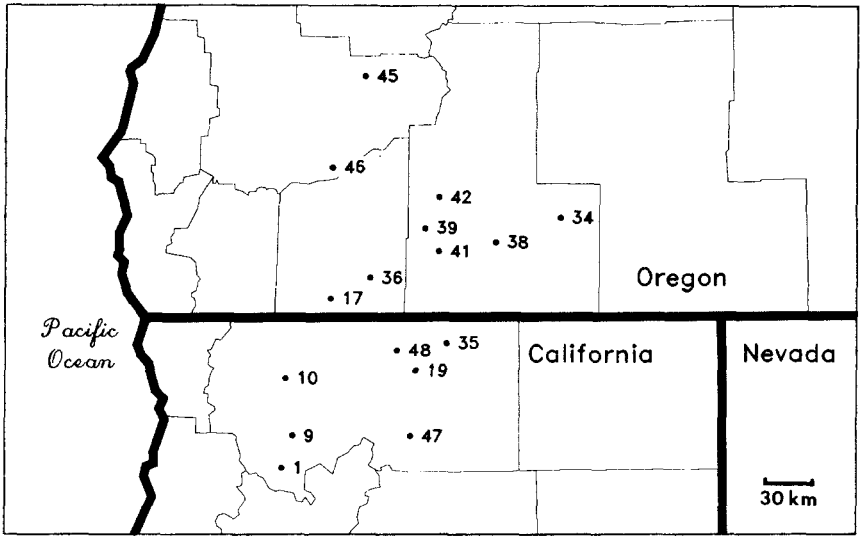

FIG. 3. Locations of study sites used in experiment 3.

TABLE 1. TREE SPECIES COMPOSITION OF *C. occidentalis* PHEROMONE STUDY SITES IN NORTHERN CALIFORNIA AND SOUTHERN OREGON USED IN EXPERIMENT 3 (SEE FIGURE 3 FOR PLOT LOCATIONS)

Plot code	Plot name	Basal Area (%)						
		Douglas-fir	White fir	Red fir	Ponderosa pine	Sugar pine	Other conifer.	Hardwoods
1	Shadow Creek	68.9	0.6	0	13.7	0	0	16.8
9	Hockaday Springs	10.8	74.1	0	4.2	8.4	1.2	1.2
10	Boulder Creek	40.9	0.7	0	27.5	5.4	17.4	8.1
17	Mt. Ashland	0	58.9	0	0	0	7.4	33.7
19	Prather Creek	15.8	47.5	0	21.7	3.3	10.8	0.8
34	Riverbed Butte	0	40.4	0	46.5	13.1	0	0
35	Juanita Lake	19.4	35.1	0	28.4	0	16.4	0.7
36	Keene Creek	49.7	43.6	0	6.7	0	0	0
38	Dry Lakes Flat	36.3	40.0	0	21.9	0	1.9	0
39	Rock Creek	10.6	50.6	8.8	0	2.4	27.6	0
41	Pelican Bay	33.9	58.9	0	4.8	0.8	1.6	0
42	Mares Egg Spring	0	100.0	0	0	0	0	0
45	Toketee	65.3	4.0	0	0	0	29.9	0.8
47	Sand Flat	0	6.6	93.4	0	0	0	0
48	Martin Dairy	2.1	72.2	0	23.5	0	2.1	0

preparation were deployed in a grid with 50 m between each trap. Each trap was hung 1.5 m high on the branch (living or dead) closest to the grid point located using a compass and chain. Traps were left up for the entire budworm flight period (ca. 4 weeks). *Choristoneura* populations were sparse at all study sites. However, at two of the sites (17 and 38), trap liners were periodically replaced to avoid saturation due to high trap catch. At the end of the flight period the number of males trapped was recorded as was the species of the branch from which the trap was hung. A tally of surrounding host tree species was made using a 10-factor prism (Dilworth and Bell, 1979) at each trap position.

Because *Choristoneura* males tend to saturate Pherocon 1CP traps used here (Sanders, 1978; Houseweart et al., 1981), data from any plot having more than three TDAL-baited traps with counts greater than 40 were excluded from further analysis. An analysis of covariance was performed on the raw counts to determine the main effects of study site, position, and host tree prism tally on trap catch. Within each of the study sites, trap catch counts were ranked. A Kendall's tau correlation coefficient (Conover, 1971) was calculated between trap catch rank and host tree prism count.

Experiment 4. In 1981, 24 pairs of adjoining open-grown Douglas-fir and ponderosa pine trees of similar height and crown proportions were located on San Antonio Ridge, New Mexico. A TDAL-baited trap was hung 1.5 m high on a branch tip facing the same arbitrarily chosen direction on each tree in a pair. After one night, the number of males trapped was recorded. A Wilcoxon signed-ranks test was used to determine if trap catch differed significantly between traps located on Douglas-fir and traps located on ponderosa pine.

RESULTS

Experiment 1. C. occidentalis catch was greater in traps located in host foliage than in those located in midair at both the 1.5-m height and at the 10-m height (Table 2). Furthermore, traps located in host foliage at 10 m attracted more males than those located in host foliage at 1.5 m. There was no significant difference in catch between traps hung in midair at 10 m and at 1.5 m.

Experiment 2. As in Experiment 1, traps located in host foliage caught more males than those located in midair (Table 3). This result was obtained in dense *C. occidentalis* populations in New Mexico and sparse *C. occidentalis* and *C. retiniana* (bait = TDAC in Table 3) populations in Oregon. Traps located in nonhost foliage also attracted more males than traps located in midair. This was observed for ponderosa pine and quaking aspen in New Mexico and ponderosa pine for both *Choristoneura* species in Oregon. Furthermore, in all cases traps located in hosts caught more males than did those

TABLE 2. MEAN NUMBER OF *C. occidentalis* CAUGHT IN TRAPS LOCATED AT TWO HEIGHTS IN MIDAIR AND TWO HEIGHTS IN HOST CROWNS AT SAN ANTONIO CANYON, NEW MEXICO, 1981

Trap height (m)	Trap position	Mean trap catch
1.5	Foliage	4.2 (b)[a]
1.5	Midair	0.9 (a)
10.0	Foliage	18.9 (c)
10.0	Midair	0.8 (a)

[a]Means followed by different letters are significantly different using a Wilcoxon signed-ranks test ($\alpha = 0.05$) (Conover, 1971).

in nonhost foliage. This difference did not appear to be as great as the difference between the foliage and midair positions however.

Experiment 3. Four of the 15 study sites were dropped from the analyses because they had more than three traps with greater than 40 moths per trap. Analysis of covariance on the raw counts indicated that neither trap position nor host tree prism tally had a significant effect on trap catch. Study site had a significant effect on trap catch which most likely reflects differences in budworm densities among the sites. Mean ranks for each trap position are given in Table 4. Kendall's tau correlation coefficient between trap catch rank and host tree prism count was −0.024, indicating no significant correlation ($P = 0.691$). No significant correlation was found when Kendall's tau was calculated for each study site separately. Furthermore, when these analyses were repeated with the data from all 15 study sites, similar results were obtained.

Experiment 4. Mean catch for traps located at 1.5 m in Douglas-fir was 25.9, and the mean for traps located in ponderosa pine was 25.3 moths per

TABLE 3. MEAN CATCHES FOR TRAPS LOCATED AT 10-M HEIGHT IN HOST FOLIAGE, NONHOST FOLIAGE, AND MIDAIR.[a]

Location	Bait	Nonhost species	*N*	Nonhost trap catch	Midair trap catch	Host trap catch
New Mexico	TDAL	PP[b]	18	17.9 (b)[c]	7.2 (a)	31.5 (c)
New Mexico	TDAL	QA	10	35.7 (b)	15.1 (a)	46.1 (c)
Oregon	TDAC	PP	7	12.4 (b)	3.9 (a)	21.0 (c)
Oregon	TDAL	PP	7	25.0 (b)	7.9 (a)	34.1 (c)

[a]Tests were conducted in July, 1981, in San Antonio Canyon, New Mexico, and in August, 1982, near Hyatt Lake, Oregon.
[b]PP = ponderosa pine, QA = quaking aspen.
[c]Means in each line followed by different letters are significantly different ($\alpha = 0.05$) using a Wilcoxon signed-ranks test (Conover, 1971).

TABLE 4. MEAN CATCH RANK (RANKED WITHIN PLOT) FOR VARIOUS TRAP POSITIONS FROM 11 STUDY SITES IN SOUTHERN OREGON AND NORTHERN CALIFORNIA, JULY–AUGUST, 1979

Position	*N*	Mean catch ranked within plot
Douglas-fir foliage	7	4.4
White fir foliage	67	5.6
Ponderosa pine foliage	11	5.2
Hardwood foliage	11	5.8
Dead branches, not in foliage	25	5.2

trap. A Wilcoxon signed-ranks test indicated that there was no significant difference in the numbers of males caught between the two trap positions. Trap saturation does not appear to be a problem in this study because mean catch was less than 30 and no traps caught more than 40 moths per trap.

DISCUSSION

Trap height has previously been recognized as an important positional factor affecting trap catch in the Lepidoptera (Cardé, 1979). We have found in *C. occidentalis* that trap catch is greater in the upper crown levels than near the ground (Table 2). This result is similar to that found by Miller and McDougall (1971) with *C. fumiferana*.

The phenomenon of increased trap catch in proximity to the host plant is common in the Lepidoptera and has been observed in *Acrolepiopsis assectella* (Plutellidae) (Rahn, 1977), *Laspeyresia pomonella* (Olethreutinae) (Phillips and Barnes, 1975; Riedl et al., 1979), *Lymantria dispar* (Lymantriidae) (Holbrook et al., 1960), *Pectinophora gossypiella* (Gelechiidae) (Sharma et al., 1971), *Platynota stultana* (Tortricidae) (Aliniazae and Stafford, 1972) and *Trichoplusia ni* (Noctuidae) (Saario et al., 1970).

We report here, for both *C. occidentalis* and *C. retiniana*, trap catch is greater when traps are positioned in foliage than when they are positioned away from any foliage (Tables 2 and 3). One explanation of this phenomenon is that there are pheromone-releasing females located in host foliage, and this causes males to remain near to these trees. However, we have found that foliage proximity increases trap catch in nonhost as well as in host trees. Since females normally do not fly before mating, it is unlikely that the increased trap catch in nonhost foliage is due to the presence of calling virgin females in the foliage. Furthermore, we have observed this phenomenon in sparse populations as well as in dense populations. In sparse populations, the density of females in any tree is so low that it is unlikely that female-released pheromones are responsible for the increased trap catch in proximity to foliage.

It is not clear what behavioral mechanism is responsible for the observed increased trap catch in proximity to host foliage. Several authors report observations of males "buzzing" or "fluttering" around host foliage during the day in epidemic *Choristoneura* spp. populations (Stark and Borden, 1965; Greenbank et al., 1979). We have observed this phenomenon in *C. occidentalis* also. However, flight to pheromones occurs almost entirely during the evening (Liebhold and Volney, unpublished), thus it is unknown what function this diurnal behavior serves. We have observed, during the period of flight to pheromones, that males tend to traverse within ca. 50 cm of the foliage surface until they initiate upwind zig-zag flight behavior, characteristic of the flight of Lepidoptera to a pheromone source (Cardé, 1979; Sanders et al., 1981). Rarely do males fly very far away from foliage. We attempted to verify that this behavior was responsible for the increased trap catch in proximity to foliage by constructing several large models that we thought might duplicate the visual image of tree crowns. However, trap catch in proximity to these models was not significantly different from trap catch in midair. Nevertheless, we suspect that it is a visual cue rather than an olfactory cue that is responsible for this phenomenon, since both host and nonhost trees produce the effect. Perhaps the image of foliage provides an essential visual stimulus in the optomotor reaction that is thought to guide moths in their anemotactic flight to pheromone sources (Kennedy and Marsh, 1974).

Trap catch was greater in proximity to host foliage than in proximity to nonhost foliage at 10 m height (Table 3). This inidcates that, in the upper crown, males either orient selectively to pheromone sources originating in host trees over those originating in nonhosts, or they become aggregated around host trees prior to attraction to pheromones. However, because the difference is not extremely large, it seems unlikely that this behavior is of importance in reproductive isolation of sympatric host-specific *Choristoneura* spp. Furthermore, we found that when traps are placed nearer to the ground (i.e., 1.5 m), there was no significant difference in catch between traps located in host foliage, nonhost foliage, and on dead branches (Table 4). These results indicate that for the development of standardized ground-level trapping techniques to be used for monitoring *Choristoneura* spp. density, it does not matter whether traps are placed in host foliage, nonhost foliage, or on dead branches.

Basal area, which is estimated by prism tallies (Dilworth and Bell, 1979), is positively correlated with foliar biomass for a given species in a given area (Kleinschmidt et al., 1980). Thus, the lack of correlation between prism tallies of host trees and trap catch indicates that males do not limit their flight to microhabitats with a high concentration of host foliage, suggesting that males can disperse a considerable distance from their site of larval development. Such behavior could tend to counter the development of reproductive isolation between groups of insects developing on separate host tree species within a single stand.

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PLANT PHENOLICS AS CHEMICAL DEFENSES: EFFECTS OF NATURAL PHENOLICS ON SURVIVAL AND GROWTH OF PRAIRIE VOLES (*Microtus ochrogaster*)

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Abstract—Very few studies have shown experimentally that plant chemical defenses actually reduce the performance of individual mammalian herbivores, much less the density of mammalian populations. We investigated the effects of representatives of three classes of plant phenolics on the survival and growth of prairie voles by incorporating the compounds into artificial diets and feeding them to weanlings for three weeks. At low levels of protein, both quercetin (a flavonoid) and tannic acid (a hydrolyzable tannin) caused reduced growth rates; no effect occurred at high levels of protein. Quebracho (a condensed tannin) inhibited feeding and thus was lethal at all levels of protein. These results indicate that plant phenolics are likely to influence the performance and dynamics of natural populations of microtine rodents by reducing the quality of available forage. The hypothesis that the primary mode of action of the phenolics is the reduction of digestibility of protein was not supported. The reduced growth caused by both quercetin and tannic acid could be attributed primarily to their toxicity. The effect of quebracho resulted from reduced intake (unpalatability).

Key Words—Plant phenolics, artificial diets, feeding trials, growth rates, *Microtus ochrogaster*, prairie voles.

INTRODUCTION

Voluminous work has shown that plant secondary compounds markedly reduce feeding, growth, reproduction, and survival of individual insects (Harris, 1960; Soo Hoo and Fraenkel, 1964; Lichtenstein et al., 1964; Harley and Thorsteinson, 1967; Shaver and Lukefahr 1969; Feeny, 1970, 1976;

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Berenbaum and Feeny, 1981; Scriber, 1981; Lincoln et al., 1982) and may act as important regulators of insect population dynamics (Lawton and McNeill, 1979; Haukioja, 1980; Schultz and Baldwin, 1982). Work with herbivorous mammals has been more limited. Most studies simply indicate a correlation between secondary compounds in plants and limited use of these plants by mammals (Nagy and Tengerdy, 1967, 1968; Oh et al., 1970; Cooper-Driver et al., 1977; Oates et al., 1977; Bryant and Kuropat, 1980; Schwartz et al., 1980). A few studies have shown that chemical defenses actually inhibit rumen microbes (Oh et al., 1967, 1968) or reduce the growth, reproduction, or survival of wild mammals (Kendall and Sherwood, 1975; Kendall and Leath, 1976; Bryant, 1981; Jung and Batzli, 1981). Although the chemicals involved were not always specifically identified, alkaloids, saponins, and phenolic compounds have been implicated.

Recently, Haukioja (1980) suggested that population cycles of microtine rodents may be generated by induction of chemical defenses in their preferred forage species, and Bryant (1981) suggested a similar mechanism to account for cycles in snowshoe hare populations. Batzli (1983a,b) conducted a theoretical analysis for lemming populations that indicated that a feedback loop between induction of plant secondary compounds and population density of herbivores would lead to oscillatory instability.

Plant chemical defenses are often classified into two groups: qualitative (toxic) or quantitative (digestibility-reducing) defenses (Feeny, 1976; Rhoades and Cates, 1976; Rhoades, 1979). According to this paradigm, qualitative compounds, such as alkaloids and cyanogens, are easily assimilated and have potent physiological effects at low dosages. Quantitative compounds, including phenolics such as tannins, disrupt digestive functions and are too large to be assimilated by animals. This interpretation has gained wide acceptance because of the capacity of tannins to complex in vitro with biopolymers, especially protein. However, little experimental evidence exists to support such a mode of action of tannins in vivo.

This paper reports the results of experiments conducted to examine the effects of plant phenolic compounds on growth and survival of voles (herbivorous rodents). We designed our experiments to test the hypothesis that polymeric phenolics in concentrations similar to those found in nature would reduce growth of weanlings by complexing with protein, thereby reducing its digestibility.

METHODS AND MATERIALS

Although artificial diets allow precise control of levels of chemicals in the diet, they do not mimic all the complex chemical interactions that probably occur when natural vegetation is consumed. Nevertheless artificial diets do allow straightforward testing of hypotheses regarding the action of indi-

vidual compounds and interactions among compounds. We conducted our experiments with an artificial diet previously developed in our laboratory for use with microtine rodents (Lindroth et al., 1984). We added representatives of three major classes of plant phenolics: quercetin (a flavonoid), tannic acid (a hydrolyzable tannin), and quebracho (a condensed tannin) to the basal diet. Harborne (1979) suggested 5% dry weight as an average value for total flavonoid content, and hydrolyzable and condensed tannins often occur in leaves at levels of 1–5% dry weight (Feeny, 1976; Rhoades and Cates, 1976; Gartlan et al., 1980). Dietary levels of phenolics used in this study, 0, 3, and 6%, spanned the normal range of these compounds in vegetation.

To detect the presence of a protein–phenolic interaction, we also manipulated levels of protein in the diets so that basal diets contained 20, 12, 8, or 5% protein. Reductions in protein concentration of these diets were compensated by increases in the level of carbohydrates and oil in proportion to the concentration of these ingredients in the 20% protein diet. The diet ingredients were mixed with demineralized water to a consistency of thick paste, spread onto sheets of aluminum foil, and dried in a forced air oven at 28° C for 48 hr. The resulting moisture content of the diets averaged about 5%. Due to a limited supply of diet components and because assays of some diets would have provided no additional useful information, not all of the possible combinations of protein and phenolic levels were used. The only source of quebracho that we could locate was a nonsulfited crude extract available from leather tanners. We used this unpurified quebracho for feeding trials because the cost of purifying large amounts was prohibitive. To check that our results were not artifacts of a toxic impurity, we purified a small quantity of quebracho using a Sephadex column (Bernays et al., 1981) and incorporated it at 3 and 6% levels into a 20% protein diet. We then compared results from these feeding trials to those from trials with the unpurified extract.

Experimental animals came from a prairie vole (*Microtus ochrogaster*) colony maintained at the University of Illinois. The colony receives ad libitum rabbit pellets and water and is continually outbred to maintain variability. Founders for the colony came from old fields near Urbana, Illinois. The growth of five pairs of weanling voles served as a bioassay of diet quality. Young were weaned at 18 days old, kept as littermates, and fed a transition diet of rabbit pellets mixed with a control artificial diet. At 20 days, young were paired with nonsiblings, weighed, and assigned to an experimental diet with the same protein level as the transition control diet. Litters were divided among the experimental diets in order to eliminate confounding genotypic or maternal effects. Experimental diets were presented in powdered-diet food jars; both food and demineralized water were provided ad libitum. Animals were maintained in plastic shoebox cages with hardwood chip bedding and cotton nesting material at room temperature (22–25° C) on a 15:9 light–dark cycle. All voles were weighed at three- to four-day intervals for three weeks.

Because excretion of uronic acids can be used as an index of hepatic

TABLE 1. BODY MASS LOSS AND SURVIVAL OF WEANLING VOLES FED QUEBRACHO DIETS[a]

Diet	No. of voles	Mass loss (g/day)	No. of deaths	Mean survival on diet (days)
20% Protein				
Crude quebracho				
1%	6	0.23 ± 0.11^{a}	5	11.0 ± 2.3^{d}
3%	10	0.88 ± 0.12^{b}	10	$5.8 \pm 0.9^{b,c}$
6%	10	1.90 ± 0.34^{c}	10	2.6 ± 0.3^{a}
Purified quebracho				
3%	6	1.81 ± 0.09^{c}	6	2.7 ± 0.2^{a}
6%	4	1.58 ± 0.32^{c}	4	2.9 ± 0.3^{a}
12% Protein				
Crude quebracho				
3%	10	$0.73 \pm 0.09^{a,b}$	10	6.5 ± 0.9^{c}
6%	10	1.50 ± 0.14^{c}	10	$3.5 \pm 0.4^{a,b}$

[a]Values represent means ± 1 SE; means within a column that bear different superscripts are significantly different (LSD test, $P < 0.05$.).

detoxication metabolism (Lake et al., 1976), a 24-hr urine sample was collected from each animal between 38 and 42 days of age. The survival of weanling voles fed quebracho diets was poor, so urine samples were collected from subadult animals that had been fed the quebracho diets for three days. Voles were placed into metabolic cages, fed an experimental diet, and given demineralized water ad libitum. Urine was acidified with sulfuric acid to prevent bacterial growth. At the end of 24 hr the urine volume was recorded, and the urine was stored frozen (−20° C) for future analysis of uronic acid content (Bitter and Muir, 1962). Glucuronolactone was used as a standard; additional details of the method will be reported elsewhere (Lindroth and Batzli, 1983).

At six weeks old, animals were sacrificed and autopsied, and livers and kidneys were freeze-dried and weighed. These organs are the primary sites of detoxication and excretion of toxins by mammals (Schuster, 1964), and their sizes have been shown to change in response to plant toxins in the diet (Jung and Batzli, 1981). Carcasses were freeze-dried and extracted with petroleum ether (30–60° C bp) to determine fat content.

Digestibility trials were conducted on the high (20%) and low (8%) protein control diets, and on the phenolic diets that caused growth suppression. For the control, 6% quercetin, and 3 and 6% tannic acid diets, subadult prairie voles were fed an experimental diet and water ad libitum during 5-day pretrial and 4-day trial phases. Effects of 3% quercetin on digestibility were not investigated since the influence of this diet on vole growth was similar to that of the 6% quercetin diet. Only data from those animals showing

TABLE 2. TWO-WAY ANOVAS FOR FINAL CONDITION OF VOLES FED QUEBRACHO DIETS

Source of variation	*df*	Sum of squares	*F*	*P*
Survival (days): protein vs. quebracho				
Protein	1	4.27	1.43	0.237
Quebracho	2	4132.90	693.10	<0.001
Interaction	2	2.23	0.37	0.689
Error	54	161.00		
Total	59	4300.40		
Survival (days): quebracho purity vs. level				
Purity	1	36.82	12.51	0.002
Level	1	51.20	17.40	<0.001
Interaction	1	18.84	6.40	0.018
Error	26	76.52		
Total	29	141.84[a]		
Mass loss (g/day): protein vs quebracho				
Protein	1	0.61	2.25	0.140
Quebracho	2	44.78	81.96	<0.001
Interaction	2	0.30	0.55	0.580
Error	54	14.75		
Total	59	60.44		
Mass loss (g/day): quebracho purity vs. level				
Purity	1	4.90	8.76	0.006
Level	1	5.15	9.21	0.005
Interaction	1	3.46	6.19	0.020
Error	26	14.54		
Total	29	21.57[a]		

[a]Sums of squares are not additive because of unequal sample sizes.

less than a 10% change in body mass during the trials were used to calculate digestion coefficients.

To compensate for poor survival of voles fed the quebracho diets, we altered the digestibility trial procedure. We ran trials on only the 3% crude quebracho, 20% protein diet because protein level did not affect survival and because rapid mortality at higher levels of quebracho precluded the use of digestibility trials. Subadult voles received a mixture of rabbit pellets and the quebracho diet ad libitum during a 5-day pretrial period. Twelve hours before the onset of the trial phase, voles were switched to the quebracho diet. The trial phase ran for three days, during which voles received the diet and water

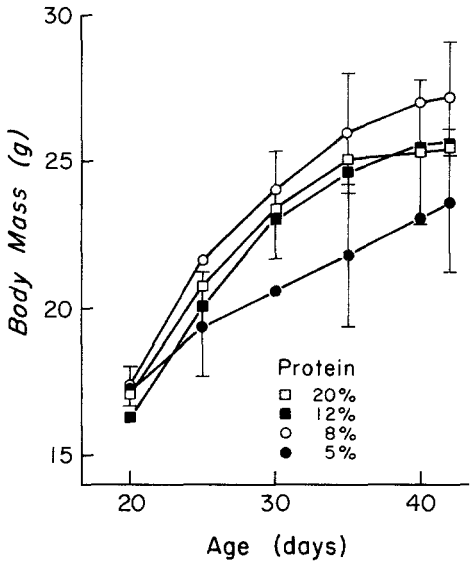

FIG. 1. Growth of weanling prairie voles fed control diets. Vertical bars indicate 95% confidence intervals; sample size for the 5% protein diet is 7, for all other treatments is 10.

ad libitum. These prairie voles lost an average of 13% of their initial body mass during the 3-day trial.

At the end of the trials feces and excess food were dried and weighed so that digestibilities could be calculated by the difference between intake and output. Samples of both food and feces were analyzed for total organic nitrogen through acid digestion and determination of ammonia by the Nessler method (AOAC, 1975).

RESULTS

Survival and Growth. All but one of the weanlings fed the various control, quercetin, and tannic acid diets survived for the duration of the feeding trials. The single exception died of unknown causes shortly after being placed on the high protein, 6% tannic acid diet. Voles fed the quebracho diets survived poorly (Table 1). Even on the high protein, 1% quebracho diet, all but one animal died. Voles fed 3% crude quebracho diets survived twice as long as did voles fed 6% crude quebracho diets. An analysis of variance showed that quebracho greatly decreased survival, but protein and protein–quebracho interaction effects were not significant (Table 2). Voles fed 3 and 6% purified quebracho survived equally poorly (Table 1); analysis of variance

TABLE 3. TWO-WAY ANOVAS FOR FINAL CONDITION OF VOLES FED QUERCETIN DIETS

Source of variation	*df*	Sum of squares	*F*	*P*
Final vole mass (g)				
Protein	1	24.09	2.27	0.137
Quercetin	2	13.37	0.63	0.536
Interaction	2	92.56	4.37	0.018
Error	54	572.18		
Total	59	702.20		
Fat indices (g fat/g lean dry body mass)				
Protein	1	0.153	3.21	0.079
Quercetin	2	0.066	0.70	0.502
Interaction	2	0.066	0.70	0.503
Error	54	2.569		
Total	59	2.854		
Relative liver mass (g dry mass/g lean dry body mass)				
Protein	1	0.00039	2.22	0.142
Quercetin	2	0.00005	0.13	0.874
Interaction	2	0.00030	0.85	0.433
Error	54	0.00960		
Total	59	0.01034		
Relative kidney mass (g dry mass/g lean dry body mass)				
Protein	1	0.00005	11.09	0.002
Quercetin	2	0.00006	6.15	0.004
Interaction	2	0.00000	0.31	0.736
Error	54	0.00025		
Total	59	0.00037		
Uronic acid excretion (log (mg/g body mass$^{0.5}$/day))				
Protein	1	0.014	0.67	0.418
Quercetin	2	29.180	715.70	< 0.001
Interaction	2	0.114	2.79	0.070
Error	54	1.101		
Total	59	30.409		

showed that effects due to quebracho purity, quebracho level, and their interaction, were significant (Table 2).

Animals grew equally well on the 20, 12, and 8% protein control diets (Figure 1), but voles fed the 5% protein diet grew significantly slower than those fed the 8% diet. Body fat indices (g fat/g lean dry body mass) of voles were negatively correlated with dietary protein content ($r = 0.38$, $P < 0.02$, $df = 35$). Our results indicate that the minimum level of dietary protein required for maximum growth on our basal diet lies between 5 and 8%. Voles

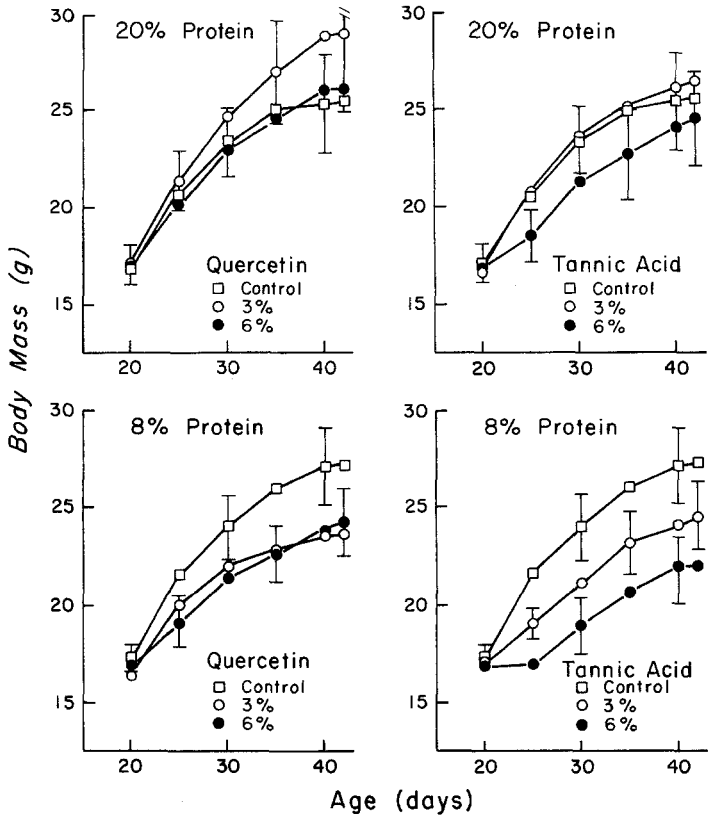

FIG. 2. Growth of weanling prairie voles fed quercetin and tannic acid diets. Vertical bars indicate 95% confidence intervals; sample size for each treatment is 10.

fed the basal artificial diets grew similarly to, but tended to deposit more body fat than, voles raised on rabbit pellet or alfalfa diets (Lindroth et al., 1984; unpublished data).

At high protein levels (20%), quercetin did not affect growth (Figure 2). In fact, voles on the 3% quercetin diet tended to gain more mass than control animals, although this difference was not statistically significant. At low levels of protein (8%), vole growth was suppressed similarly by both the 3 and 6% quercetin diets. An analysis of variance of final vole body mass (Table 3) indicated that the main effects of protein and quercetin were not significant but that the interactive effect of these components was. Thus the effect of quercetin was stronger at low levels of protein. Quercetin did not significantly affect fat deposition by animals fed either high protein (and therefore low carbohydrate) or low protein (and therefore high carbohydrate) diets (Table 3).

At high levels of protein, tannic acid did not significantly suppress

TABLE 4. TWO-WAY ANOVAS FOR FINAL CONDITION OF VOLES FED TANNIC ACID DIETS

Source of variation	*df*	Sum of squares	*F*	*P*
Final vole mass (g)				
Protein	1	7.08	0.77	0.385
Tannic acid	2	107.52	5.83	0.005
Interaction	2	56.00	3.04	0.056
Error	54	497.78		
Total	59	668.38		
Fat indices (g fat/g lean dry body mass)				
Protein	1	0.099	1.68	0.200
Tannic acid	2	0.094	0.80	0.456
Interaction	2	0.026	0.22	0.803
Error	54	3.177		
Total	59	3.396		
Relative liver mass (g dry mass/g lean dry body mass)				
Protein	1	0.00055	1.91	0.172
Tannic acid	2	0.00024	0.42	0.656
Interaction	2	0.00066	1.15	0.323
Error	54	0.01538		
Total	59	0.01683		
Relative kidney mass (g dry mass/g lean dry body mass)				
Protein	1	0.00006	11.11	0.002
Tannic acid	2	0.00017	15.75	0.001
Interaction	2	0.00000	0.06	0.938
Error	54	0.00029		
Total	59	0.00052		
Uronic acid excretion (log (mg/g body mass$^{0.5}$/day))				
Protein	1	0.645	25.13	<0.001
Tannic acid	2	27.923	544.44	<0.001
Interaction	2	0.746	14.54	<0.001
Error	54	1.385		
Total	59	30.699		

growth of voles. Growth was also unaffected at the 12% protein level, so these results will receive no further consideration. But at low protein levels tannic acid substantially reduced growth (Figure 2). The overall effect of tannic acid was strongly negative (Table 4), but the interactive effect of protein and tannic acid on growth was only marginally significant ($P = 0.056$) (Table 4). Accumulation of body fat by animals was not significantly affected by tannic acid at any level of protein (Table 4).

TABLE 5. MEAN (± 1 SE) CONSUMPTION AND DIGESTIBILITY OF ARTIFICIAL DIETS BY SUBADULT PRAIRIE VOLES[a]

Diet	Body mass (g)	Ingested dry matter (mg/g BM/day)	Dry matter digestibility (%)	Digested dry matter (mg/g BM/day)	Protein digestibility (%)	Digested protein (mg/g BM/day)
20% Protein						
Control (6)	23.9 ± 1.4	187 ± 11^{b,c}	61.1 ± 1.8^{b}	114 ± 6^{b,c}	87.9 ± 1.7^{d}	32.8 ± 2.1^{c}
3% quebracho (7)	23.3 ± 1.5	35 ± 4^{a}	55.8 ± 2.0^{a}	20 ± 2^{a}	76.4 ± 1.4^{b}	5.2 ± 0.6^{a}
8% Protein						
Control (6)	27.0 ± 1.2	184 ± 8^{b,c}	59.1 ± 0.8^{a,b}	108 ± 4^{b}	81.5 ± 1.0^{c}	12.0 ± 0.5^{b}
6% quercetin (6)	23.1 ± 1.1	215 ± 8^{c}	61.4 ± 1.0^{b}	132 ± 10^{c}	81.4 ± 0.6^{c}	13.2 ± 0.4^{b}
3% tannic acid (6)	26.7 ± 2.0	178 ± 11^{b}	61.6 ± 1.2^{b}	110 ± 9^{b}	77.6 ± 0.4^{b,c}	10.7 ± 0.7^{b}
6%tannic acid (6)	23.5 ± 1.8	214 ± 20^{c}	61.6 ± 1.4^{b}	132 ± 12^{c}	70.8 ± 2.2^{a}	11.3 ± 0.8^{b}

[a]Values in each column (performance category) that bear different superscripts are significantly different (LSD test, $P < 0.05$). Sample sizes are shown in parentheses.

Voles lost mass on all of the quebracho diets (Table 1). Animals fed the 6% crude quebracho diets lost mass twice as fast as animals on the 3% diets and eight times as fast as those on the 1% diet. Losses were similar at both the 20 and 12% levels of protein; analysis of variance showed the main effect of protein was not significant (Table 2). Voles fed diets with purified quebracho (3 and 6%) lost mass at a rate similar to that of voles on diets with 6% crude quebracho (Table 1). The main effects of quebracho purity, quebracho level, and their interaction, were all significant (Table 2).

Consumption and Digestion of Diets. Poor growth by voles fed quercetin or tannic acid at low levels of protein was not due to reduced consumption rates (Table 5). Rather, animals fed the 6% phenolic diets tended to consume more food than animals fed the control diets. Because neither quercetin nor tannic acid affected dry matter digestibility of the diets, voles fed the low protein, 6% phenolic diets consumed significantly more digestible dry matter than voles fed the corresponding control diet. Intake by voles fed the 3% quebracho diet was markedly reduced, and dry matter digestibility was also lower than on the control diet. Voles fed the high protein control diet digested significantly more protein than voles on the low protein diets. Apparent protein digestibility was not affected by quercetin, and it was lowered only 3.9 and 10.7% by 3 and 6% tannic acid, respectively, compared to the control diet. Protein digestibility for voles on the quebracho diet was 11.5% less than for those on the control diet. Because intake by voles fed the 6% phenolic diets was elevated, these animals digested amounts of protein similar to that digested by voles fed the low protein control diet.

Assimilation of Phenolics. Relative liver sizes were not affected by the presence of phenolics in the diets, but relative kidney sizes were (Tables 3 and 4). Relative kidney mass of animals on the 8% control diet (0.0135 g/g lean dry body mass) were significantly less than that of animals on the 20% protein control diet (0.0155 g/g lean dry body mass). Within each level of protein, kidneys of voles fed diets containing 3% phenolics were not significantly enlarged. Kidney sizes of animals fed 6% quercetin at high and low levels of protein increased 16 and 17%, respectively, over animals fed the control diets, and those of voles fed 6% tannic acid at high and low levels of protein increased 23 and 30%, respectively, over those of voles on the control diets.

In general the presence of quercetin or tannic acid in the diet greatly elevated excretion of uronic acids by prairie voles (Figure 3). These increases were somewhat more than proportional to the concentration of phenolic in the diet. Animals fed the quercetin diets tended to excrete more uronic acids at low levels of protein than at high levels of protein, but this difference was not significant (Table 3). However, the same trend was more striking and highly significant for voles fed tannic acid diets (Table 4). Voles fed the quebracho diets did not exhibit significantly greater excretion of uronic acids. The results for uronic acid are discussed in greater detail elsewhere (Lindroth and Batzli, 1983).

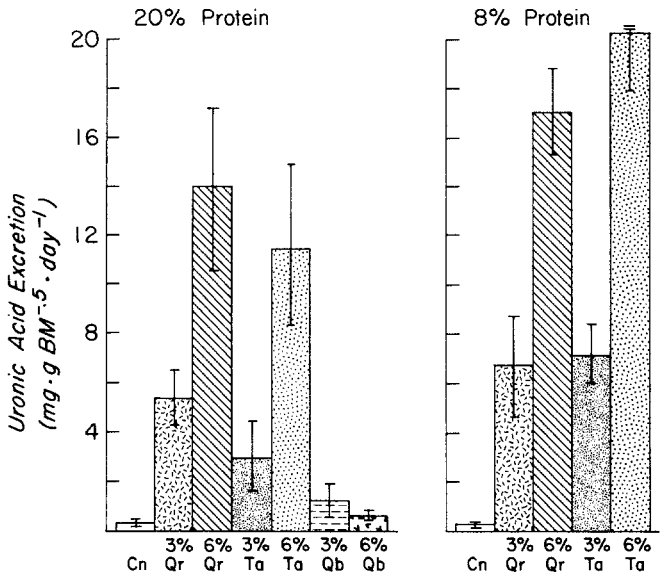

FIG. 3. Uronic acid excretion of prairie voles fed control (Cn), quercetin (Qr), tannic acid (Ta), or quebracho (Qb) diets. Vertical bars show 95% confidence intervals. Sample size is 6 for quebracho treatments and 10 for all others.

DISCUSSION

Tannins and related phenolics are considered to be the most widespread, and possibly the most important defensive plant secondary metabolites (Rhoades, 1979; Swain, 1979). Generalist herbivores such as voles are certain to encounter a diversity of phenolics in their potential food plants. Results from this study clearly indicate that plant phenolic compounds can adversely affect the performance of individual prairie voles. Representatives of three general classes of phenolics all reduced survival and/or growth. The magnitude of effect was influenced by several factors, including the type and amount of phenolic compound and the level of protein in the diet. Quebracho, a condensed tannin, had the most profound effect on vole performance, causing death at dietary levels at which the other phenolics did not even reduce growth. Tannic acid, a hydrolyzable tannin, suppressed growth to a greater extent than did quercetin, a flavonoid. These results differ from those of Joslyn and Glick (1969), who showed that tannic acid inhibited the growth of laboratory rats to a greater extent than did quebracho.

The capacity of phenolics to bind with protein in vitro is well known; enzymes bound in this manner show a marked reduction in activity, and proteins complexed in this fashion are much less susceptible to digestion by

proteases (Goldstein and Swain, 1965; Swain, 1979). Simple phenolic compounds, such as quercetin, are not considered "true tannins" because they rarely form stable copolymers in the presence of protein (Swain, 1979). Still, these compounds can be potent enzyme inhibitors and thereby disrupt vital cellular processes (Harborne, 1979). We found no evidence to suggest that quercetin disrupted digestive processes in prairie voles; rather, the evidence suggests a toxic mode of action. Quercetin reduced growth of voles when fed with low levels of protein, but did not reduce protein digestibility (Table 5). Animals on low protein diets consistently detoxicated more quercetin than animals on high protein diets, but this trend was not significant (Figure 3). Low protein diets are known to decrease the ability of animals to metabolize toxins (Dauterman, 1980), and voles on such diets may experience a greater toxic effect of quercetin even though this effect was not strongly reflected in the uronic acid output. Wagner (1977) showed quercetin to be a strong inhibitor of many enzyme systems, including ATPase and RNA polymerase, and Fewtrell and Gomperts (1977) found quercetin to disrupt calcium ion transport. Thus our results are also consistent with the interpretation that when animals are fed low protein diets toxic effects occur because less protein is available for enzyme functions.

Tannic acid did have a minor effect on digestive processes (Table 5), but it is unlikely that this was the cause of growth suppression. Voles fed the low protein, 6% tannic acid diet also had greater food intake so total digested protein was not significantly different from controls. Again, this is contrary to the results of Joslyn and Glick (1969), who found 5% tannic acid in artificial diets to substantially depress food consumption by rats. That digestible protein intake did not vary among animals fed control and tannic acid diets suggests that toxic effects of this phenolic or its derivatives were responsible for reduced growth. Bernays and Chamberlain (1980) found that tannic acid is hydrolyzed in the gut of the tree locust (*Anacridium melanorhodon*). Given the sensitivity of hydrolyzable tannins to acid or alkaline conditions (Roux et al., 1980); this reaction probably also occurs in the gastrointestinal tracts of voles. Tannic acid releases gallic acid upon hydrolysis, and rats have been shown to assimilate dietary gallic acid (Booth et al., 1959). Our results for uronic acid excretion (Figure 3) suggest that prairie voles absorbed more tannic acid derivatives at low levels of dietary protein than at higher levels of protein, and this difference is the best explanation for the differences in growth response. If tannic acid was indeed binding to dietary protein, more of this phenolic may have been hydrolyzed and assimilated when diets contained low amounts of protein.

Quebracho significantly reduced protein digestibility, but this compound inhibited feeding even more strongly (Table 5). At equal concentrations, purified quebracho was probably even less palatable than crude quebracho, thus resulting in greater weight loss (Tables 1 and 2). Because consumption

of quebracho diets was so low, the uronic acid results provide little insight into the assimilation and detoxication of this compound.

Our results agree with several recent studies with insect herbivores that also question the action of plant phenolics as inhibitors of digestive processes. Digestibility of diets to several locust species (Acrididae) was not affected by either tannic acid (Bernays et al., 1980) or quebracho (Bernays et al., 1981). Lincoln et al. (1982) showed that a phenolic leaf resin significantly depressed larval survivorship, growth rates, and size at diapause in the checkerspot butterfly (*Euphydryas chalcedona*), but it did not affect protein digestion.

The fact that voles fed the low protein, high phenolic diets tended to have greater food consumption deserves attention. Although higher relative consumption by these animals may have resulted in part from their smaller size (Table 5), phenolics did not reduce food consumption as might be expected for a defensive compound. Price et al. (1980) explained this pardox for phytophagous insects by proposing that longer periods of eating lead to longer exposure to enemies (predators or parasitoids). This may reduce survival of herbivores and, ultimately, total damage to the plants. Since herbivorous mammals are also exposed to predators while eating, this concept may be extended to them as well.

In summary, it is clear that plant phenolics can substantially affect the individual performance of prairie voles, although it does not appear that the effects are achieved simply by disruption of digestive processes. The question remains as to whether such plant defenses also influence the population dynamics of mammalian herbivores in natural habitats, and we are currently investigating that possibility.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS. XV. Identification of Several Acids in Scales of *Heliothis zea*[1] Moths and Comments on Their Possible Role as Kairomones for *Trichogramma pretiosum*[2,3,4]

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Abstract—Acids found in moth scales of laboratory-reared *Heliothis zea* (Boddie) moths are hexanoic, heptanoic, octanoic, nonanoic, 2- (or 3-) furan carboxylic, phenylacetic, benzoic, sorbic, and 4-hydroxybenzoic acid. The last two of these acids are preservatives added to the artifical diet as sorbic acid and methyl-*p*-hydroxybenzoate. Female *Trichogramma pretiosum* Riley exhibited increased rates of parasitization of *H. zea* eggs in the presence of some of these compounds in laboratory experiments. Exposure to a mixture of all of these compounds did not increase parasitization, and the elimination of acids from the crude moth-scale extract did not reduce parasitization by the wasps.

Key Words—Acids, moth scales, *Heliothis zea*, kairomones, parasitoids, biological control, *Trichogramma pretiosum*.

[1]Lepidoptera: Noctuidae.
[2]Hymenoptera: Trichogrammatidae.
[3]In cooperation with the University of Georgia College of Agriculture Experiment Stations, Coastal Plain Station, Tifton, Georgia 31793.
[4]Mention of a commercial or proprietary product in this paper does not constitute endorsement by the USDA.

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INTRODUCTION

Lewis et al. (1972) reported that scales left by ovipositing *Heliothis zea* (Boddie) moths contain kairomones that stimulate host searching by the egg parasitoid, *Trichogramma evanescens* Westwood. Tricosane was the most active hydrocarbon of a series of hydrocarbons identified from *H. zea* scales by Jones et al. (1973). These hydrocarbons caused higher rates of parasitization on *H. zea* eggs by *T. evanescens*. Tricosane, by itself, and hexane extracts of the moth scales increase rates of parasitization by wild *Trichogramma* spp. in the field (Lewis et al., 1975a,b). In contrast to the above, with *Trichogramma pretiosum* Riley as the test insect, tricosane elicited no response, while the hexane extract of moth scales did elicit a response. The acetone fraction of the hexane extract of *H. zea* moth scales, eluted from a silica gel, increased rates of parasitization by *T. evanescens*, indicating substances other than hydrocarbons may function as kairomones (Jones et al., 1973). As we investigated fractions other than hydrocarbon fractions, it became evident that acids could be spread throughout most of the fractions. Also the parasites were observed to respond when exposed to acids on filter paper.

METHODS AND MATERIALS

Moth scales were obtained from a laboratory colony of *H. zea* (Burton, 1969; Young et al., 1976) at the Southern Grains Insects Research Laboratory. The scales of the mixed sexes were obtained as a by-product of the mating pairs which lay the eggs that sustain the colony.

The scales, which are allergenic, are collected routinely from mating cages by vacuum through a centrifugal dust collector (Harrell and Perkins, 1971) into commercial vacuum cleaner bags in a clean handling room designed to minimize personnel exposure to airborne scales.

The scales (80–220 g/batch) were extracted sequentially by percolation with hexane, chloroform, ether, acetone, methanol, and water in a large, coarse-fritted Buchner funnel or a chromatographic column. About 1 liter of each solvent was used for each 100 g of moth scales. The extracts were bioassayed for effect on *T. pretiosum* behavior. Hexane, chloroform, ether, and acetone fractions were extracted with 2 N sodium hydroxide. The basic extracts were washed three times with ether, acidified with HCl, and reextracted with ether. The ether extracts were dried over anhydrous sodium sulfate, concentrated in a rotary evaporator to a volume of ca. 20 ml, and treated with excess of an ethereal solution of diazomethane to produce methyl esters. The methyl esters were quantitatively analyzed by gas chromatography on a Hewlett-Packard 5840 gas chromatograph using a 30-m glass capillary column coated with methyl silicone (OV-1) fluid and helium as carrier gas (linear flow velocity, 15 cm/sec). Qualitative analysis was

accomplished with a Hewlett-Packard 5985 gas chromatograph–mass spectrometer (GC-MS), using a 4-ft × 1/4-in. glass column packed with 100–120 mesh Gas Chrom Q coated with 2% OV-101 with He as carrier gas flowing at 20 ml/min. The data system attached to the mass spectrometer allowed comparison of the obtained spectra with the EPA-NIH spectral library for identification of the methyl esters.

Infrared analyses were run on a Beckman IR-4210 spectrometer equipped with a 4× beam condenser.

The *Trichogramma* stock used in this study originated from Hermosillo, Mexico, and crosses successfully with a stock from Los Mochis, Mexico (Gonzales and Allen, 1975; Division of Biological Control, University of California, Riverside, California 92501, unpublished results), identified as *T. pretiosum* (Oatman et al., 1970). These parasitoids were reared at the Southern Grain Insects Research Laboratory according to the procedure of Lewis and Redlinger (1969) on *H. zea* eggs at ca. 26°C and 70% relative humidity.

The *H. zea* eggs used for the bioassays and for rearing the *Trichogramma* were obtained from laboratory cultures. The eggs were processed with a sodium hypochlorite wash as described by Burton (1969), irradiated with 25 krad (^{60}Co source) when 8–36 hr old, and stored at ca. 10°C prior to use.

The 1:1000 dilution of a hexane extract of *H. zea* moth scales (MSE) was formulated according to the procedure of Jones et al. (1973).

The bioassays were conducted for ca. 1 hr on a laboratory counter, under fluorescent lights, in 150 × 15-mm glass Petri dishes with six *H. zea* eggs and two (1-day-old) female *T. pretiosum* per dish. Treatment solutions (1 ml/dish) were applied using an aerosol chromatographic sprayer (Lewis et al., 1972). The dishes were arranged in a randomized complete block design to eliminate the effect on the parasites of phototropic response and other environmental inhomogeneities in the assay arena.

To determine the extent of parasitization for each treatment, the *H. zea* host eggs from the bioassay were crushed on a microscope slide and the number of parasite eggs were counted (Lewis and Redlinger, 1969).

Bioassay data were subjected to an analysis of variance and Duncan's multiple-range test. Arcsin transformations were performed on percentages prior to analysis.

RESULTS

Analysis of Extracts of H. zea *moth scales.* The extractable material comprised 8.2% of the weight of the moth scales. The hexane and methanol extracts contained the largest percentages by weight which were distributed as follows: hexane, 41.3%; chloroform, 9.0%; ether, 2.2%; acetone, 4.4%; and methanol, 43.1%.

Since the hexane extract (Soxhlet, 4 hr), from which the MSE is made,

was known to be active in the bioassay, it was not surprising that the hexane eluent was also active. Sorbic acid was found to be present in the chloroform fraction and in preliminary observations was examined with interest by the parasites.

It seemed likely if other acids were present and were active, that activity might be found in many fractions because of the propensity of acids to "tail" on polar adsorbents such as silica gel. Furthermore heptanoic acid has been reported as a kairomone for *Orgilus lepidus*, a wasp parasite of the potato tuberworm (Hendry et al., 1973). The volatile nature and strong odor of the ether extract was consistent with the presence of acids. For these reasons we pursued the identification of the acids in the ether extract.

A cursory bioassay showed the ether extract to be biologically active. The odor of the extract was mostly removed by extraction with sodium hydroxide solution. On acidification and reextraction, the odor returned. Infrared spectra of the crude ether extract showed two types of carbonyl.

Gas chromatograph–mass spectral analysis of the methyl esters of the acids was straightforward, except that it was not possible to distinguish between the methyl esters of 2- and 3-furoic acids because of virtually identical patterns of fragment ions.

All solvent extracts of scales were shown to be active in increasing the rate of parasitization of *H. zea* eggs by cursory laboratory bioassays. Activity was also present in all fractions after extraction with base to remove acids. Some of the fractions were found to be very complex mixtures. For example, after passage through silica gel with solvents of graded polarity, one eluent (40% chloroform in hexane) showed 231 peaks on glass capillary GC. The major component of this mixture comprised 6% of the total by electronic integration. The acidic fractions as methyl esters were also complex, as shown by glass capillary GC. However, a much simpler picture is shown by the total ion chromatogram when using a packed column in the gas chromatograph–mass spectrometer. With only major components showing on a packed column, the methyl esters of the acids were found to be those listed in Table 1.

Since the initial discovery of kairomones for *Trichogramma* species showed an homologus series of hydrocarbons to be active, we also tested acids that were part of the homologous series of fatty acids.

Bioassay 1. In this series of bioassays, each acid (100 μg/dish) was compared to MSE and to an untreated control. Each run consisted of 20 replications with four responsive runs per acid. A responsive run was defined as a run in which the difference between an untreated control and the standard MSE-treated dishes (run simultaneously with the acids) must have been at least 20 percentage points. Only runs meeting the above criterion of response by the parasites were included in the statistical analyses. Since the experiment was conducted during the winter, in a building under emergency energy restrictions, temperature/humidity fluctuations may have affected respones of the

TABLE 1. ACIDS IN *H. zea* MOTH SCALES[a]

Compound identified	% of total known compounds	Quantity (ng/insect)	% of total mixture	Retention time (min) of ester
Hexanoic acid	2.56	3	1.55	8.40
2- or 3-Furoic acid	5.82	8	3.53	9.55
Sorbic acid	22.91	30	13.88	10.44
Heptanoic acid	3.27	4	1.98	10.65
Benzoic acid	17.69	23	10.71	11.96
Octanoic acid	2.72	4	1.65	12.63
Phenylacetic acid	15.43	20	9.34	13.41
Nonanoic acid	1.82	2	1.10	14.34
p-Hydroxybenzoic acid Methyl-*p*-methoxy-benzoate	5.54	37	3.35	16.51
Methyl-4-hydroxy-benzoate	22.25		13.48	19.23
Total			60.57	

[a]Analyzed by GC-MS as methyl esters.

parasites. In several years of conducting bioassays standard MSE has given the most consistent response.

The results in Table 2 show that the female parasitoid responds positively to some, but not all, of the acids. A positive response was obtained from the 2-furoic, 3-furoic, hexanoic, and benzoic acids. The response to 3-furoic acid was not significantly different from that for the hexane extract of moth scales. Positive responses were also obtained with butyric acid and valeric acids that were not found in the scales. No response was obtained to the other acids present in the scales.

Bioassay 2. This bioassay was conducted to determine the kairomonal activity of the combined acids in the proportion found in the ether extract of scales (see Table 1), except that *p*-hydroxybenzoic acid was not included since its presence was attributed to the addition of a preservative, methyl-*p*-hydroxybenzoate, to the artificial diet. The acids were applied in an ether–hexane (1:1) solution containing a total of 72 μg/ml/dish with the percentage for each acid being expressed in μg; i.e., hexanoic, 2.56 . . .; benzoic, 17.69 μg . . .; nonanoic, 1.82 μg as in Table 1.

The assay allowed comparison of the response of female parasitoids to the combined acids, MSE, and an untreated control. The results, means of 80 replications, show no influence on the parasitization rates was obtained from the combination of acids, 55.1%, vs. control, 50.0%, whereas a simultaneous significant response to the crude extract, MSE, 71.7%, was obtained.

TABLE 2. PERCENT PARASITIZATION, IN PETRI-DISH BIOASSAYS, BY *Trichogramma pretiosum* RILEY FEMALES IN PRESENCE OF VARIOUS ACIDS[a]

Compound tested	Test mean	MSE	Control
Benzoic	48.2a	70.0b	35.2c
Phenylacetic	43.7a	62.0b	35.6a
2-Furoic	50.2a	63.1b	34.9c
3-Furoic	62.0a	69.3a	47.0b
Propionic	47.9a	69.3b	44.3a
Butyric	63.0a	76.8b	47.0c
Valeric	57.5a	71.8b	47.6c
Hexanoic	54.0a	63.8b	39.1c
Heptanoic	28.7a	46.2b	21.0a
Octanoic	25.2a	56.6b	28.0a
Nonanoic	36.3a	68.9b	45.7a
Sorbic	29.4a	55.0b	28.1a

[a]Means followed by different letters, for each test compound, are significantly different ($P < 0.05$) as determined by Duncan's multiple-range test. Eighty replications, except for butyric acid; 79 replications.

Bioassay 3. This experiment was conducted to determine the importance of acids in eliciting the response by *T. pretiosum* to MSE. The standard hexane extract was extracted with 10% NaOH solution in a separatory funnel to remove the acids. The hexane layer was dried over sodium sulfate and bioassayed with standard hexane extract and untreated control by the Petri-dish method used previously.

The results, means of 180 replications, show that the deacidified extract, 68.6%, did not decrease parasitization by *T. pretiosum* from MSE, 67.2%, and both were significantly higher than the control, 50.0%.

DISCUSSION

Our bioassays do not indicate a major kairomonal role for the identified acids. The bioassays do indicate that the wasps are capable of perceiving the acids. This indication is further supported by visual observations of intense antennation and klinokinesis in the presence of the acids by the parasitoids. The same behavior was noted when the wasps were previously observed at close range in the presence of both the hydrocarbons and MSE. The highly constrained arena of the laboratory bioassay used may be inadequate to measure subtleties of behavior that might clarify a possible role for the acids. It may be that the effect of the hydrocarbons overrides any effect of the acids in the response of the wasps to the deacidified MSE.

We believe it is adaptively advantageous for generalist parasitoids such

as *Trichogramma* species to be able to perceive a wide range of compounds and to correlate these cues with the presence of host eggs.

This study demonstrates the need for further examination of the moth scales, the need for a more thorough understanding of the behavioral acts involved in host location, and the specific roles played by semiochemicals.

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EFFECTS OF DODECYL ACETATE AND *Z*-10-TRIDECENYL ACETATE ON ATTRACTION OF *Eupoecilia ambiguella* MALES TO THE MAIN SEX PHEROMONE COMPONENT, *Z*-9-DODECENYL ACETATE

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Abstract—Attraction of *E. ambiguella* males to *Z*-9-dodecenyl acetate (*Z*9–12: Ac), alone and in combination with dodecyl acetate (12: Ac) or *Z*-10-tridecenyl acetate (*Z*10–13: Ac) was tested in both wind-tunnel and field experiments. In the laboratory, response to *Z*9–12: Ac reached a distinct dosage optimum at which attraction was nearly as good as to live females. Addition of 12: Ac, a minor component of female glands and effluvia, had no effect at low doses of *Z*9–12: Ac and only marginally improved attraction at the optimum. However, inclusion of 12: Ac with an overdose of *Z*9–12: Ac fully restored activity. In the field, *Z*9–12: Ac alone was not very attractive at any dose; catches markedly increased by adding 12: Ac and climbed with increasing dose of each compound up to 10 mg. Optimum ratio of *Z*9–12: Ac to 12: Ac was 1: 1 to 1: 5. Another compound, *Z*10–13: Ac, had the same effect as 12: Ac in the laboratory and the field but was effective at a lower dose. In the field, inclusion of the synergist permits use of high cap loads to attract more males, perhaps from greater distances.

Key Words—*Z*-9-Dodecenyl acetate, *Z*-10-tridecenyl acetate, dodecyl acetate, sex pheromone, synergism, behavior, wind tunnel, field trapping, European grape moth, *Eupoecilia ambiguella*, Lepidoptera, Tortricidae.

INTRODUCTION

Volatiles from sex pheromone glands in Lepidoptera often contain one or two main attractant components accompanied by a number of chemically related minor constituents. The latter may in part represent biologically inactive materials formed during biosynthesis but, in some cases, are known to exert an important effect, either by augmenting the biological activity of the phero-

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mone blend or by eliciting distinct behavioral effects of their own. Analysis of the behavioral function of each component is important for an understanding of the chemical language used during courtship and, in particular, for successful use of pheromones in pest control.

The final evaluation of pheromone blends usually involves field tests with natural populations. Wind tunnels permitting detailed analysis of moth behavior (Farkas and Shorey, 1972) proved useful in evaluating effects of changing blend composition (Baker et al., 1981) and of inhibitors (Preiss and Priesner, 1978) in the laboratory. Tunnels with moving floors permitted demonstration of optomotor responses (Kennedy and Marsh, 1974) and, subsequently, differentiation of response to enantiomers (Miller and Roelofs, 1978). Factors affecting mating disruption have been studied by Sanders (1981, 1982).

The following experiments with the European grape moth, *Eupoecilia ambiguella* Hb. (Lepidoptera, Tortricidae), were made to study possible behavioral functions of components of the female sex gland and to investigate in some detail attraction and synergism previously observed in field screenings. We were also interested in determining to what extent the attraction of grape moth males to live females could be duplicated by substituting the synthetic blend or if further chemical analyses were in order.

Z-9-Dodecenyl acetate (*Z*9-12:Ac) was identified as a main component of the sex pheromone (Arn et al., 1976). Secondary components include the corresponding *E* isomer in very small amounts, the corresponding *Z* alcohol, and the saturated straight-chain acetates of 12, 16, 18, and 20 carbons (Arn, 1981). While the latter four appear to act together in augmenting male attraction to *Z*9-12:Ac, dodecyl acetate (12:Ac) is the strongest synergist of all. Added in a fivefold excess to the monoene, this compound leads to a five- to sixfold catch increase, and caps with this mixture outcatch live females by a factor of 2–3; 12:Ac alone is not attractive. (Arn et al., 1979). A similar effect is observed with *Z*-10-tridecenyl acetate (*Z*10-13:Ac) in the field. From high-resolution mass fragmentograms at *m/e* 61, the latter compound, a homolog of the main pheromone component, was suspected present in *E. ambiguella* female effluvia, but its identity awaits confirmation (Arn et al., 1982).

METHODS AND MATERIALS

Chemicals and Dispensers. Chemical purity was 98% or better by capillary gas chromatography on Silar 10C. *Z*9-12:Ac and 12:Ac were purchased from Socar AG, Dübendorf, Switzerland. The former was purified by argentation chromatography (Heath et al., 1977) to contain 0.2% *E* which corresponds approximately to the proportion found in the females. *Z*10-13:Ac at 0.1% *E* was supplied by Dr. Simon Voerman, Institute for Pesticide Re-

search, Wageningen, The Netherlands. Hexadecyl, octadecyl, and eicosyl acetate were purchased from NuChekPrep Inc, Elysian, Minnesota; hexadecane, ethyl dodecanoate, and methyl tridecanoate from Fluka AG, Buchs, Switzerland.

Red rubber caps (Arn et al., 1979) were used as dispensers for wind-tunnel and field experiments. Chemicals were placed in the cavity of the cap in 20–100 μl hexane and the solvent allowed to evaprate. Fresh caps were made up weekly for the wind tunnel; the same caps remained in use for the duration of a test in the field.

Rearing of Insects. A continuous laboratory culture of *E. ambiguella* was maintained for 10 years on a diet developed for the codling moth (diet 1 of Mani et al., 1978) based on sawdust, wheat products, and casein. A modification of the agar-based diet of Hansen and Zethner (1979) was subsequently found more productive and time-saving for rearing the insects used in this study. It contained the following ingredients (by weight): agar agar 25, wheat germ 94, alfalfa meal 25, casein 44, brewer's yeast 19, Wesson's salt 12.5, sunflower oil 2.5, sucrose 37, sorbic acid 2, cholesterol 1.25, methylparaben 1.25, ascorbic acid 20, Vanderzant's vitamin mixture 7.5, 10% formaldehyde 1.4, aureomycine 2.5, water 750. The solidified mass was grated into shreds 3 mm wide and 3 cm long to increase the surface area. Infested trays were held at a 18:6 hr light-dark cycle with the scotophase starting at 2200 hr, at 24° C and 56% relative humidity. Mature larvae readily left the medium to enter corrugated paper strips for pupation. High humidity (above 65%) and moderate temperature (20° C) favor *E. ambiguella* oviposition. For this, 1-liter polystyrene containers holding ca. 100 male and female moths were lined with crumpled polyethylene bags. The moths were fed 10% sucrose solution from a wick and sprayed with water regularly. Turnover time for one generation was 5 weeks.

Insects used in the wind-tunnel tests were sexed as pupae and held in the same room as the wind tunnel to allow adjustment to photoperiod conditions.

Wind Tunnel. The tunnel was made of Plexiglas (50 × 50 × 170 cm) with access ports at the upwind and downwind ends. Since preliminary experiments with *E. ambiguella*, a night flier, gave no indication of an optomotor response and differences in behavior with changing blends could be readily observed, the tunnel was used without a moving floor (Figure 1).

Air from the temperature- and humidity-controlled room was pushed through an Airopac glass fiber filter and a Camcarb charcoal filter (both from Camfil A.B., Trosa, Sweden), then through layers of organdy and metal screen (1 mm^2), to smooth the flow, before entering the chamber.

Odor-laden air leaving the wind tunnel was taken up by a 35-cm-diam. metal port at the downwind end connected to a fan and passed through a second charcoal filter before reentering the room. No significant change in male response, which might occur due to contamination by chemicals, could be observed over eight months of experimentation.

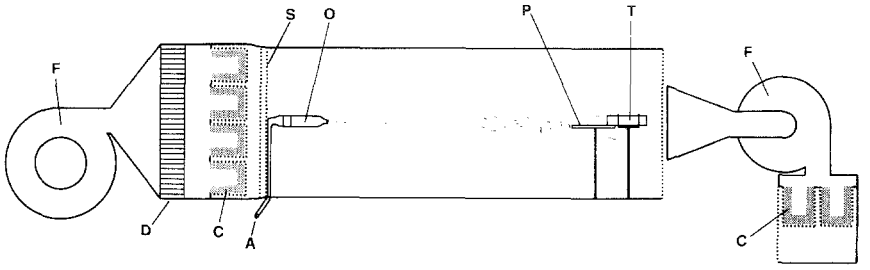

FIG. 1. Wind tunnel used in laboratory bioassays. F, centrifugal fan, D, dust filter, C, charcoal filter, S, screens, A, air inlet, O, odor source containing 3 live females or rubber cap with test chemicals, P, platform, T, male holding tube.

The females or rubber caps containing the test chemical were placed in a glass tube 150 mm long with a 29-mm standard joint at one end and the outlet tapered to a pipet tip of 4 mm ID pointing down the tunnel. The tube was attached to a socket centered at the upwind end of the tunnel. Air from a gas cylinder was introduced via a silicone rubber tubing at such a rate that the exit speed from the pipet tip was the same as that of the surrounding air.

A 15-cm^2 galvanized steel platform was mounted on a rod 30 cm from the downwind end of the chamber, at a level ca. 2 cm below the center of the odor plume.

Smoke created with $TiCl_4$ was used to visualize the air flow. Measurements of air speed with smoke puffs matched those of a Thermo-Air model 442 thermoelectric anemometer (E. Schildknecht Ing. SIA, CH-8625 Gossau, Switzerland), which was used to check localized air flow. The plume leaving the odor source became turbulent 20 cm from the tip but did not exceed 20 cm in diameter at the downwind end of the wind tunnel. The speed of the outlet fan was adjusted so that the plume was taken up fully, but the general air flow in the tunnel remained unaffected. Air speed was 30 cm/sec.

The experiment room was kept at a 16:8 hr photoperiod with lights off at 0930 hr. Temperature and relative humidity were 24° C, 56% with lights on, and 20° C, 82% with lights off. The insect holding area received 3000–6000 lux from daylight fluorescent tubes during photophase and 0.7–1.0 lux from incandescent lights during scotophase. The wind tunnel received diffuse overhead light from 220-V incandescent light bulbs operated at 160 V; intensity along the flight axis was 3.3–4.4 lux.

Preliminary experiments showed that under these conditions grape moth females began to call 3.5 hr after dark and continued for ca. 4 hr. Forty to 70% of females were found calling between 4.5 and 6.5 hr after lights off (1400–1600 hr). This period, during which male response was consistently highest, was chosen for observations.

Behavioral Observations. On the test day, individual 3-day-old males were placed in 25 × 150-mm glass tubes closed with nylon gauze before the

onset of the dark phase. Observations started 4.5 hr after lights off and lasted for 2 hr.

To start a test, a holding tube with the upwind gauze removed was placed on a wire rack with the open end resting on the downwind edge of the platform. The subsequent behavior was recorded: incidence of takeoff, upwind flight, and landing on the source. Times from start of test to takeoff and to end of observation were taken with a stopwatch. Observation was terminated and the male discarded when the latter (1) did not take off within 60 sec, (2) touched down on the odor source, or (3) touched another part of the wind tunnel.

Each treatment was tested with at least 50 males divided over 3 days. The sequence of treatments was randomized. The level of male activity was determined on each test day with positive controls; results from only one of 49 test days were eliminated as response to the control remained below 60%. Testing stopped after 2 days of observation with 20 males when the dose tested was clearly outside the optimum range.

For statistical analysis, percent males completing each behavioral step was transformed using the arcsin (Sokal and Rohlf, 1969) and submitted to analysis of variance followed by Duncan's multiple-range test. Flight times were transformed to logarithms and submitted to analysis of variance followed by Student-Newman-Keuls multiple-range test.

Field Experiments. Trapping tests were conducted in vineyards of Yvorne and Maienfeld in western and eastern Switzerland, respectively. Tetra traps with flaps (Arn et al., 1979) were placed at levels of 1–5 m; distances between treatments were 1.5–2 m. Positions of treatments were systematically varied in each replicate series to compensate for possible effects of wind direction, trap interaction, and unequal population density in the vineyard. At Yvorne, the six to eight treatments of a test were placed in two parallel rows; replicates were separated by 100–200 m. At Maienfeld, all traps were placed in two long rows with replicates following one another, and positions moved at each reading. In this case, three buffer traps, baited with 100 μg Z9–12:Ac and 100 μg 12:Ac, were placed at either end of each row to prevent moth buildup in the terminal traps of the test.

Catches were recorded every 2–7 days; data were transformed to $\log(x + 1)$ and submitted to two-way analysis of variance followed by Duncan's multiple-range test.

RESULTS

Wind Tunnel Experiments. Typical male response to female odor or equivalent chemical attractant can be characterized as wing fanning while leaving the tube and moving towards the upwind edge of the platform, takeoff, upwind flight in the odor plume, and touchdown on the tip of the odor

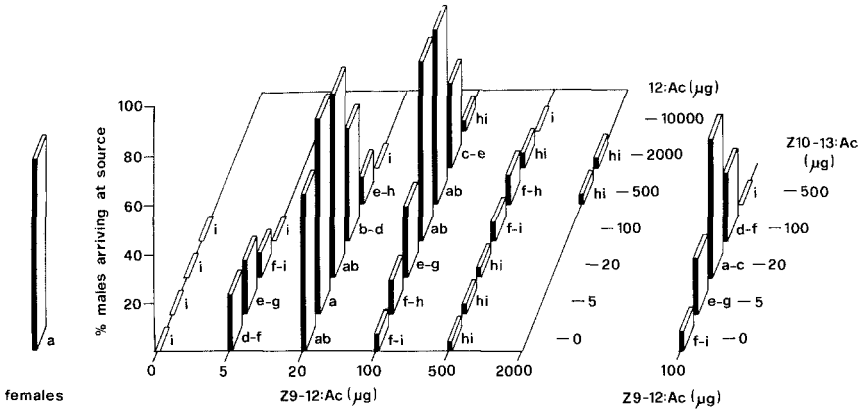

FIG. 2. Responses of *E. ambiguella* males to live females and to various dosages and blends of test chemicals in the wind tunnel. Values in the same test followed by the same letter are not significantly different at P = 0.05.

source. After touchdown, a male usually fanned from a second up to a minute before departing. Usually, males which had touched down once fell back downwind and, if not eliminated from the wind tunnel, made several successfull approaches to the source. Some males went into erratic flight after leaving the platform and eventually were eliminated. Others left the plume before reaching the source and, in some cases, reached the source in a further attempt.

In one test series, cap loads of Z9-12:Ac and 12:Ac were varied over 30 treatments. Figure 2 shows the overall response expressed as percent males arriving at the odor source. With Z9-12:Ac alone, male respone was 23% at a load of 5 μg, peaked at 20μg (64%), and dropped again to low levels (7%) at 100 μg and beyond. Maximum response to Z9-12:Ac alone was not significantly lower than to virgin females.

Addition of 12:Ac had different effects at different levels of Z9-12:Ac. At suboptimal dose of Z9-12:Ac, it led to a decrease in attraction; at the optimum dose of Z9-12:Ac, 12:Ac caused a small increase before becoming inhibitory.

A striking effect of 12:Ac on attraction was observed when Z9-12:Ac was present above the optimum level. At 100 μg Z9-12:Ac, adding 12:Ac restored activity to the same level as that of virgin females (78%); responses were between 71 and 73% at ratios of the two compounds of 1:1 and 1:5, higher levels of 12:Ac being inhibitory. An optimum was also apparent at 500 μg of each compound, but the overall response was very low.

Behavioral steps are broken down in Figure 3 for the most pertinent cuts across the preceding diagram. Takeoff, upwind flight, and landing clearly follow the same maxima and are influenced in the same way by the

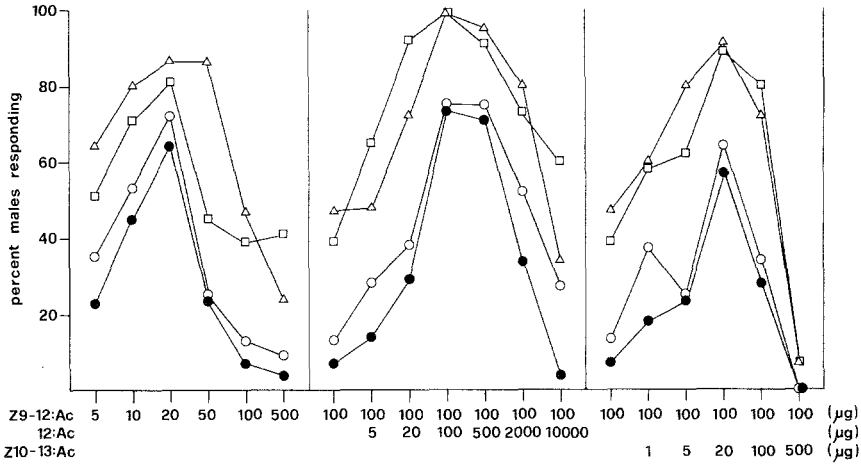

FIG. 3. Wind-tunnel responses of *E. ambiguella* males broken down into behavioral steps. △ takeoff, □ upwind flight, ○ landing at source as percent of males succeeding in the previous event, ● landing as percent of total males in test.

composition of the odor. Curves for takeoff and upwind flight are generally higher and wider, suggesting that conditions for these two steps are less critical than for landing.

A more detailed account of responses at maxima and other treatments included for comparative purposes is given in Table 1. The optimum dose of Z9-12:Ac combined with the smallest amount of 12:Ac tested (5 µg) was superior to 20 µg Z9-12:Ac alone in terms of percent of all insects arriving at the source and to the 100:100 blend for percent of upwind fliers arriving at the source. This may reflect the importance of small amounts of 12:Ac observed in female extracts and effluvia. Addition of 100 µg 12:Ac to an overdose of Z9-12:Ac (100 µg) restores activity to optimal levels as judged by all criteria; this blend only fell short of female activity in terms of arrival at the source, but the difference was not great.

Time to takeoff was the same for all treatments. However, flight time to the source was longer in the presence of 12:Ac than without, which may suggest that this compound induces a searching behavior. This contrasts with the findings for the cabbage looper (Bjostad et al., 1980) where the time spent by males searching for the source decreased in the presence of 12:Ac. Data on males flying upwind but not reaching the source are fewer but seem to follow the same trend.

Figures 2 and 3 also include responses obtained with Z10-13:Ac. Adding this compound to 100 µg Z9-12:Ac restored activity to nearly the same level (57%) as did 12:Ac, but the maximum was reached at a lower dose (20 µg). The curve for the individual behavioral steps follows the same pat-

TABLE 1. WIND-TUNNEL RESPONSES OF *E. ambiguella* MALES TO VIRGIN FEMALES AND VARIOUS ATTRACTANT BLENDS[a]

Treatment	No. of males in test	Taking off (%)	Fliers going into upwind flight (%)	Upwind fliers arriving at odor source (%)	Of all tested, arriving at odor source (%)	Time to takeoff (sec)	Flight arrivals (sec)	Upwind flight time of nonarrivals (sec)
Females	170	93 a	94 ab	85 a	78 ab	26.3	25.1 a	39.9
Z9-12:Ac, 20 μg	188	87 a	81 b	72 ab	64 bc	23.8	25.2 a	49.7
Z9-12:Ac, 20 μg + 12:Ac, 5 μg	75	96 a	95 ab	88 a	80 a	19.9	34.9 bc	50.3
Z9-12:Ac, 100 μg	278	47 b	39 c	13 d	7 d	25.0	22.1 ab	34.1
Z9-12:Ac, 100 μg + 12:Ac 100 μg	240	97 a	97 a	75 bc	73 abc	22.2	35.6 c	50.6
Z9-12:Ac, 100 μg + Z10-13:Ac, 20 μg	70	91 a	89 ab	64 c	57 c	21.2	25.7 abc	42.7

[a]Numbers in the same column followed by the same letter are not significantly different at $P = 0.05$.

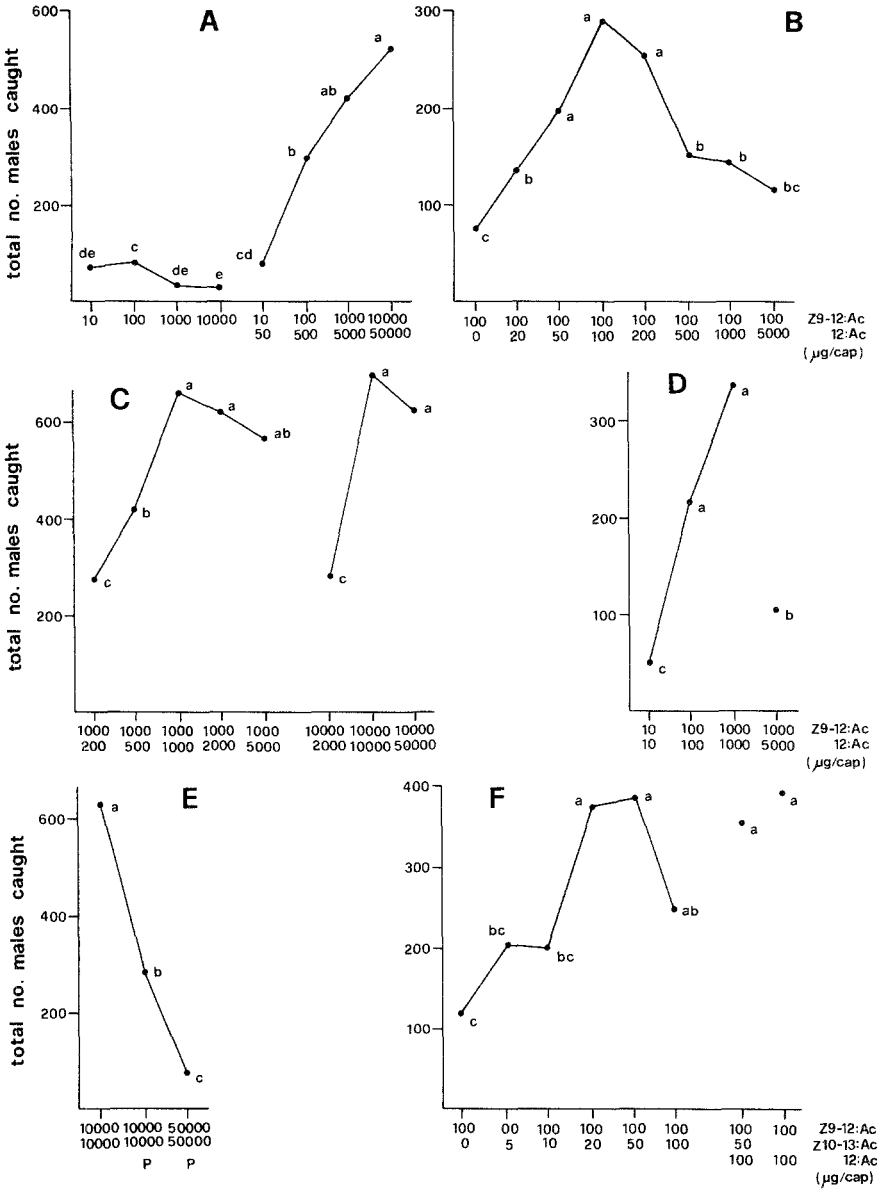

FIG. 4. Catches of *E. ambiguella* males with various dosages and blends of test chemicals (10 replicates). A, B, F: Yvorne, May 7–June 2; C: Maienfeld, July 1–16; D, July 16–27; E, July 12–27; 1982. Values in the same test followed by the same letter are not significantly different at $P = 0.05$. Formulations on rubber caps, except P = polyethylene tubing.

tern as for landings, as observed with 12:Ac; however, the maxima were reached at considerably lower levels.

Three other saturated acetates found in female extracts and effluvia were tested in combination with 100 μg Z9-12:Ac and 3 additional compounds with structural similarities and in the same molecular weight range as 12:Ac but not found to occur in *E. ambiguella*. Doses tested were 1-100 μg for hexadecyl acetate, eicosyl acetate, and methyl tridecanoate, 1-2000 for hexadecane, 5-2000 for ethyl dodecanoate, and 1-10,000 for octadecyl acetate, with 20 replicates per treatment. No activity similar to that of 12:Ac was observed; male response was in no case greater than 30% total arrivals, not significantly different from the response to 100 μg Z9-12:Ac alone.

Field Tests. Results of field experiments are given in Figure 4 (A-F). Z9-12:Ac on its own did attract some *E. ambiguella* males but was not very active. As observed in previous experiments (Arn et al., 1979), addition of 12:Ac resulted in a drastic catch increase of up to 16 times in some cases (A). At a load of 100 μg Z9-12:Ac per cap, optimum attractant-to-synergist ratio was 1:1 (B). At cap loads 10-100 times higher, the optimum was still at 1:1, but some tests also gave excellent catches at a ratio of 1:5 (C); in others this ratio was beyond the optimum (D).

In contrast to the attraction of Z9-12:Ac alone, blends of Z9-12:Ac with 12:Ac became more attractive with increasing dose up to a level of 10 mg/cap (A). Since higher cap loads were impractical, dispensers with higher release rates were made from polyethylene tubing. The half-life of these dispensers was about 3 weeks. In this formulation, a level of 10 mg of each component was clearly overdosed; further decrease of attraction was observed at a load of 50 mg (E).

The results with Z10-13:Ac fully confirmed the wind tunnel observations. With Z9-12:Ac at 100 μg, Z10-13:Ac was maximally effective at 20-50 μg/cap; catch increase was three-fold, confirming previous reports (Arn et al., 1982). Adding both 12:Ac and Z10-13:Ac at the optimum level led to no further catch increase (F).

DISCUSSION

In laboratory and field experiments 12:Ac and Z10-13:Ac have a positive influence on male attraction when added to Z9-12:Ac. The two compounds are mainly effective when Z9-12:Ac is present at a relatively high level. In the wind tunnel, adding either compound to an overdose of Z9-12:Ac restores activity to the level of calling females. In the field, they both increase attraction of Z9-12:Ac; while Z9-12:Ac alone, at any dose, caught relatively few insects, a sharp rise of catches was observed with dose in the presence of 12:Ac, leading to a 16-fold increase of catches at the optimum.

Despite the vast differences between conditions of the two test methods, maximum responses are reached at similar cap doses in the laboratory and the field. In the latter, however, the cap remains attractive at higher doses which are inhibitory in the wind tunnel. The laboratory test is more stringent since the criterion used is male landing on the very tip of the odor source, whereas in the field an approach within some 10 cm is sufficient for capture. Furthermore, while the plume in the wind tunnel is rather sharply delimited, the irregular air flow prevailing under natural conditions may permit the moth to avoid zones of overdose and to follow optimum stimulus dosage boundaries, probably even close to the source.

The effect of secondary components on attraction observed here appears to differ from those of other investigators (e.g., Baker et al., 1981) in two important respects: Z9–12:Ac is attractive on its own, and no positive effect of the additives is apparent at low doses of Z9–12:Ac. Both 12:Ac and Z10–13:Ac are effective in restoring attractiveness of Z9–12:Ac at overdose. As a hypothesis, one could assume a competitive effect between Z9–12:Ac and the synergists at the receptor level, preventing overload. Z10–13:Ac appears to be more effective since it produces a similar effect at lower doses than 12:Ac; The optimum attractant-to-additive ratio is 1:1 to 1:5 for 12:Ac and 5:1 to 2:1 for Z10–13:Ac.

In this context it is interesting that the molecular structure of Z10–13:Ac is identical to Z9–12:Ac, the only difference being an additional methylene group towards the acetate end of the molecule. On the other hand, Z-9-undecenyl acetate with the same structure from the acetate end up to the double bond, but with a methylene group less at the hydrocarbon end, is attractive on its own (unpublished). Molecules showing close structural similarities to the main attractant may display relatively good receptor fit (cf. Priesner et al., 1977).

We do not know at present how the observed phenomena relate to those of male attraction to living females. In our attractive mixtures, the optimal amounts of additives are far higher than what could be present in the female effluvia. Perhaps 12:Ac at these high levels mimics in part the additional saturated acetates of 16, 18, and 20 carbons found in female extracts and effluvia. Added singly in wind-tunnel tests, these compounds showed no effect; however, field tests indicate that they can augment catch when added together (Arn, 1981, and unpublished).

The results appear to indicate that presence of either 12:Ac or Z10–13:Ac permits use of high release rates at which Z9–12:Ac alone would not be attractive, to draw in more males, perhaps even from greater distances. This can be discussed in the light of the threshold hypothesis (Roelofs, 1978). Accordingly, 12:Ac and Z9–12:Ac would both serve to increase the disorientation threshold and, as a result, widen the "attraction area" towards higher loads. Our findings may have consequences for the use of pheromones in pest control since recent results (Sanders, 1981, 1982) support the view that

the better attractant may also be the better disruptant. It is conceivable that in the grape moth or other species, additional compounds can be found which augment trap catch in a similar way to that reported here.

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BEHAVIORAL EFFECTS OF SECONDARY COMPONENTS OF SEX PHEROMONE OF WESTERN SPRUCE BUDWORM (*Choristoneura occidentalis*) FREE.[1]

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Abstract—In a laboratory wind tunnel, upwind flight and close-range orientation to a pheromone source by male *C. occidentalis* were facilitated by the addition of the secondary components *E*/*Z*11-tetradecen-1-ol acetate (89:11) and *E*/*Z*11-tetradecen-1-ol (85:15) to low source concentrations of the primary components, *E*/*Z*11-tetradecenal (92:8). Male responses to the blends tested never equalled their responses to virgin females. The primary components alone, when released at a rate similar to that of a "calling" female, never elicited male upwind flight or source location. However, the addition of the secondary components enhanced these behavioral sequences.

Key Words—*Choristoneura occidentalis*, western spruce budworm, Lepidoptera, Tortricidae, pheromones, behavior, wind tunnel.

INTRODUCTION

The sex pheromone of the western spruce budworm *Choristoneura occidentalis* Freeman has been reported to be similar to that of the spruce budworm *C. fumiferana* Clem. (Sanders, 1971; Weatherston et al., 1971). Recent work by Cory et al. (1982) demonstrated that a mixture of 92% (*E*)- and 8% (*Z*)-11-tetradecenal (*E*/*Z*11-14:Ald) was emitted from female *C. occidentalis*, and this mixture was shown to be at least as attractive as females in

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the field. Silk et al. (1982) have published similar results, but in contrast, they also reported the presence of significant quantities of *E*- and *Z*-11-tetradecen-1-ol acetates (*E*/*Z*11–14:Ac) and *E*- and *Z*-11-tetradecen-1-ols (*E*/*Z*11–14:OH) (and their saturated analogs) in both female gland washes and effluvia. This study examines the effects of *E*/*Z*11–14:Ac and *E*/*Z*11–14:OH on the sexual communication system of the western spruce budworm.

METHODS AND MATERIALS

Bioassays. Flight bioassays were conducted in a sustained-flight wind tunnel (after Kennedy and Marsh, 1974; Miller and Roelofs, 1978). The all-glass tunnel had a working section 2.4 × 0.9 × 0.9 m with a striped floor pattern positioned 5 cm below the floor (Alford et al., 1983).

Male and female *C. occidentalis* pupae (diapausing strain) were supplied through a laboratory rearing program at Forest Pest Management Institute, Canadian Forestry Service, Sault Ste. Marie, Ontario. Pupae were sexed and held in 4-liter cardboard cartons at 21° C on a 16:8 light–dark cycle. Newly emerged adults were segregated daily, and the sexes were kept in separate holding chambers. Males, 2–4 days old, were flown during the 1.5 hr prior to lights off, at a light intensity of 4–5 lux (Alford et al., 1983).

Male moths were held individually in 3 × 6-cm cylindrical metal screen cages for at least 12 hr prior to their use. Each caged male was introduced into the downwind end of the tunnel and held in the air stream (50 cm/sec) for 5 min to allow for adjustment to tunnel conditions. The test chemicals were suspended 15 cm above the center of the tunnel 2 m upwind of the males. Observations of single males began immediately after introduction of each chemical stimulus. The following behavioral events were recorded: wing fanning, flight, upwind flight (of >25.0 cm), contact with the source, and copulatory attempts directed at the source. Test stimuli were used for a maximum of 5 min and were random in their order of evaluation.

Chemicals. All components were obtained from Chemical Samples Co., Columbus, Ohio and were >98% pure with no detectable quantities of the respective opposite geometrical isomers (as determined by capillary GLC, 40 m SP 1000 SCOT column, Silk et al., 1980).

All test chemicals were prepared in *n*-hexane at concentrations of 5 ng/μl. These were *E*- and *Z*11–14:Ald, *E*- and *Z*11–14:Ac, and *E*- and *Z*11–14:OH at *E*/*Z* ratios of 92:8, 89:11 and 85:15, respectively (Silk et al., 1982). Chemicals were pipetted onto 1 × 4-cm filter paper strips to give the appropriate source concentrations (Table 1). The solvent was allowed to evaporate for 5 min and the strips were then sealed individually in small vials and each used within 10 min.

TABLE 1. BEHAVIORAL EFFECTS: SOURCE CONCENTRATIONS OF ALDEHYDES AND EFFECTS OF ADDING ACETATES AND/OR ALCOHOLS TO ALDEHYDE SOURCES

	No. of subjects	No. wing fanning	Initiating flight (%)[a]	Flying upwind (%)	Contacting lure (%)	Attempting to copulate at lure (%)
Females	23	21*[b]	100 *	100 *	100 *	90.5*
10 ng Ald	83	63	15.9 †[c]	6.3 †	3.2 †	0 †
25 ng Ald	35	32	71.9*†	68.8*†	56.3*†	0 †
50 ng Ald	16	14	78.6*	78.6*	57.1 †	0 †
10 ng Ald +						
5 ng Ac	21	16	62.5*†	43.7*†	25.0 †	0 †
10 ng Ac	47	41	85.4*†	80.5*†	48.8*†	2.4 †
20 ng Ac	27	23	87.0*	69.6*†	39.1*†	4.3 †
35 ng Ac	16	15	53.3*†	46.7*†	26.7*†	0 †
10 ng Ald +						
5 ng OH	15	10	70.0*†	50.0*†	30.0 †	0 †
15 ng OH	15	11	63.6*†	36.4*†	18.2 †	0 †
35 ng OH	38	29	86.2*†	65.5*†	51.7*†	3.4 †
50 ng OH	30	28	92.9*	67.9*†	53.6*†	0 †
75 ng OH	21	20	65.0*†	50.0*†	30.0*†	0 †
10 ng Ald +						
5 ng Ac + 5 ng OH	25	21	85.7*	81.0*†	61.9*†	19.0*†
10 ng Ac + 15 ng OH	18	14	78.6*	71.4*†	35.7*†	7.1 †
10 ng Ac + 35 ng OH	41	36	94.4*	91.7*†	69.7*†	2.8 †
10 ng Ac + 50 ng OH	24	22	95.5*	95.5*	63.6*†	4.5 †
10 ng Ac + 75 ng OH	14	12	66.7*	25.0 †	25.0 †	0 †
20 ng Ac + 35 ng OH	21	18	88.9*	62.5*†	16.7 †	0 †
20 ng Ac + 50 ng OH	20	16	87.5*	56.3*†	12.5 †	0 †
20 ng Ac + 75 ng OH	15	12	50.0*†	25.0 †	0 †	0 †

[a]Values presented are percentages of the number of males wing fanning.
[b]Values followed by asterisks are significantly different ($P < 0.05$) than corresponding values of behavioral effects with 10 ng Ald alone; determined by test for differences between two proportions in a binomial distribution.
[c]Values followed by † are significantly different ($P < 0.05$) than corresponding values of behavioral effects with females; determined as in *b*.

RESULTS

Initial tests determined the upwind flight threshold of *C. occidentalis* males to the aldehyde components (i.e., 92:8 *E*/*Z*11–14:Ald) as demonstrated by Cory et al. (1982) to be necessary for trap capture. A 10-ng source concentration was below, or near, the flight threshold in the wind-tunnel system (Table 1); only 6% of the males that initiated wing-fanning subsequently flew upwind, while 69% and 79% of activated males flew upwind when exposed to 25-ng and 50-ng sources, respectively. Brief wing-fanning was observed in ca. 15% of males exposed to 5-ng sources of the aldehydes.

Subsequent tests utilized the 10-ng source concentration with admixture of the acetates and alcohols (Table 1). Males exhibited no response when exposed to the acetates or alcohols alone (one male initiated wing-fanning when exposed to an acetate source). The acetate–aldehyde mixture elicited the greatest activity in the two-component tests when they were present at equal source concentrations (10 ng). The addition of the alcohols to 10 ng sources of the aldehydes elicited the greatest activity when present at a source concentration range of 35–50 ng.

Similar levels of activity were observed with the three-component combinations of 10 ng Ald + 10 ng Ac + 35 and 50 ng OH. These two treatments elicited levels of upwind flight comparable to virgin females (Table 1). However, no combination tested was as effective as females in eliciting contact and copulatory attempts at the source. Interestingly, the three-component treatment of 10 ng Ald + 5 ng Ac + 5 ng OH elicited a significant increase in copulatory attempts compared to 10 ng Ald alone or to females. However, this occurred during one test period only and was not observed in subsequent trials.

DISCUSSION

The 10ng filter paper source used in this study released the aldehydes at a rate similar to that of "calling" female *C. occidentalis* (Silk, unpublished data), yet only a few males initiated flight to this synthetic source and none directed copulatory attempts toward the source, in contrast to the males' responses to females. Increased release rates (source loadings) of the aldehyde components alone increased the number of males flying upwind to contact the pheromone source, but tests run for the purpose of defining component ratios to which males are most responsive should be conducted at release rates approximating that used by females (Roelofs, 1978). The inability of previous trapping studies (Cory et al., 1982) to ascertain the participation of the acetates and alcohols in the sex pheromone communication of *C. occidentalis* may have been due to relatively high release rates

E/*Z*11–14:Ald (0.1% PVC pheromone sources). Additionally, traps only yield data on relative trapping efficiencies and are thus inefficient by themselves in assessing the total pheromone blend.

In our study, the use of aldehyde release rates approximating that of a female yielded a discriminating bioassay for demonstrating that the *E*/*Z*11–14:Ac's and *E*/*Z*11–14:OH's influenced the responsiveness of *C. occidentalis* males by increasing the number of males initiating upwind flight and increasing contact with the pheromone source. At equal loading rates on filter paper, the unsaturated aldehydes, acetates, and alcohols have a release ratio of ca. 1.0:0.2:0.1, respectively (Silk, unpublished data). Consequently, males used in this study were not exposed to these three groups of compounds in the exact proportion found in the effluvia from females (1.0:0.3:0.8, Silk et al., 1982). However, the best three-component treatment (Table 1) had relative release ratios of the three compounds close to the female effluvial rate and ratios (ca. 1.0:0.2:0.5).

It appears that both the unsaturated acetates and alcohols emitted by "calling" virgin *C. occidentalis* (Silk et al., 1982; Cory et al., 1982) are important components of the sex pheromone of this insect. In addition, the saturated analogs present in female effluvia (Silk et al., 1982), although not considered in this study, should be examined for possible behavioral activity as has been observed in the congeneric eastern species, *C. fumiferana* (Alford et al., 1983). We are in the process of further calibrating pheromone dispensers in order to develop sources with release rates and component ratios (for all three component classes) equaling that from virgin females to more accurately define the sex pheromone of *C. occidentalis*.

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ROLE OF PHEROMONE COMPONENTS IN EVOKING BEHAVIORAL RESPONSES FROM MALE POTATO TUBERWORM MOTH, *Phthorimaea operculella* (ZELLER) (LEPIDOPTERA: GELECHIIDAE)[1,2]

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Abstract—Responses of the male potato tuberworm moth, *Phthorimaea operculella* (Zeller), to two sex pheromone components and female crude extract were studied. Both in a wind tunnel and the field, males were better able to locate a source consisting of a 1:1 mixture of (*E,Z*)-4,7-tridecadien-1-yl acetate and (*E,Z,Z*)-4,7,10-tridecatrien-1-yl acetate than a source consisting of the triene alone. The addition of the diene increased the time spent in the vicinity of the pheromone source, time spent on the source itself, and also increased the average number of visits to the source per individual when compared to the triene alone. The triene elicited high levels of locomotor activity and may play a major role in eliciting earlier (long-range) steps in the behavioral sequence of sexually activated moths. The diene appeared to influence later (short-range) behavior. The behavioral responses of males to a 1:1 mixture of the diene and triene were similar to those elicited by female crude extract.

Key Words—Potato tuberworm moth, *Phthorimaea operculella*, (*E,Z*)-4,7-tridecadienyl acetate, (*E,Z,Z*)-4,7,10-tridecatrienyl acetate, insect sex pheromone, Lepidoptera, Gelechiidae.

INTRODUCTION

The use of pheromones in plant protection for monitoring populations and for direct control through air permeation has been studied intensively in the hope that this may reduce, at least partially, problems arising through the

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application of broad-spectrum insecticides. However, although knowledge of the chemical structures of compounds involved in sexual communication in moths has increased greatly, the role of individual pheromone components is little understood (Roelofs and Cardé, 1977).

After several partial identifications (Fouda et al., 1975; Roelofs et al., 1975; Yamaoka et al., 1976), two components of the sex pheromone of females of the potato tuberworm moth, *Phthorimaea operculella* (Zeller) (Gelechiidae) (PTM), were identified as (*E,Z*)-4,7-tridecadien-1-yl acetate (*E,Z*4,7–13:Ac) and (*E,Z,Z*)-4,7,10-tridecatrien-1-yl acetate (*E,Z,Z*4,7,10–13:Ac) (Persoons et al., 1976). The objective of the present study was to investigate how these pheromone components influenced the sexual behavior of males of this serious cosmopolitan pest of potatoes.

METHODS AND MATERIALS

Insects. Experimental insects were reared in a laboratory culture derived from larvae collected from potato plots at Ginninderra Experimental Station, CSIRO Division of Plant Industry (ACT). The larvae were reared on potato tubers.

Female Extracts. For quantification of pheromone components, the distal abdominal segments together with the ovipositor of 100 two- to three-day old females were excised between the second and fourth hours of the dark period (15.5:8.5 light–dark regime, at 25° C), and extracted in cyclohexane (ca. 1 ml) for 24 hr. The supernatant extract was then removed and filtered through Na_2SO_4 and a layer of cottonwool. The volume of the extract was reduced in a nitrogen stream to 0.1 ml. Injections were made into a Varian aerograph model 1400 gas chromatograph (4-m × 2-mm glass column, 5% Carbowax 20 M on Gas-chrom Z, 80/100 mesh, helium at 25 ml/min, 180°) which was connected to a VG model 70/70 mass spectrometer set to scan repeatedly over m/z 79, which is a common ion for both *E,Z*4,7–13:Ac and *E,Z,Z*4,7,10–13:Ac. Retention times of peaks from the extract coincided well with those from synthetic standards. Comparison of peak heights allowed the pheromone component content of the female extract to be calculated.

Synthetic Pheromone Components. The synthetic components were obtained from the University of Southampton, U.K. The diene sample (PTM1) had a nominal purity of 95% and the triene (PTM2) 85%. The samples were reanalyzed at CSIRO by gas chromatography on a Varian aerograph model 2100 (SCOT, glass 55 m × 0.5 mm, Silar 10C, nitrogen, 150°) and by gas chromatography–mass spectrometry (4-m × 2-mm glass column, 10% Silar 10C on AW-DMCS chromosorb W 80/100 mesh, helium at 25 ml/min, 158°). The major peak (triene) from PTM2 accounted for 77.6%, with the next largest component (at 10.7%) being a diene.

The standard solution was prepared to contain 5 ng of PTM1 and 5 ng of

PTM2 in each microliter. The calculation of the amounts in the extracts took into account the measured proportion of diene and triene components in the synthetic samples.

Laboratory experiments. Laboratory wind tunnel studies were made with males that had been held under a 15.5:8.5 light–dark regime at 25%. A simple wind tunnel (112 cm long, 22 cm ID) was constructed from transparent PVC sheets. Filtered air from a remote compressor was conveyed through the tunnel via a plastic funnel, at a rate of about 12 liters/min (approx. 20 cm/sec in the tunnel). The end of the funnel was covered with a cloth to reduce turbulence in the air entering the tunnel. Air was continuously exhausted from the experimental chamber to avoid contamination. The pheromone source was placed in the middle of the upwind quarter of the tunnel, on a glass Petri dish, located on a Plexiglas plate (21.5 × 17 cm). The observations were made for a maximum of 15 min for any one treatment (one moth/observation) and began 6 hr after the onset of the scotophase. When the treatment was changed, the glass Petri dish was also replaced and the tunnel purged with clean air for at least 30 min.

Individual 2- to 5-day-old males were placed in a 15 × 5-cm wire screen tube open at one end. The tube was positioned with its long axis parallel with the wind flow in the middle of the downwind end of the tunnel with the open end facing upwind. Males, in response to pheromone, could escape from the tube via the open end and fly towards the odor source. The distance between the release point and the odor source was about 100 cm. Verbal descriptions of the behavior of the male were recorded with a cassette recorder. Individual observations were terminated if a male remained inside the release screen tube for 60 sec after positioning of the tube. Males leaving the release tube were scored for exhibiting the following behavioral steps: (1) leaving the release tube; (2) wing-fanning while walking; (3) jumping (short bouts of flight); and (4) contacting the source. In addition, the time spent on the Plexiglas plate holding the source and on the source itself was also measured. Observations in the source area terminated when a male remained stationary for 30 sec. Male moths were used only once and then discarded.

The pheromone dispenser was a 1-cm length of red rubber tubing (6 mm OD, 4 mm ID), which was treated with 20 μl volume of cyclohexane solution, containing 200 ng of the synthetic diene or triene (or both, depending on the treatment), or 20 female equivalents (FE) of the female extract. New dispensers were prepared every second day. Between experiments, dispensers were stored individually in sealed glass vials at −20°.

Field Experiments. Studies of male behavior close to an attractant source in the field were conducted using a flat, circular white arena of plywood with a radius of 80 cm. The pheromone dispenser was located in the middle of the arena on a small glass Petri dish. The arena was marked with a circle at a radius of 10 cm from the center. The arena was placed on the ground in a potato field (Ginninderra Experimental Station, CSIRO Division of Plant

Industry, ACT). Visual observations of male behavior were made with the assistance of a night-vision device (NVD) (Javelin model 226). Verbal recordings of observations of male behavior were made using a portable cassette recorder. The observer was stationed about 2 m crosswind of the pheromone source.

Observations were conducted on 5 days between 2000 and 2300 hr in January and February, and on 3 days between 1900 and 2100 hr in March, 1981. Although potato tuberworm moths were present in the field, numbers were low, and it was therefore necessary to release batches of laboratory-reared males (2–5 days old) 4–5 m downwind from the pheromone source. These males emerged under natural light : dark conditions and were held until use in a glasshouse (25–35°). After the release of one batch, observations were made for 15–20 min; after this time the bait (dispenser plus Petri dish) was changed. The dispensers and the treatments were the same as those described in the laboratory experiments. Observations started when a male entered the field of vision of the NVD (about 30–40 cm from the source). The males were scored for entering the circle (10 cm radius) around the source and for contacting the source. Time spent inside the 10-cm-radius circle and on the source was also recorded.

RESULTS

Quantity of Pheromone per Female The mean quantity of each of the known pheromone components in the extract was found to be 3.5 ng/female for *E,Z*4,7–13 : Ac and 3.7 ng/female for *E,Z,Z*4,7,10–13 : Ac. This supported the findings of Persoons et al. (1976), who found that the ratio of the two compounds in female extracts was about 1 : 1. The 20 FE of female extract, used in some of the experiments, was estimated to contain about 140 ng of pheromone, while the other pheromone sources contained 200 ng of synthetic compounds.

Sexual Behavior of Males in Wind Tunnel Tests. In the wind tunnel experiments, the male, when activated by a pheromone stimulus (female extracts or both pheromone components), generally ran to the upwind end of the release cage and then flew from the cage in an upwind direction. However, this flight was not continuous; after a very short time males alighted on the walls of the tunnel and spent considerable time running, with or without wing fanning. Running was frequently interrupted by short "jumping" flights. Sometimes these jumps were almost perpendicular to the substrate so that the insect remained close to its starting point. In other cases, jumps were oriented upwind and carried the insect close to the pheromone source. Most frequently males reached the Plexiglas plate on which the pheromone source was situated by jumping from the wall of the tunnel. Once on the plate, males walked or ran on its surface until they had located the

source, whereupon they touched it with their antennae and climbed upon it. However, no copulatory movements were observed. Wing fanning while walking on the plate was observed throughout the approach to, and while climbing onto, the pheromone source. In the more effective treatments a male would walk off the pheromone source and contact it again several times without leaving the Plexiglas plate. Males that finally lost interest in the source either settled down and sat motionless on the Plexiglas plate, or jumped from the vicinity of the source, generally in an upwind direction. Such males usually settled down after some time on the walls or the linen cloth at the upwind end of the tunnel.

Most males became active and left the release cage in an upwind direction when exposed to the triene alone, diene + triene, or female extract (Table 1). These treatments also elicited sexual responses in most individuals in the form of wing fanning while walking. A significantly lower number of males responded to the diene alone.

No males located the source when this was treated with diene alone. Similarly, with the triene alone, only 2 of 30 males contacted the pheromone source. Significantly higher numbers found the source when this contained diene + triene or female extract (numbers at the latter two treatments did not differ significantly from one another). The same applied to the average number of visits to the source per individual. While there were very few visits to the source containing triene alone, significantly greater numbers came to the diene + triene or female extract treatments (Table 1).

The average time spent in the vicinity of the pheromone source and on the source itself was also significantly longer with the diene + triene and female extract than in the two other treatments. The average number of jumping flights was much lower with the diene alone than in the other three treatments, but there was also a significant difference between the triene alone and female extract.

Sexual Behavior of Males in Field Tests. In the field, the first males appeared in the field of vision of the NVD within 30–60 sec of their release. Further males arrived for about 10 min. Almost all arrivals were running or walking on the surface of the arena, and only a few individuals were seen to fly or jump.

Very few males came to the diene alone. When the bait consisted of triene alone, males arrived very soon after release, running vigorously in a more or less straight line upwind. Most of them did not stop or start to search for the source, but ran directly through a segment of the 10-cm circle around the source and disappeared upwind.

With sources containing diene + triene or female extract, males appeared with equal rapidity after having been released, but their approach was slower and less direct than in the tests with the triene alone. Males walked along a zig-zagging route, especially in the vicinity of the source. They climbed upon and usually spent some time on the source after this had been contacted.

TABLE 1. RESPONSES OF POTATO TUBERWORM MOTH MALES TO SYNTHETIC PHEROMONE COMPONENTS AND FEMALE PHEROMONE EXTRACT IN LABORATORY WIND TUNNEL[a]

Treatment (ng)	Leaving release cage (%)	Sexually excited (%)	Locating pheromone source (%)	Average number of jumps	Number of visits to source	Average time (sec) spent	
						In vicinity of source	On pheromone source
*E,Z*4,7-13:Ac(100)	60 a	50 a	0.0 a	1.4 a	0.0 a	4.8 a	0.0 a
*E,Z,Z*4,7,10-13:Ac(100)	86.6 b	86.6 b	6.6 a	6.0 b	0.1 a	9.3 a	0.7 a
*E,Z*4,7-13:Ac + *E,Z,Z*4,7,10-13:Ac(200)	93.3 b	90.0 b	70.0 b	7.2 bc	2.8 b	52.8 b	16.9 b
Female extract (20 FE)	100.0 b	96.6 b	63.3 b	8.2 c	2.3 b	52.0 b	15.0 b

[a]Means followed by the same letter within one column do not significantly differ at the 5% level (Duncan's NMRT). Data were transformed $\sqrt{x+1}$ for analysis, and the arcsin transformation used for percentages. In each treatment a total of 30 males were observed.

TABLE 2. RESPONSES OF POTATO TUBERWORM MOTHS MALES TO SYNTHETIC PHEROMONE COMPONENTS AND FEMALE PHEROMONE EXTRACT IN THE FIELD[a]

Treatment (ng)	Entering 10-cm circle around source (%)	Locating pheromone source (%)	Number of visits to source	Time (sec) spent		Number of males observed
				Inside 10-cm circle	On source	
*E,Z*4,7–13:Ac (100)	80.0	20.0	0.2 a	15.4 a	0.4 a	5
*E,Z,Z*4,7,10–13:Ac (100)	95.6	39.1	0.5 a	22.4 a	2.1 a	23
*E,Z*4,7–13:Ac + *E,Z,Z*4,7,10–13:Ac (200)	95.9	70.8	1.8 b	55.0 b	8.1 b	24
Female extract (20 FE)	100.0	58.8	1.2 b	51.1 b	6.0 b	17

[a]Means followed by the same letter within one column do not significantly differ at the 5% level (Duncan's NMRT, data were transformed $\sqrt{x+1}$ for analysis). Percentage figures refer to percentage of individuals arriving on arena that entered 10-cm cirle or located source.

It was not uncommon for the source to be revisited by the same male on several occasions. After one or more visits to the source, males either left the field of vision in an upwind direction, or settled down, sometimes very close to the source, and remained motionless. The image of the NVD at the light intensities prevailing during the observations was coarse-grained and scintillated rather strongly. Therefore, it was not possible to observe whether males exhibited wing fanning while walking, or attempted to copulate with the source.

Most of the males appearing in the field of vision entered the 10-cm circle around the source for each of the treatments (Table 2). Fewer males were able to locate the pheromone source when this contained diene alone or triene alone, than when the source contained both compounds or female extract. The number of visits to the source per individual was also higher with the diene + triene and female extract. Males spent significantly more time per visit inside the 10-cm circle when the source contained diene + triene or female extract. More time was also spent on the source in these two treatments, but there was no significant difference between female extract and triene alone.

DISCUSSION

In the wind tunnel experiments the diene alone activated fewer males than the other treatments. Although in the field experiments the exact number released and recaptured for each treatment was not recorded, the low number of males approaching the diene alone suggested that the wind tunnel and field results were similar.

In laboratory studies, T. Ono (personal communication) has found that the response threshold of male PTM to the diene is much higher than that to the triene. This is further supported by the results of field trapping experiments where the diene alone captures fewer moths than the triene alone or mixtures of the two compounds (Persoons et al., 1976; Voerman and Rothschild, 1978).

In the present study, treatments containing the triene evoked more jumps from the males in wind tunnel tests than the diene alone. This suggests that the triene may initiate and maintain flight during approaches to a pheromone source in a natural field situation. Sustained upwind flight could not be elicited from PTM males in the wind tunnel, although such flight appears to be the usual response to a pheromone stimulus in a number of other Lepidoptera tested in wind tunnels (Cardé and Hagaman, 1979; Castrovillo and Cardé, 1979; Baker and Cardé, 1979). The lack of activity in PTM males in the present tests may have been related to the relatively small size and simplicity of the wind tunnel used. However, males ran or walked in most experiments. In the field observations most males were seen walking within

a distance of 30–40 cm from the pheromone source. T. Ono (personal communication) also found that a searching walk was necessary before PTM males could successfully locate the pheromone source. In one glasshouse experiment, very few males were able to locate a pheromone source suspended at a height of 1 m, while large numbers of males visited a source placed on a side wall at the same height.

In the present wind tunnel and field observations, males remained in the vicinity of, and on, the source for longer periods, when this contained diene + triene rather than the triene alone, which suggested that the diene increased the persistence of the male in searching for the source. This was further reflected by the greater number of visits to the source per individual.

Results obtained with the female extract were very similar to those with the diene + triene mixture in all experiments.

The males' failure to attempt copulation with the pheromone source in the wind tunnel may have been due to the absence of suitable contact stimuli provided, for example, by moth scales (Ono, 1977).

The results of the various experiments undertaken in the present study suggest that the triene acts as a long-range cue eliciting "earlier" steps (arousal, upwind flight) in the sequence of mating behavior while the diene is involved in later behavioral steps (landing, searching). In the few other moth species, in which the function of the sex pheromone components has been studied so far, components influencing late behavioral steps evoked no behavioral response when presented alone to males. Examples include dodecyl alcohol in *Grapholitha molesta* Busck. (Cardé et al, 1975; Baker and Cardé, 1979), dodecyl acetate in *Argyrotaenia velutinana* Walk. (Baker et al., 1976) and *Trichoplusia ni* Hbn. (Bjostad et al., 1980; Linn and Gaston, 1981). The diene component of the PTM sex pheromone differs from the above examples in that it may elicit responses early in the behavioral sequence even if presented alone, as has been shown in experiments other than those discussed here (Roelofs et al., 1975; Persoons et al., 1976; Voerman and Rothschild, 1978).

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PHEROMONE PRODUCTION BY AXENICALLY REARED *Dendroctonus ponderosae* AND *Ips paraconfusus* (COLEOPTERA: SCOLYTIDAE)[1]

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Abstract—Mountain pine beetles, *Dendroctonus ponderosae* Hopkins, and California five-spined ips, *Ips paraconfusus* Lanier, were reared axenically from surface-sterilized eggs on aseptic pine phloem. After 24 hr in host logs, axenic female *D. ponderosae* and male *I. paraconfusus* produced the aggregation pheromones, *trans*-verbenol (*D. ponderosae*), and ipsenol and ipsdienol (*I. paraconfusus*). Emergent, axenically reared male *D. ponderosae* contained normal amounts of the pheromone *exo*-brevicomin. Axenic female *D. ponderosae* treated with juvenile hormone or exposed to vapors of α-pinene, produced the pheromone *trans*-verbenol. By 25–35 days after eclosion, axenic females exposed to α-pinene vapors produced over six times as much *trans*-verbenol as wild females, suggesting that while microorganisms in wild females may produce *trans*-verbenol, they may also inhibit production of the pheromone or use it as a substrate.

Key Words—*Dendroctonus ponderosae*, *Ips paraconfusus*, Coleoptera, Scolytidae, axenic rearing, monoterpenes, aggregation pheromones, *trans*-verbenol, *exo*-brevicomin, ipsenol, ipsdienol.

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INTRODUCTION

Do scolytid beetles (Coleoptera: Scolytidae) or their symbiotic microorganisms synthesize aggregation pheromones? This question has been addressed by various research projects yielding controversial results.

Bacillus cereus Frankland and Frankland, isolated from the guts of *Ips paraconfusus* (Lanier), cultured, and exposed to α-pinene, produced the aggregation pheromone, *cis*-verbenol, as well as *trans*-verbenol, a pheromone in *Dendroctonus* spp. (Brand et al., 1975). Mycangial fungi from *Dendroctonus frontalis* Zimmerman convert *cis*- and *trans*-verbenol to verbenone (Brand et al., 1976) and also produce sulcatol, an ambrosia beetle pheromone (Byrne et al., 1974), and the corresponding ketone (Brand and Barras, 1977). On the other hand, male *I. paraconfusus* (Borden et al., 1969; Hughes and Renwick, 1977) and female *Dendroctonus ponderosae* Hopkins (Conn, 1981), treated topically with juvenile hormone (JH), produce their respective aggregation pheromones, implying at least partial neural–hormonal control of pheromone production. Moreover, *trans*-verbenol was found in the hemolymph of female *D. ponderosae* 24 hr after they were exposed to α-pinene vapors, suggesting that if the hemolymph is microbe free, the beetles themselves convert inhaled monoterpenes to terpene alcohols in the hemolymph (Hughes, 1973).

A recent study revitalized the case for at least partial microbial involvement. *I. paraconfusus* males fed streptomycin lost the capability to convert the monoterpene myrcene into the aggregation pheromones, ipsenol and ipsdienol, but retained the ability to convert α-pinene to *cis*-verbenol (Byers and Wood, 1981). However, no attempt was made to confirm that the streptomycin-treated beetles were microbe free. Detailed studies of pheromone biogenesis (Fish et al., 1979; White et al., 1979; Hendry et al., 1980) have not contributed to a resolution of the controversy, because they did not separate the beetles from their associated microorganisms.

The development of techniques for rearing bark beetles axenically (Whitney and Spanier, 1982) has provided an opportunity to address each type of organism separately. We report that axenically reared *I. paraconfusus* and *D. ponderosae* can produce their aggregation pheromones completely in the absence of the normal, extracellular complement of symbiotic microorganisms.

METHODS AND MATERIALS

Following Whitney and Spanier's (1982) procedure, *I. paraconfusus* and *D. ponderosae* eggs were surface sterilized with mercuric chloride. They were individually reared axenically to adulthood in glass vials on aseptic pine phloem with a dead, aseptic yeast supplement. Rarely, cultures with bacterial contamination were found; these were discarded. Some pupae were transferred aseptically to a diet of pine phloem alone.

Wild *I. paraconfusus* were collected as they emerged from laboratory infested ponderosa pine logs; *D. ponderosae* were collected as they emerged from infested lodgepole pine logs obtained (with one exception) from Riske Creek or Pavillion, British Columbia. Wild beetles were held at 2–4° C on moist paper until used in experiments.

Whitney and Spanier (1982) exhaustively confirmed that their rearing method was truly axenic. Confirmation that our adult beetles were free of extracellular microorganisms was achieved by culturing crushed beetles on malt-agar plates, allowing them to walk over the agar plates, and by culturing the frass and diet in the rearing unit. In all cases there was no evidence of fungi or bacteria. Examination of many axenic *D. ponderosae* by scanning electron microscopy revealed that, unlike wild beetles, no microorganisms were visible on their exposed cuticle. These tests, however, do not disclose obligatory, intracellular microorganisms (Buchner, 1953; Boush and Coppel, 1974). Some axenically reared adults were allowed to crawl on bark from infested logs for 48 hr to pick up and feed on the microorganisms present.

Six experiments were conducted on axenically reared, post teneral beetles (Table 1). We examined *I. paraconfusus* males for their ability to produce ipsenol and ipsdienol in host logs. *D. ponderosae* males were assessed for their ability to produce *exo*-brevicomin, a multifunctional pheromone (Rudinsky et al., 1974; Pitman et al., 1978), and females for their ability to produce in host logs the aggregation pheromone, *trans*-verbenol (Pitman et al., 1968; Pitman, 1971). Beetles were allowed to bore in host logs only where the bark had been shaved off and the exposed surface wiped with hydrogen peroxide. Female *D. ponderosae* were also tested for pheromone production following exposure in a closed glass jar to the vapors of α-pinene (2.2×10^{-4} g/cc for 24 hr) or 3-carene (9.9×10^{-5} g/cc for 24 hr), and after topical treatment on the abdominal venter with 100 μg of juvenile hormone (JH) III (10,11-epoxyfarnesenic acid methyl ester, Calbiochem-Behring Corp., La Jolla, Calif.).

Pheromone production was assessed by excising the abdomens from experimental beetles and macerating them in twice-distilled pentane over Dry Ice. Single beetle extracts were spiked with 2- and 3-octanol as internal standards, and analyzed by gas-liquid chromatography (Hewlett Packard 5830A) in the direct injection mode [i.e. splitless injection] on a glass capillary column (30 m $\times$ 0.66 mm ID) coated with SP-1000 (Supelco, Inc., Bellafonte, PA).

RESULTS AND DISCUSSION

Both wild and axenic male *I. paraconfusus* produced both ipsenol and ipsdienol (Table 1, Exp. I). This result is in conflict with the results of Byers and Wood (1981) in which male *I. paraconfusus* fed streptomycin were unable to convert myrcene to ipsenol and ipsdienol, although they retained the capacity to convert α-pinene to *cis*-verbenol. It suggests that streptomycin

TABLE 1. PHEROMONE PRODUCTION IN WILD AND AXENIC *Ips paraconfusus* AND *Dendroctonus ponderosae* FOLLOWING VARIOUS TREATMENTS

Exp.	Species	Sex	Treatment	No. Beetles	Mean amount of beetle-produced volatiles (ng/beetle)[a]	
I.	*Ips paraconfusus*	M	24 hr in ponderosa pine		Ipsenol	Ipsdienol
			Wild	5	204 a	14 a
			Axenic	5	103 a	8 a
			Axenic, exposed to microbes	7	159 a	31 a
			Axenic, no yeast as adults	2	20 *	1 *
			Axenic, no yeast as adults, exposed to microbes	5	27 a	1 a
II.	*Dendroctonus ponderosae*	M			*exo*-Brevicomin	
			Wild	8	230 a	
			Axenic	8	100 ab	
			Axenic, no yeast as adults	8	30 b	
III.	*Dendroctonus ponderosae*	F	24 hr in lodgepole pine		*trans*-Verbenol	
			Wild	7	117 b	
			Axenic	7	89 b	
			Axenic, exposed to microbes	7	56 b	
			Axenic, no yeast as adults	7	19 a	
			Axenic, no yeast as adults, exposed to microbes	7	63 b	

					trans-Verbenol	3-caren-10-ol
IV.	*Dendroctonus ponderosae*	F				
			Wild, exposed to air	8	0 a	3 a
			Wild, exposed to α-pinene vapors	8	490 c	2 a
			Wild, exposed to 3-carene vapors	8	80 b	44 b
			Axenic, exposed to air	8	0 a	0 a
			Axenic, exposed to α-pinene vapors	8	9900 d	0 a
			Axenic, exposed to 3-carene vapors	8	0 a	13 b
V.	*Dendroctonus ponderosae*	F			*trans*-Verbenol	
			Wild, exposed to air	21	10 a	
			Wild, exposed to α-pinene vapors	40	207 c	
			Axenic, exposed to α-pinene vapors			
			5 days posteclosion	9	58 b	
			15 days posteclosion	10	137 b	
			25 days posteclosion	10	1216 d	
			35 days posteclosion	8	1357 d	
VI.	*Dendroctonus ponderosae*	F			*trans*-Verbenol	
			Wild, methanol	8	0 a	
			Wild, JH III in methanol	8	695 c	
			Axenic, methanol	8	3 a	
			Axenic, JH III in methanol	8	25 b	

[a]Values for treatments in each experiment followed by same letter are not significantly different, Mann-Whitney U test, $P < 0.05$. Asterisk following values for yeastless axenic *I. paraconfusus* in Exp. I indicates too few beetles for statistical test.

may have selectively eliminated gut microflora or that its real impact was a selective metabolic effect on one pheromone synthesis system, but not the other.

Axenically reared *D. ponderosae* males contained 100 ng of *exo*-brevicomin/beetle, not significantly different from wild males (Table 1, Exp. II). The low amount of *exo*-brevicomin (30 ng) in the males denied a dead yeast supplement in the adult diet may be due to a deficiency of nutrients, e.g., sterol, normally provided by microorganisms (Kok, 1979). We conclude that *exo*-brevicomin is synthesized by the beetle.

Axenic female *D. ponderosae* were definitely able to produce *trans*-verbenol during a 24-hr feeding period in lodgepole pine logs (Table 1, Exp. III). This result supports Byers and Wood's (1981) hypothesis that *I. paraconfusus* is capable of producing *cis*-verbenol from α-pinene without microbial involvement. There was no increase in pheromone production by axenics exposed to microbes. Such a result would have been evidence for addition of a microbial to a beetle production system. Only the axenics denied yeast as adults produced significantly less *trans*-verbenol (19 ng/beetle) than beetles in the other four treatment categories. When these beetles were exposed for 48 hr to microbes from their natural habitat, pheromone production reached normal levels (63 ng/beetle), apparently due to a nutritional supplement acquired during the 48-hr exposure.

Axenically reared *D. ponderosae* females exposed to vapors of α-pinene produced 9900 ng of *trans*-verbenol/beetle, 20 times as much as wild females exposed to α-pinene (Table 1, Exp. IV). However, in a subsequent experiment, under identical conditions, axenically reared females from a different locality (Manning Park) produced no *trans*-verbenol. This result led to two hypotheses: (1) that different populations have variable capacities to convert α-pinene to *trans*-verbenol, and (2) that although all axenic beetles used had passed the teneral stage, many had not completely matured to maximal pheromone-producing capacity. Both hypotheses proved valid.

A test of wild beetles from Riske Creek, Pavillion, and Manning Park, disclosed that after 24-hr exposure to α-pinene vapors, the Manning Park beetles produced 76 ng of *trans*-verbenol/beetle ($N = 11$), whereas Riske Creek and Pavillion beetles, respectively, produced 245 ng/beetle ($N = 7$) and 219 ng/beetle ($N = 11$). The Manning Park population had sustained heavy parasitism by a nematode, *Sphaerulariopsis hastata* Kahn, a possible cause of reduced vigor (Khan, 1957a,b; Thong and Webster, 1975; MacGuidwin et al., 1980). Subsequent tests were conducted only with Riske Creek or Pavillion beetles.

An experiment with axenically reared females at varying posteclosion ages disclosed a striking increase in their capacity to produce *trans*-verbenol when exposed to α-pinene (Table 1, Exp. V). *Trans*-verbenol production rose from approximately half that in wild beetles by 5-day females to six

times the level in wild beetles by 25-day or older females. Because posteclosion age was not recorded in other experiments, it is possible that comparisons of axenic with wild beetles might alter if beetles of other ages were used.

We conclude that over a 3- to 4-week maturation period, female *D. ponderosae* gradually develop the enzymatic capacity (White et al., 1980) to convert α-pinene to *trans*-verbenol. Thus, wild beetles which had experienced this maturation period prior to emergence and, by emerging, had identified themselves as being mature, were biochemically competent, whereas 5-day axenics were not (Table 1, Exp. V). A similar phenomenon occurs with regard to the development of enzymatic capacity in the flight muscles of adult *Locusta migratoria* L. (Bücher, 1965). A lack of enzymatic capacity for pheromone production would explain why immature male *I. paraconfusus* were unattractive to other beetles when introduced to a new host (Wood and Vité, 1961; Vité and Gara, 1962; Borden, 1967), and why 1-day posteclosion *Dendroctonus frontalis* Zimmerman females were less capable of converting α-pinene to *trans*-verbenol than 5-day females (Bridges, 1982).

We hypothesize that microorganisms in the hindguts of wild female *D. ponderosae* may curtail the rate of pheromone production by the beetles, may compete for and tie up much of the available α-pinene, or may utilize *trans*-verbenol as a substrate, metabolizing it to other products that we do not see on our chromatograms. In any of the above cases, *trans*-verbenol would accumulate, as observed, in mature, axenically reared females to a greater extent than in wild females (Table 1, Exp. IV and V). The microorganisms in wild beetles might regulate the pheromone content, preventing it from reaching excessive levels and disposing of residues following mating and cessation of pheromone production. These hypotheses are supported by experiments on the boll weevil, *Anthonomis grandis* Boheman. Weevils that were relatively bacteria-free produced much more pheromone than heavily contaminated ones (Gueldner et al., 1977; Wiygul et al., 1982). Our data do not rule out the possibility that there are two pheromone production modes, one by the beetle and one by symbiotic microorganisms. In this case, *trans*-verbenol would be an intermediate product in the overall metabolic breakdown of α-pinene by the microorganisms, as well as a beetle-produced substrate for further microbial breakdown.

When exposed to 3-carene, wild female *D. ponderosae* produced 80 ng of *trans*-verbenol/beetle (Table 1, Exp. IV). The 3-carene was 95% pure and contained no α-pinene. It is not a precursor of *trans*-verbenol. Therefore, when a wild female attacks a new host, any monoterpene may act as a token stimulus that induces the beetle to convert a sequestered α-pinene derivative (Hughes, 1975) to *trans*-verbenol. The lack of *trans*-verbenol production by axenic females exposed to 3-carene may constitute evidence that we had eliminated a microbial production system through the axenic rearing process. Both wild and axenic females exposed to 3-carene produced significant

amounts of 3-caren-10-ol (Table 1, Exp IV), a common terpene alcohol in this species (unpublished result). No 3-caren-10-ol was produced following exposure to α-pinene.

Twenty-four hours after a topical application of JH, axenic female *D. ponderosae* had produced significantly more *trans*-verbenol than control beetles treated with 1 μl of methanol, but less than the wild beetles (Table 1, Exp. VI). Therefore, axenically reared females apparently retain the capacity to sequester an intermediate α-pinene derivative and to convert it to *trans*-verbenol in response to a hormonal stimulus. The lesser production by axenic than wild females may be because the axenic females were too young (Table 1, Exp. V).

Our experiments have shown that axenically reared bark beetles in two species can produce their aggregation pheromones normally when they are allowed to bore into their hosts. For the mountain pine beetle they have also demonstrated that axenic males contain *exo*-brevicomin and that axenic females can produce *trans*-verbenol in the absence of their host, if they are exposed to the vapors of an appropriate pheromone precursor or are given a hormonal stimulus. Several novel hypotheses, e.g., the possible dietary role played by yeasts in enabling the beetles to produce pheromones and the postulated microbial breakdown of beetle-produced terpene alcohols in the hindgut, should be investigated further.

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ETHYL (*Z*)-9-HEXADECENOATE A SEX PHEROMONE OF *Syndipnus rubiginosus*,[1] A SAWFLY PARASITOID[2]

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Abstract—A female-produced sex pheromone of *Syndipnus rubiginosus* Walley (Hymenoptera: Ichneumonidae), a parasitoid of the yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae), was isolated and identified from hexane extracts of 250 virgin females. Column chromatography (Florisil), gas chromatography, mass spectrometry, high performance liquid chromatography, and ozonolysis indicated the structure was ethyl (*Z*)-9-hexadecenoate. The optimum male response is at 300–1000 ng (3–10 FE). No cross-attraction between *S. rubiginosus* and the sympatric sawfly parasitoid *S. gaspesianus* (Provancher) could be demonstrated.

Key Words—Sex pheromone, Hymenoptera, Ichneumonidae, *Syndipnus rubiginosus*, ethyl (*Z*)-9-hexadecenoate, parasitoid, *Pikonema alaskensis*, mass spectra, ozonolysis.

INTRODUCTION

Since the first identification by Butenandt et al. (1959), sex pheromones have been demonstrated in hundreds of species, most of which are destructive species in the orders Lepidoptera and Coleoptera. The sex pheromones that have been identified for Hymenoptera, for the most part, have also been in

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destructive species (Ikan et al., 1969; Jewett et al., 1976; Bartelt et al., 1982b). The identification of the pheromone of the honeybee (Butler and Fairey, 1964), is an exception.

Of the references to sex pheromones of parasitic Hymenoptera, most refer only to evidence of a sex pheromone. Such evidence has been demonstrated for the family Aphelinidae (Rao and Debach, 1969; Khasimuddin and Debach, 1975), Chalcididae (Leonard and Ringo, 1978; Simser and Coppel, 1980), Cynipidae (Read et al., 1970), Pteromalidae (King et al., 1969; Yoshida, 1978; Van den Assem et al., 1980), Scelionidae (Schwartz and Gerling, 1974), Braconidae (Genieys, 1925; Fink, 1926; Murr, 1930; Parker, 1931; Whitting, 1932; Hagen, 1953; Schlinger and Hall, 1960; Bousch and Baerwald, 1967; Cole, 1970; Read et al., 1970; Lewis et al., 1971; Weseloh, 1976; Tagawa, 1977; Askari and Coppel, 1978; Askari and Alishah, 1979), and Ichneumonidae (Champlain, 1921; Cole, 1970; Vinson, 1972; Robacker et al., 1976; Coppel and Mertins, 1977). The sex pheromone of only one parasitic hymenopteran has been isolated and identified. Robacker and Hendry (1977), using laboratory bioassays, isolated neral and geranial from *Itoplectis conquisitor* (Say). No field results have been reported.

Syndipnus rubiginosus Walley (Hymenoptera: Ichneumonidae) is a commonly reported parasitoid of the yellowheaded spruce sawfly, *Pikonema alaskensis* (Rohwer) (Hymenoptera: Tenthredinidae) (Forbes, 1949; Bradley, 1951; Raizenne, 1957; Rau, 1976; Houseweart and Kulman, 1976; Thompson and Kulman, 1980). Rau (1976) reported that *S. rubiginosus* attacks late (4th–6th) instar yellowheaded spruce sawfly larvae.

This paper describes the isolation and identification of a sex pheromone of *S. rubiginosus*. Field trials were conducted with synthetic pheromone, and cross-attraction between *S. rubiginosus* and the related species *S. gaspesianus* (Provancher) was studied also.

METHODS AND MATERIALS

Insects. Yellowheaded spruce sawfly larvae were collected and handled as described by Bartelt et al. (1982a). Emerged *S. rubiginosus* were separated by sex. [Males have a yellow face and females have a black face (Walley, 1940).] Virgin females (2–4 days old) were killed by immersion in hexane and stored at −20° C. Males were held at 15° C and provided with honey and water until used in bioassays.

Greenhouse Bioassay. To monitor purification steps, a greenhouse bioassay cage containing 45 male *S. rubiginosus* was used (Bartelt et al., 1982a). Pheromone preparations were placed on 4-cm watch glasses, at the rate of ca. 1 female equivalent (FE) per plate. After the solvent had evaporated, the plates were positioned in the upwind end of the cage, in a row perpendicular to the air flow, and just above small (5 dm) spruce trees.

Qualitatively, a positive response included: positive anemotaxis, down-

wind hovering in front of plates, landing on plates, abdomen raising, wing fanning, and copulatory attempts with nearby males. Male response to various test materials was quantified by counting the number of males "hovering" within 2 cm directly in front of a watch glass every 15 sec over a 2-min period. The 2-min totals counts were analyzed as balanced incomplete block designs. The number of treatments, blocks, replications, and block size varied with the experiment.

Field Bioassays. Field studies were conducted in white spruce plantations near Grand Rapids, Minnesota, during June 1981–1982. Test materials were evaluated in the field by catches of males in Pherocon® II traps.

Test materials were placed on 4-cm watch glasses and the solvent allowed to dry before placement in the traps. One hundred nanograms of the antioxidant, 2,5-di-*tert*-pentylhydroquinone, was also added to each watch glass. The traps were secured to branches of 3–5 m tall white spruce trees about 1.5 m from the ground and about 5 m apart.

In 1981, field bioassays of Florisil fractions were run using ca. 1 FE per trap. These were tested in a randomized complete block design (RCB) containing five plots, each with one replication of the 10 treatments. Traps were checked after 2 days. The 1982 field test of various dosages (0, 10, 30, 100, 300, and 1000 ng) were tested in a RCB design, containing five plots, each with one replication of the six treatments. The experiment was repeated on five consecutive days.

In the cross-attraction experiments between *S. rubiginosus* and *S. gaspesianus*, traps contained virgin females held in brass screen cages 4 cm long and 2 cm diameter. Control traps were empty. The experiment was a RCB design, with five plots, each with two replications of the three treatments. The experiment was repeated on four consecutive days.

Chemical Analyses. Virgin females were either extracted by a hexane wash in an Erlenmeyer flask or with hexane in a Soxhlet extractor. The extract was combined with the hexane used to kill the females, concentrated under vacuum, and stored at −20°C until used for bioassay or chemical analysis.

The concentrated crude extract was fractionated by column chromatography on Florisil (2.5% water by weight). The column (20 × 2.0 cm) (void volume ca. 40 ml) was eluted consecutively with 80-ml volumes of hexane, then 2.5, 5.0, 7.5, 10, 25, 50% ethyl ether in hexane (by volume), ethyl ether, acetone, and methanol. The effluent was collected in 10 fractions of 80 ml each and stored at −20°C until used for bioassay or chemical analysis.

Gas-liquid chromatography (GLC) was performed with a Hewlett Packard 5830A, equipped with a flame ionization detector, effluent splitter, and thermal gradient collector (similar to that described by Brownlee and Silverstein, 1968). The glass column (1.9 m × 1 mm ID) contained 5% Silar 5CP on 80/100 Gas Chrom Q and was eluted with N_2 at 20 ml/min. For collections, the temperature program was 100° for 1 min, then 5°/min to 250°. For analysis of ozonolysis products and aldehyde standards, the tem-

perature program was 50° for 5 min, then 10°/min to 250°.

High-performance liquid chromatography (HPLC) was carried out with a Waters Associates M6000A pump and R401 differential refractometer detector. Samples (1–2 μg) were injected onto a 100 × 4 mm, 20% $AgNO_3$ on silica column and eluted with toluene at 1.0 ml/min. Retention times were compared to standard esters.

Mass spectra were obtained on a LKB-9000 gas chromatograph–mass spectrometer equipped with a 1.0-m × 2-mm ID 10% Alltech CS-5 column and electron ionization energy of 70 eV.

Ozonolysis was conducted as described by Beroza and Bierl (1967). Samples (2 μg) were ozonized in 20 μl of CS_2 for 2 min at −70°. The ozonides were reduced with triphenylphosphine.

Synthetic ethyl palmitoleate [ethyl (*Z*)-9-hexadecenoate, 99% pure] was obtained from Sigma Chemical Company (St. Louis, Missouri 63178).

RESULTS

The 1981 field evaluation of the Florisil fractions shows that a 5% ether in hexane Florisil fraction (prepared from an extract of 20 virgin females) caught an average (N = 5) of 9 males/trap in a 2-day test (Table 1). The other fractions had catches that did not differ significantly from zero.

In greenhouse bioassays (winter 1981–1982), 5% and 7.5% ether in hexane fractions (prepared from an extract of 250 virgin females) were more attractive than the other fractions. The results are shown in Table 1. The 7.5%

TABLE 1. FIELD AND GREENHOUSE BIOASSAY OF FLORISIL FRACTIONS[a]

Fraction	1981 Field mean 2-day catch[b]	Greenhouse mean response[c]
Hexane	0.2a	0a
Ether in hexane		
2.5%	0a	0a
5.0%	9.0b	4.9b
7.5%	0.6a	6.0c
10.%	0a	0a
25.%	0a	0a
50.%	0a	0a
Ether	0.2a	0a
Acetone	0a	0a
Methanol	0a	0a

[a]Field and greenhouse bioassays were done with two separate sets of Florisil fractions.
[b]Mean catch is given (N = 5); different letters denote significant differences at the 0.05 level by the least significant difference (LSD) method.
[c]Mean response is given (N = 9).

fraction was significantly more attractive than the 5% fraction. These two were compared by GLC and contained peaks of similar retentions, but the 7.5% fraction had larger amounts. The 5% Florisil fraction used for the 1981 field bioassays also contained the same peaks as the other active Florisil fractions.

The 7.5% Florisil fraction was collected in 13 fractions from the GLC (Figure 1). The collected fractions were tested in the greenhouse, and only one fraction, GLC fraction 4, showed any attractiveness. It had a mean response ($N = 4$) of 7.0, and all other fractions had responses of zero.

The mass spectrum obtained for GLC fraction 4 (GLC inlet, 70 eV), is shown in Figure 2. The parent ion (M) was observed at m/e 282. The M − 45, M − 46, M − 88 peaks observed were consistent with an ethyl ester of a 16-carbon fatty acid with one double bond.

To determine the double-bond location, GLC fraction 4 was ozonized, and the GLC retention times of the products were compared to those of standard aldehydes. The GLC chromatogram of the ozonized GLC fraction 4 contained peaks at 5.89, equal to the retention time of heptanal, and at 17.99, equal to the retention time of ethyl-9-oxononanoate. These data indicated

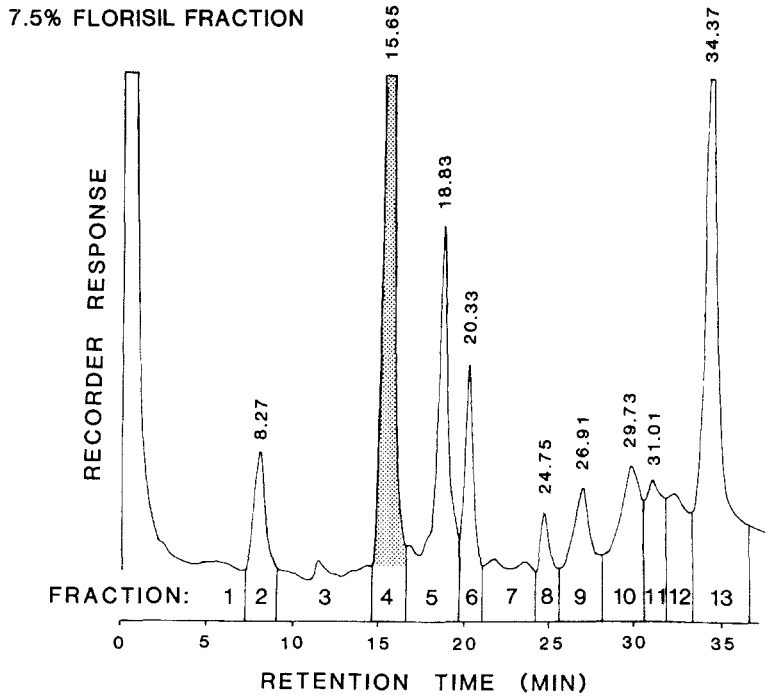


FIG. 1. Gas-liquid chromatogram of active Florisil fraction.

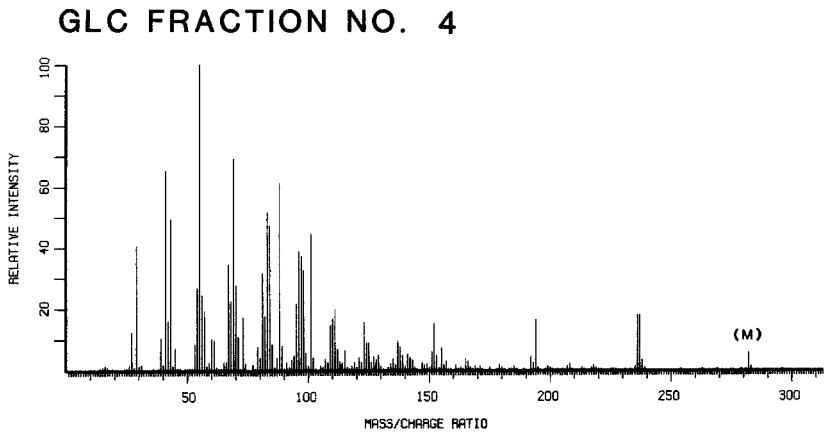


FIG. 2. Mass spectrum of GLC fraction 4.

that the double bond was in the 9 position and that the compound was an ethyl ester of 9-hexadecenoate. Ozonolysis of synthetic ethyl (*Z*)-9-hexadecenoate gave an identical GLC chromatogram.

To determine the configuration about the double bond, the 7.5% Florisil fraction was subjected to HPLC on a $AgNO_3$ silica column. Peaks appeared in the chromatogram (Figure 3) at 1.3 (hexane solvent), 1.8, and 3.2 ml after

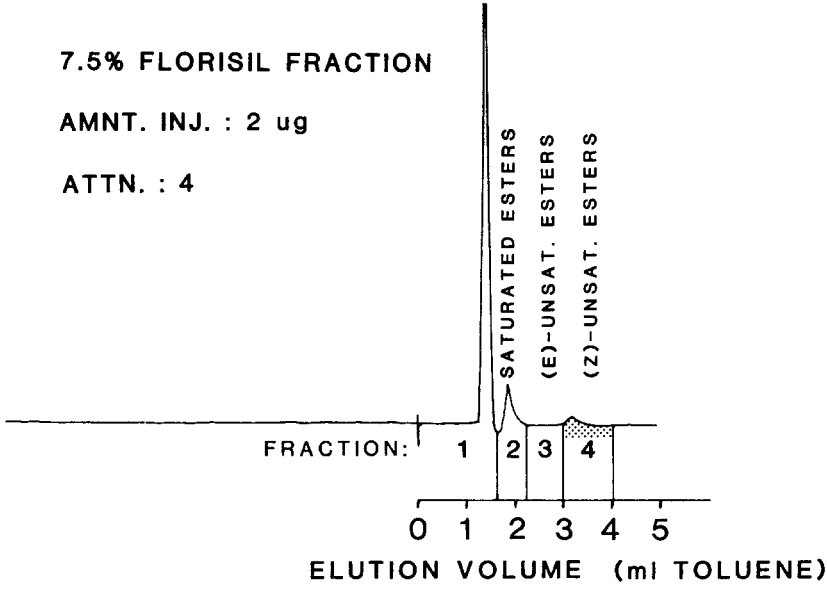
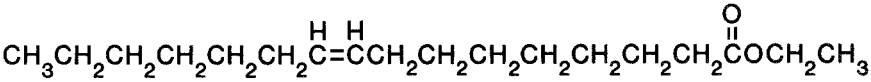


FIG. 3. High-performance liquid chromatogram of active Florisil fraction.



ethyl (Z)-9-hexadecenoate

(ethyl palmitoleate)

FIG. 4. Structure of ethyl (*Z*)-9-hexadecenoate.

injection. The peak at 1.8 ml was equal to the retention time of a saturated ester, and the peak at 3.2 ml was equal to the retention time of a *cis* monoene ester. The material was collected in four fractions (Figure 3) and bioassayed in the greenhouse. Only one fraction, HPLC fraction 4, had activity. It had a mean response ($N = 6$) of 7.7, and all other fractions had responses of zero. This fraction had the retention time of a *cis* ester, so the compound was tentatively identified as ethyl (*Z*)-9-hexadecenoate (or by its trivial name, ethyl palmitoleate) (Figure 4).

Synthetic ethyl palmitoleate gave identical GLC retention time, mass spectrum, ozonolysis products, and HPLC retention time as the female-derived material.

One FE (100 ng) of synthetic ethyl palmitoleate was tested in the greenhouse and elicited a significantly higher response (8.6) than a control (0.0). The synthetic ethyl palmitoleate elicited all of the characteristic male behaviors as elicited by female-derived materials.

In the field, various dosages of synthetic ethyl palmitoleate were tested by catches of males in Pherocon II traps. Dosages of 0.1 FE and 0.3 FE did catch males but did not differ significantly from controls, which caught none (Table 2). Attraction to 1 FE (2.96) was significantly higher than to controls, 0.1 FE, or 0.3 FE. Three FE and 10 FE caught significantly more males (5.32

TABLE 2. COMPARISON OF VARIOUS DOSES OF SYNTHETIC ETHYL PALMITOLEATE IN THE FIELD

Dose (female equivalents)	Mean 1-day catch of *S.r.* males[a]
Control	0a
0.1 FE (0.01 μg)	0.28a
0.3 FE (0.03 μg)	0.56a
1.0 FE (0.1 μg)	2.96b
3.0 FE (0.3 μg)	5.32c
10. FE (1.0 μg)	6.0c

[a]Mean catch is given ($N = 25$); different letters denote significant differences at the 0.05 level by the least significant difference (LSD) method.

TABLE 3. CROSS-ATTRACTION BETWEEN *S. rubiginosus* AND *S. gaspesianus*

Mean 4-day catch	Bait		
	S.r.♀	*S.g.*♀	Control
S. rubiginosus males	3.1b	0a	0a
S. gaspesianus males	0.1a	19.5c	0.2a

[a]Mean response is given ($N = 10$); different letters denote significant differences at the 0.05 level by the least significant difference (LSD) method.

and 6.0) than 1 FE, but 3 FE and 10 FE did not differ significantly from each other. Although no direct comparison of virgin females to synthetic pheromone was made, traps baited with virgin females and synthetic pheromone (3 FE) in adjacent plots caught averages of 1.3 ($N = 20$) and 4.6 ($N = 10$) males per day, respectively.

The cross-attraction study shows that females of each species attract only conspecific males, and that catches of the other species seem to be accidental, in that they do not differ significantly from controls (Table 3).

DISCUSSION

We conclude that ethyl palmitoleate is a sex pheromone of *S. rubiginosus*, and, alone, it can elicit responses from males. The compound is active in relatively small amounts (100 ng can attract males in the field), and the optimum male response is to about 300 to 1000 ng. *S. gaspesianus* males are not attracted to ethyl palmitoleate and are not attracted to virgin female *S. rubiginosus*, but they are highly attracted to virgin female *S. gaspesianus*. This suggests the existence of a different sex pheromone in *S. gaspesianus*.

Various means have been suggested for monitoring parasitoid populations to aid in management programs. Lewis et al. (1971) developed a female-baided trap; Hrdy and Sedivy (1979) suggested using chemical attractants to detect both the pest and its parasitoids; and Robacker et al., (1976) suggested the use of parasitoid pheromones.

With the identification of sex pheromones of both the yellowheaded spruce sawfly (Bartelt et al., 1982b) and its parasitoid *S. rubiginosus*, it is possible to monitor both by means of chemical attractants and use the information in management decisions.

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VOLATILE FOOD ATTRACTANTS FOR *Oryzaephilus surinamensis* (L.) FROM OATS[1,2]

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Abstract—The sawtoothed grain beetle, *Oryzaephilus surinamensis* (L.) (Coleoptera: Cucujidae), is attracted to certain volatile components that occur in whole and rolled oats as determined by a laboratory pitfall chamber bioassay. More than 100 components were detected in the attractive carbonyl-containing fractions; 14 of these, making up 60% of the total, were identified and bioassayed. Although hexanal, heptanal, octanal, (*E*)-2-heptenal, and 2-furaldehyde, at doses ranging variously from 1 to 100 μg, were all significantly attractive, only 1/10 to 1/100 as much (*E*)-2-nonenal or (*E,E*)-2,4-nonadienal was necessary to produce comparable insect response. In addition, propanal and formaldehyde (previously reported in oats but not detected by us) were bioassayed and found to be attractive.

Key Words—*Oryzaephilus surinamensis*, Coleoptera, Cucujidae, oats, attractants, (*E*)-2-nonenal, (*E,E*)-2,4-nonadienal, 2-furaldehyde, formaldehyde, propanal, hexanal, heptanal, octanal, (*E*)-2-heptenal.

INTRODUCTION

Oryzaephilus surinamensis (L.) (Coleoptera: Cucujidae), the sawtoothed grain beetle, is an economically important worldwide pest of both stored

[1]Presented at the North Central Branch meeting of the Entomological Society of America in St. Louis, Missouri, March 15–17, 1983.
[2]The 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.

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grains and processed cereal products (Howe, 1956; Loschiavo and Smith, 1970). Under the integrated pest management concept of insect control, early detection of insects in storage facilities is crucial to prevention of extensive damage. Therefore, identification of host-food attractants and pheromones of *O. surinamensis* might be expected to lead to new, more effective detection and control programs (Burkholder, 1977).

Recent research in our laboratory has demonstrated (Freedman et al., 1982; Mikolajczak et al., 1983) that oat triglycerides composed primarily of palmitic, oleic, and linoleic acids stimulate aggregation of the sawtoothed grain beetle when bioassayed in a two-choice, open-arena Petri dish chamber (Loschiavo et al., 1963). Mixtures of these three compounds as free fatty acids also induce aggregation. While these results are significant, the relatively nonvolatile nature of these stimuli and the type of bioassay used imply that the observed response is due to an arrestant or feeding stimulant (Dethier et al., 1960). The data do not account for the demonstrated true (acting over a distance) attractancy of oats and oat extracts for the sawtoothed grain beetle (Pierce et al., 1981; Freedman et al., 1982). Pierce et al. (1981) reported that *O. surinamensis* and *O. mercator* (merchant grain beetle) were attracted to possible pheromones produced by both sexes of both species as well as to volatiles from the culture medium, but the responsible materials were not identified. Our current objectives were to detect, identify, and bioassay volatile chemical stimuli in oats that attract *O. surinamensis*.

METHODS AND MATERIALS

Insect Rearing. Sawtoothed grain beetles were reared on a diet of Quaker Old-Fashioned rolled oats and brewers' yeast (95:5 wt/wt) in 0.95-liter glass jars in an environmental chamber maintained at 27°C and 60% relative humidity with a 16:8 light–dark cycle. Test insects were of mixed sexes, 4–6 weeks old, and were starved 5–7 days prior to the bioassay.

Pitfall Bioassay. A two-choice pitfall olfactometer similar to that described by Phillips and Burkholder (1981) was employed in this study for all pure compounds. Manipulations of the insects in setting up the tests were conducted under a red safelight, and the bioassays were done in total darkness for 1 hr. Four replicates using 25 beetles each were done simultaneously for every test stimulus. A 2000-μg dose of crude oat extract was assayed daily as a positive control. Test samples were dissolved at appropriate concentrations in redistilled solvent, and 10 μl of the solution was applied to the test filter paper disks which were placed in the bottom of the appropriate vials; 10 μl of solvent only was applied to the control disks.

Responses of the beetles to treated disks vs. control disks were converted to "percent response" (Tamaki et al., 1971; Nara et al., 1981), which is defined as $100\ (T - C)/N$; T and C are the number of beetles in the vials

containing the treated and control disks, respectively, and N is the total number of insects used. Significance of response was determined by the chi-square test computed as $\chi^2 = (T - C)^2/(T + C)$.

Petri Dish Bioassay. All crude column chromatographic fractions were bioassayed using the Petri dish procedure previously described by Loschiavo, et al. (1963) and modified by Freedman et al. (1982).

Chemicals for Bioassays. Chemicals were either purchased commercially or synthesized [benzyl acetate and (*E*,*E*)-2,4-nonadienyl acetate] by standard procedures and, if necessary, were purified by preparative gas chromatography to greater than 95% purity. Purified samples were placed in vials, blanketed with nitrogen, sealed with Teflon-lined caps, and stored at −20° C. All solvents used for extractions or as control samples were routinely redistilled.

Preparation of Volatile Concentrate. Rolled oats (9 kg) was steeped four times, for 12–16 hr each time, in 12-liter portions of pentane. The combined extracts were concentrated to 1.5 liters with a 30 × 1 cm Vigreux column while the distillation flask was heated with a warm water bath. All other distillations were also done by this procedure. Another comparable crude extract was prepared in the same manner using dichloromethane (CH_2Cl_2) as the solvent.

Most of the solvent remaining in the pentane extract was removed on a rotary evaporator without heating. The concentrated pentane extract was then transferred to a 5-liter, 3-necked flask, 2 liters of water was added, and the volatile portion was removed by simultaneous steam distillation–extraction using a Likens–Nickerson head (Likens and Nickerson, 1964). This operation was carried out at ambient pressure for 2.5 hr with 125 ml of CH_2Cl_2 as solvent. As much solvent as possible was removed by distillation, and the residual CH_2Cl_2 was distilled off as an azeotrope with methanol; the methanol was then azeotropically distilled with pentane, thus leaving the steam volatiles concentrated in 5 ml of pentane solution suitable for column chromatography. An identical steam distillation–extraction procedure was applied also to the CH_2Cl_2 extract of rolled oats.

Column Chromatography. A chromatographic column (1.0 cm ID) was packed to a depth of 22 cm with 60–200 mesh silica gel that had previously been thoroughly extracted with diethyl ether and air dried. The volatile concentrate (pentane solution, 5 ml) derived from the pentane extract was applied to this column and then was eluted with the solvents shown in Figure 1. Ten milliliters was collected in each of fractions 1–25 and 40 ml in each of the last five fractions. All fractions were concentrated to 3 ml (in pentane) by distillation; methanol was azeotropically distilled with pentane. The corresponding volatile concentrate from CH_2Cl_2 extraction of oats was also resolved into 30 chromatographic fractions in precisely the same manner. Vials containing these materials were stored at −20° C. All 60 fractions were bioassayed by the Petri dish procedure.

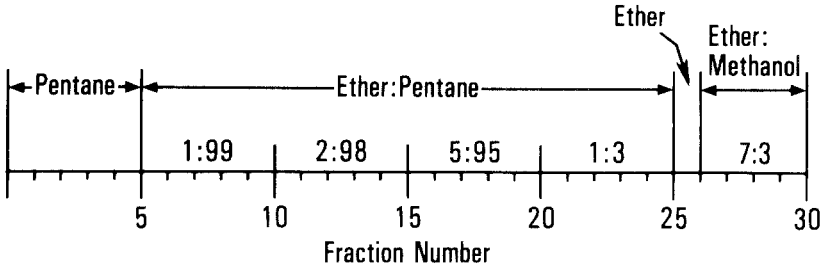

FIG. 1. Column chromatography of rolled oats volatiles.

Gas Chromatography (GC). A Varian 3700, dual flame ionization detector-gas chromatograph having both capillary and packed column capabilities was used for all GC procedures. Analytical separations were achieved with a 15-m × 0.32-mm ID fused silica bonded DB-1 capillary column having a 1.0 μm film thickness (J & W Scientific, Inc.). It was operated at 40, 60, or 75° C for 3 min, then programed at 3, 5, or 7° C/min to 270° C and held. Other parameters were: injector, 270° C (injector split ratio, 30:1); detector, 280° C; and helium flow, 2.6 ml/min at 10 psi inlet pressure. Kovats retention indices were calculated according to Van den Dool and Kratz (1963) using hydrocarbon references.

Preparative GC separations were done on a 175-cm × 4-mm ID glass column packed with 10% CS-10 on 100–120 mesh Chromosorb W-AW (Alltech Assoc.). Operating conditions were: column temperature programed from 60 or 80° C to 270° C at 7 or 12° C/min and held; injector, 270° C; detector, 280° C; and helium flow, 30 ml/min at 20 psi inlet pressure. The column effluent was split by a variable splitter (ratio set at 10:1); one portion proceeded to the detector and the larger portion went to a third detector base mounted on the instrument, from which fractions were collected by passing the effluent through 0.5 × 2.5-cm columns of 60–80 mesh Tenax-GC (Alltech Assoc.) made from glass disposable Pasteur pipets. The Tenax was conditioned by Soxhlet extraction for 16 hr with diethyl ether, drying, and then heating in a glass tube under a 30 ml/min helium flow at 275° C for 16 hr. Collected materials were recovered by passing 10 ml of pentane through the Tenax traps and then concentrating the resulting solutions to an appropriate volume by distillation and finally by a very slow stream of nitrogen. Samples were sealed and stored at −20° C when not in use.

Gas Chromatography–Mass Spectrometry (GC–MS). A glass GC column 183 cm × 2 mm ID packed with 3% Silar-5CP on 80–100 mesh Gas Chrom Q (Applied Science) coupled to a Kratos MS 30 mass spectrometer was used for GC-MS analyses. The column temperaturewas held at 50° C for 4 min, then was programed to 270° C at 10° C/min. Mass spectra were obtained at 70 eV.

TABLE 1. QUALITATIVE RESPONSE[a] OF THE SAWTOOTHED GRAIN BEETLE TO COLUMN CHROMATOGRAPHIC FRACTIONS OF PENTANE-EXTRACTED OAT VOLATILES

Fractions	Response
1–3	Repellent
4–11	Inactive/repellent
12–16	Attractive[b]
17–24	Inactive
25–27	Attractive
28–30	Inactive

[a]Petri dish bioassay.
[b]Fraction 13 was inactive.

RESULTS

The crude steam distillates of both the pentane and CH_2Cl_2 extracts of rolled oats were significantly repellent (% response −22 and −69, respectively) to the sawtoothed grain beetle at the concentration that was applied to the chromatographic column.

Table 1 presents a qualitative summary of beetle responses observed for the column chromatographic fractions from the pentane extract. The attraction listed for fractions 25–27 was weaker (but not significantly so) than that produced by the carbonyl-containing fractions 12–16. Bioassay data for these carbonyl fractions are listed in Table 2, and they indicate that all fractions except 13 were significantly attractive at the concentrations tested. GC analyses revealed that many components were present in each fraction.

Bioassay and GC data for the column chromatographic fractions derived from CH_2Cl_2-extracted volatiles were comparable to those in Table 1; hence

TABLE 2. SAWTOOTHED GRAIN BEETLE RESPONSE[a] TO CARBONYL-CONTAINING FRACTIONS FROM PENTANE-EXTRACTED OAT VOLATILES

Fractions	% Response[b]
12	25**
13	16[c]
14	33**
15	29**
16	37**

[a]Petri dish bioassay.
[b]**Indicates significance at $P < 0.01$, based on χ^2 analysis. Based on 8 replicates.
[c]Based on 4 replicates.

each of these 30 fractions was combined with the corresponding fraction from the pentane-extracted volatiles.

Capillary GC analysis of the total carbonyl-containing mixture, obtained by combining aliquots of fractions 12–16, provided evidence of at least 100 components. Fourteen of these carbonyl compounds, accounting for 60% of the mixture, were identified and are listed in Table 3 along with their percentage in the mixture, GC retention indices, and pitfall chamber bioassay data for the pure chemicals at three or four dose levels. Beetle response data are also listed for two compounds, propanal and formaldehyde, that were reported by Hrdlicka and Janicek (1964) but which we could not detect in these oat volatiles. Compounds in Table 3 are grouped loosely according to decreasing attractancy; thus, (*E*)-2-nonanal, (*E,E*)-2,4-nonadienal, formaldehyde, and 2-furaldehyde (furfural) are the most potent attractants of this group of carbonyl-containing compounds, and the last four materials listed exhibit no attractancy.

Capillary column GC retention indices for all 14 compounds in oat

TABLE 3. *Oryzaephilus surinamensis* RESPONSE[a] TO CARBONYL COMPOUNDS IDENTIFIED IN ROLLED OAT VOLATILES

Compound	% in carbonyl fraction (GC area %)	GC retention index[b]	% Response[c] to			
			100 μg	10 μg	1 μg	0.1 μg
(*E*)-2-Nonenal[d]	7	1136	−77**[e]	32**	30**	27**
(*E,E*)-2,4-Nonadienal[d]	1	1183	−36**	13	32**	24**
Formaldehyde			49**	50**	49**	6
2-Furaldehyde	Tr	804	−47**	48**	36**	5
Heptanal[d]	2	880	51**	38**	−2	
Propanal			38**	24**	4	
(*E*)-2-Heptnal	2	930	38**	22**	7	
Hexanal[d]	30	780	23**	33**	10	
(*E*)-2-Hexenal	1	824	12	28**	14	
Octanal[d]	2	982	53**	16	−2	
(*E,E*)-2,4-Decadienal	2	1285	−71**	5	24*	2
Benzaldehyde[d]	5	923	−86**	20*	−8	6
Nonanal[d]	5	1081	8	18	16	8
(*E,E*)-2,4-Hexadienal	1	1021	6	15	6	
Benzyl acetate[d]	1	1137	−29**	14	11	8
(*E,E*)-2,4-Nonadienyl acetate[d]	Tr	1348	−24**	1	−21*	−20*

[a]Pitfall chamber bioassay.
[b]DB-1 column, 15 m × 0.32 mm (J&W Scientific, Inc.).
[c](*) and (**) indicate significance at $P < 0.05$ and $P < 0.01$, respectively, based on χ^2 analysis.
[d]Detected by GC-MS.
[e]50 μg.

volatiles (Table 3) coincide with those obtained by analysis of authentic specimens. Mass spectral evidence, where indicated, is consistent with the designated structures.

DISCUSSION

Two separate batches of rolled oats were extracted, one with pentane and one with the more polar CH_2Cl_2, to determine which solvent was more effective in extracting volatiles, whether marked variations occurred in the composition of extracted volatiles, and to what extent beetle responses to the column chromatographic fractions derived from both extracts were comparable. The crude steam distillate from the CH_2Cl_2 extract was somewhat more repellent than that from the pentane extract, probably because CH_2Cl_2 extracted more material, 6.1% compared to 5.2% for pentane, and the observed repellency is simply a matter of the solutions being too concentrated in certain compounds. This explanation may also apply to the apparent repellency observed for some of the first 11 column chromatographic fractions. Differences were noted in the relative proportion of many of the constituents; however, no profound variations in the prinicpal components present were found, and corresponding chromatographic fractions from the two extracts produced generally comparable beetle responses. Although fractions 12 and 14 (Table 2) were attractive, fraction 13 was not; this occurred because 12 contained about three times as much total material as 13 and was also considerably richer in hexanal than 13. Fraction 14 was the first fraction that contained (*E*)-2-nonenal.

Our initial experiments indicated that some aldehydes were consistently repellent at levels of 100 μg or greater, hence this value was chosen as the upper limit to be bioassayed. However, it is clear from Table 3 that the insects are more sensitive to large doses of some compounds than they are to others. For example, all of the saturated aldehydes except nonanal are significantly attractive at a 100 μg dose, whereas the same amount of most of the unsaturated and aromatic compounds induces a decidedly repellent effect.

Propanal, hexanal, heptanal, and (*E*)-2-heptenal all produce significant attraction at levels of 10–100 μg; (*E*)-2-hexenal and octanal are attractive at 10μg and 100 μg doses, respectively. Two other compounds, formaldehyde and furfural, gave 49% and 36% response, respectively, at the 1-μg dose level. Most attractive of all were (*E*)-2-nonenal and (*E*,*E*)-2,4-nonadienal; these two compounds exhibited strong attractancy at the 0.1-μg dose level which is only 1/10 to 1/100 the amounts of the other active compounds required to attract the beetles. In addition, only (*E*)-2-nonenal (at 0.1–10 μg) and formaldehyde (at 1–100 μg) effectively induce attraction over a 100-fold range of concentrations. Bioassay results not shown in Table 3 demonstrated that (*E*)-2-nonenal at 0.01 μg and formaldehyde at 1000 μg were both non-

attractive. (*E,E*)-2,4-Nonadienyl acetate produced anomalous results in that it was repellent at 100, 1, and 0.1 μg doses but was neutral at 10 μg.

Most of the aldehydes identified here are likely the result of oxidative cleavage (Murray et al., 1976) of oleic, linoleic, linolenic, and other unsaturated fatty acids that occur in oat lipids (Sahasrabudhe, 1979). All of the compounds presented in Table 3, except (*E,E*)-2,4-nonadienyl acetate and benzyl acetate, have been previously identified in oat volatiles (Heydanek and McGorrin, 1981a,b; Hrdlicka and Janicek, 1964).

Propanal and formaldehyde, although we could not detect them in this work, were bioassayed because they had previously been reported by Hrdlicka and Janicek (1964) to occur in "slightly steamed oat flakes." Neither the procedures by which the compounds were identified nor the quantities detected were reported. In any case, the fact that formaldehyde was attractive is both interesting and puzzling. A freshly opened bottle of commercial 37% formaldehyde in water, containing 10–15% methanol, was used for the bioassays, and dilutions were made as required with acetone. Control filter paper disks were treated with acetone containing the appropriate concentrations of methanol and water. Formaldehyde exists in a number of forms in solution, including less than 0.1% of monomeric formaldehyde (Walker, 1964). Two other possible forms, trioxane and dimethoxymethane, exhibited no activity in the bioassay. Similarly, formic acid, which can be formed by oxidation of formaldehyde in solution, produced no discernible attraction of the beetles.

The Petri dish bioassay, which was utilized previously for relatively nonvolatile materials (Freedman et al., 1982; Mikolajczak et al., 1983), seemed also to be effective for dilute, complex mixtures of volatiles such as the column chromatographic fractions. However, when this technique was applied to the pure individual compounds in Table 3, most of them appeared to be inactive. The insects tended to wander about in the chamber and not aggregate at either the treated or control disk. This apparent lack of interest is probably the result of chemosensory fatigue or disorientation of the insects caused by rapid saturation of the chamber atmosphere with these volatile materials. The pitfall chambers, in which the treated disk is somewhat removed from the immediate area where beetles are introduced into the apparatus, eliminated this problem.

Our results indicate that the attractancy of rolled oats for the sawtoothed grain beetle is not due to the effect of any one constituent, but is rather the net result of a number of stimuli. Limited experiments with whole oats as they come from the field reveal that they contain much the same array of carbonyl compounds; hence the whole grain's attractancy is probably due to a similar group of volatile constituents.

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HOST SELECTION BY *Blepharipa pratensis* (Meigen), A TACHINID PARASITE OF THE GYPSY MOTH, *Lymantria dispar* L.

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Abstract—The host selection process of *Blepharipa pratensis* (Meigen), a tachinid parasite of the gypsy moth, *Lymantria dispar* L., was investigated. Once in the host's habitat, and following contact with a recently damaged leaf edge (cut, torn, eaten), the fly orients perpendicular to the edge and moves back and forth with the front tarsi grasping the damaged edge. Oviposturing (oviposition intention) may occur. Leaf exudates appear to arrest the fly on the leaf and increase tarsal examination (searching). If an edge of a gypsy moth-eaten leaf is contacted, oviposition usually occurs. Significantly more eggs are laid when host-browsed foliage is encountered, compared to mechanically cut or damaged foliage, indicating response to a cue left by the host during feeding. The number of host-damaged leaf clusters in an area significantly enhances oviposition there; in field-cage tests, significantly more eggs (7911) were laid in simulated-crown areas with all clusters browsed, compared to the adjacent areas containing $\frac{1}{2}$ browsed (4200 eggs) and undamaged clusters (2209 eggs). A host selection sequence is suggested and discussed.

Key Words—Gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, *Blepharipa pratensis*, Diptera, Tachinidae, host selection, contact chemical, parasite behavior.

INTRODUCTION

Blepharipa pratensis (Meigen) a large tachinid parasite of the gypsy moth, *Lymantria dispar* L., deposits microtype eggs on foilage of its host's food plant. It was introduced to the United States in 1906 in an effort to establish it where the gypsy moth was rapidly proliferating. Subsequent importations and

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releases occurred between 1907 and 1933 (reviewed by Hoy, 1976). *B. pratensis* is now found throughout the major infest area of the gypsy moth and, based on collections of host material and recovery of parasites, it is considered one of the most effective biological control organisms (Herting, 1960; Pschorn-Walcher, 1974; Howard and Fiske, 1912). Recent investigations into the population dynamics and behavior of *B. pratensis* (Godwin and ODell, 1981), and the development of a technique for laboratory culture of the parasite (ODell and Godwin, 1979a), provide a basis for considering methods to enhance the dependability of *B. pratensis* as a biological control agent.

The behavioral patterns involved in host selection of entomophagous insects are the major determinants of their efficiency as a controlling agent (Lewis et al., 1975). Study of these patterns is providing new strategies for increasing the reliability of entomophages in pest management programs (Gross, 1981). Several reports summarize the life history of *B. pratensis*, and others discuss the incidence of parasitism relative to host population fluctuations (Sisojevic, 1975), but there have been only general observations on the behavior of the adult parasite and the process of host selection. Godwin and ODell (1981) reported that the majority of microtype eggs laid by *B. pratensis* were deposited on foliage in the upper crown of trees and that oviposition occurred during the day when gypsy moth larvae usually were resting in sites away from the crown. ODell and Godwin (1979a) noted that, in the laboratory, egg deposition by *B. pratensis* appeared to increase when gypsy moth-browsed leaves were placed in cages.

Studies of other tachinids that lay microtype eggs on foliage indicate that the leaf damage by the feeding host is an important cue used in selecting the oviposition site. Hassel (1968) reported that leaf damage and the resulting exudation are important in the selection of oviposition sites by *Cyzenis albicans* (Fall.), a tachinid parasite of the winter moth, *Operophtera brumata* (L.). Dowden (1934) observed that *Zenilla libatrix* (Panzer), a tachinid parasite of the gypsy moth and brown-tail moth, *Nygmia phaeorrhoea* (Donovan), oviposits on almost any type of leaf; if edges of the leaf are cut, the flies readily oviposit along these cuts.

The laboratory and field studies reported here were conducted to determine the importance of host-feeding damage on the selection of oviposition sites by *B. pratensis*, and behavioral patterns that affect host selection.

METHODS AND MATERIALS

Adult *B. pratensis* were reared from puparia recovered from gypsy moth pupae collected in Centre County, Pennsylvania, and New Lisbon, New Jersey, during the summers of 1974, 1975, and 1976. Parasite larvae, pupae,

and adults were held by the technique described by ODell and Godwin (1979a). Adult females were placed individually with males and observed for mating. Males were removed after mating and the females maintained individually in plastic bag cages. When egg laying commenced, flies were transferred, individually, to new cages each morning so that daily oviposition could be assessed.

In laboratory tests to characterize the effect of damaged leaves on the oviposition behavior of *B. pratensis*, two northern red oak, *Quercus rubra* L., leaves, with the stems immersed in water, were placed in each fly cage ca. 12 days after adult eclosion and just prior to the beginning of oviposition. Sixty female flies were divided into three groups and their respective cages provisioned daily: group I with leaves browsed by fourth-stage gypsy moth larvae during the previous 12 hr; group II with leaves cut with scissors just prior to placement in cages; and group III with undamaged leaves.

Oviposition begins 10–14 days after eclosion. During the preoviposition period, and extending for 2–3 days after, adult mortality is relatively high (ODell and Godwin, 1979a). To avoid the possibility of results being biased by a near-death condition of *B. pratensis*, only those flies that had laid eggs for five or more days were included in the study. The data recorded for each fly included age when first egg was laid, age at death, and the number of eggs laid on leaves and on the bag; less than 2% are found on other surfaces. The data were analyzed by analysis of variance (ANOVA) and Wilcoxon Rank Sum Test.

Field-cage experiments were conducted in two 6.1 × 2.5 × 3.1-m screen cages located in an open field at the Northeastern Forest Experiment Station's Forest Insect and Disease Laboratory field station in Branford, Connecticut. Red oak leaf clusters (3–6 leaves per cluster) were placed individually in water-filled vials and arrayed in wooden troughs hung horizontally 2.1 m off the ground.

To investigate the relationship between the proportion of leaf clusters browsed by the gypsy moth (considered here as a measure of host density), and *B. pratensis*'s selection of oviposition sites, clusters of leaves were arrayed in the cage to simulate a 2.4 × 3.4-m crown area, with five cluster columns and 12 cluster rows spaced 0.6 and 0.3 m apart, respectively (Figure 1). Within the simulated crown there were three areas of different host-browse density, each containing 20 leaf clusters; 100% = all clusters browsed; 50% = 10 browsed and 10 undamaged clusters arranged so that they alternated by row and column; and 0% = all clusters undamaged (Figure 1). The position of any particular host-density area within the crown was randomized for each of five releases conducted in 1977: one on June 15, and two each on June 22 and June 28. Browsed clusters were obtained by caging two fifth- or sixth-instar gypsy moths with each cluster the night prior to the test.

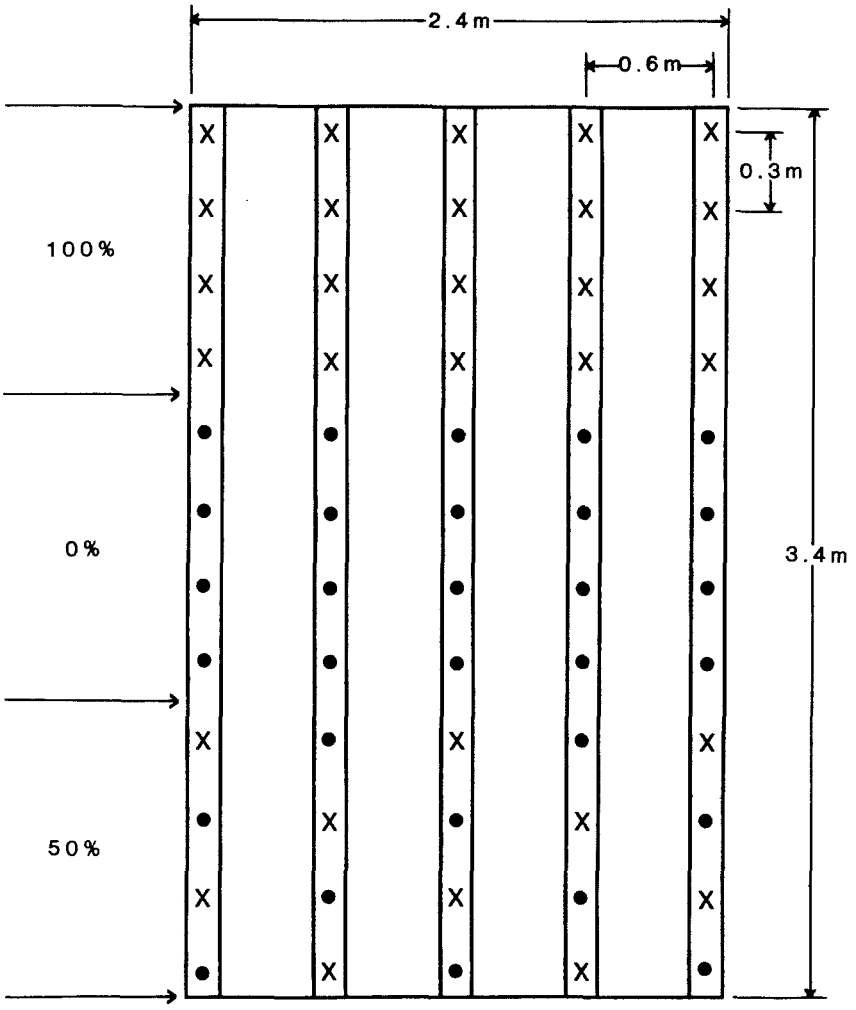

FIG. 1. Simulated tree crown containing 60 red oak leaf clusters: X = clusters browsed by gypsy moth larvae; ● = undamaged clusters. Clusters are maintained in vials with water and arrayed in three areas of host-browsed cluster density; 100% = all clusters browsed; 50% = 10 browsed and 10 undamaged clusters; 0% = all clusters undamaged. The position of areas was randomized for each release.

Flies were released between 1200 and 1400 hr on the first day of each trial and recaptured the following day at 1600 hr (all hours reported are Eastern Standard Time). Each fly was placed on a gypsy moth-browsed leaf just prior to release to enhance search activity. Five flies were used in the first release and two in each of the other trials. Leaf clusters were collected at the end of each trial and placed individually in plastic bags for examination in the laboratory.

The number of eggs was counted and recorded by cluster and location on the leaf (damaged and undamaged edge, or nonedge). The total leaf area per cluster was measured with a portable area meter (Lambda Instruments Corporation). The effect of cluster leaf area on the selection of an oviposition site was examined by calculating the correlation coefficient between cluster leaf area of each cluster in the five 100% treatment blocks and the number of eggs on each respective cluster. Analysis of variance was used to discern treatment effects. In both field and laboratory experiments, flies were observed for response to damaged leaf edges and oviposition behavior.

RESULTS

Within the plastic cages, single female *B. pratensis* typically migrated between the leaf cluster and lighted areas on the top of the bag, perusing each surface with its proboscis and stopping to feed or drink when honey (on plastic bag) or water was encountered. During the apparent search of the leaf surface, both sides were visited and tarsal contact with the leaf edge occurred frequently. When the front tarsi contacted a leaf edge that had been damaged (cut, torn, or eaten), an immediate and predictable behavioral response was elicited. The fly oriented perpendicular to the damaged edge and moved back and forth, apparently further examining the edge with the tarsi. After two or three passes over the edge, the fly grasped the edge with the front tarsi, the body elevated slightly, and the tip of the ovipositor was brought forward ventrally to the damaged margin. Although encounter with an eaten edge usually resulted in egg deposition, eggs usually were not laid when cut or torn edges were contacted. For example, one fly ovipostured 240 times on cut leaves during a 1-hr period, but only 85 eggs were laid. Eggs are also deposited by lowering the ovipositor to the leaf surface while the fly is walking. During peak oviposition, eggs were deposited rapidly on all areas of the leaf and on the plastic bag, particularly in well-lighted areas. A sticky substance deposited during oviposition adheres the eggs to the substrate.

Table 1 summarizes the results of the laboratory case tests. *B. pratensis* females caged with gypsy moth-browsed leaves laid significantly more eggs per female, and more eggs per oviposition day, than those cages with cut or undamaged leaves, but there was no significant difference in number of eggs laid between females caged with cut leaves and those caged with undamaged leaves. The place of oviposition (leaves or bag) did not differ significantly within or between treatments (Table 1), nor was there a significant difference in age of flies at initiation of egg laying, number of oviposition days, or age of flies at death.

In a preliminary field-cage test, flies that were not exposed to gypsy moth-browsed leaves prior to release ($N = 8$) flew directly to the top of the cage. Those that were exposed ($N = 8$) flew directly to leaves and began to

TABLE 1. LABORATORY TESTS TO DETERMINE OVIPOSITION RESPONSE OF *Blepharipa pratensis* TO CUT, BROWSED, AND UNDAMAGED LEAF CLUSTERS

Leaf treatment	N[a]	Activity periods[b]		Number of eggs laid[c]			
		1	2	Bag	Leaves	Cage total	Ave. eggs/♀/day[d]
Undamaged	12	14.3a[e]	14.7a	8954 (664.9)[f]	2120 (329.3)	10,714a (892.8)	60.8a
Cut	11	16.6a	8.4a	5252 (296.3)	3212 (510.5)	8464a (639.0)	92.0a
Eaten	10	13.8a	12.5a	9611 (604.5)	9017 (550.8)	18,628b (910.6)	149.0b*

[a]Number of female *B. pratensis*, one per cage.
[b]Average number of days of adult activity per female; period 1 = eclosion to initiation of oviposition, and period 2 = initiation of oviposition until death.
[c]Comparisons between locations from each treatment were made using Wilcoxon Signed Rank Test. No difference (at 5% level) was indicated.
[d]Average eggs laid per female per treatment divided by the total number of oviposition days (per treatment).
[e]Within columns, items followed by a different letter are significantly different from the others; * = significance < 0.01.
[f]Standard deviation.

TABLE 2. FIELD TEST TO DETERMINE OVIPOSITION RESPONSE OF *Blepharipa pratensis* TO VARIOUS DENSITIES OF HOST-BROWSED LEAF CLUSTERS = HOST DENSITY

Trial (N)	100		50		0	
	Eggs ($\bar{X}$)	%	Eggs ($\bar{X}$)	%	Eggs ($\bar{X}$)	%
5	1582.2 ± 389.31	55	840.0 ± 237.4	29	441.8 ± 40.36	15

Analysis of variance on natural logarithms of number of eggs

Source	DF	SSE	F[a]
Cluster density	2	2.4464	27.72**
End of cage	1	0.0382	0.864 ns
Density × cage end (treatment + edge)	2	0.1187	1.345 ns
Between cages	4	1.8934	10.728*
Total eggs laid (covar. in blocks)	1	1.6627	37.686***
Cage effects	3	0.2307	1.743 ns
Error (within cage)	5	0.2206	—

[a]* = significant at the 0.05 level, ** = 0.01 level, *** = 0.001 level; ns = not significant.

peruse leaves rapidly and lay eggs as they flitted from cluster to cluster. All flies used in the tests exhibited the preconditioned behavior. This locomotor behavior carried them up and down cluster rows several times each hour. Periodically, they stopped to preen and drink from vials containing the leaf clusters. Flies seldom left the crown area.

Table 2 summarizes the results of the field-cage tests. In all five trials, the proportion of browsed leaf clusters significantly affected the number of eggs laid within that block; the position of treatment within the array was not a significant variable. Eggs were found on 299 of the 300 clusters. Generally, clusters that bordered the 100% treatment had more eggs than others within the same treatment (0% or 50%). We observed that flies did not go beyond the end of the simulated crown. Since the turning behavior associated with change of direction might influence how many eggs were laid on end treatments, this variable was included in the ANOVA (Table 2). No effects were indicated.

Only 5% of the 10,496 eggs deposited on browsed leaf clusters were found on chewed leaf edges. Most, 79%, were laid on the general surface of the leaf, with the remainder, 16%, found on undamaged edges. Correlation coefficients for cluster leaf area vs cluster eggs for the five 100% treatment blocks were: $r = 0.2864$, 0.1517, 0.2569, 0.2470, and 0.6603. Thus, leaf area of clusters previously browsed by gypsy moth appears to have little, if any, influence on the number of eggs laid there.

DISCUSSION

Our observations indicate that female *B. pratensis* are stimulated when their front tarsi contact recently damaged oak leaf edges. Contact with a cut, torn, or host-damaged leaf edge elicits a host location response characterized by arrestment of flight, rapid examination of the leaf, search of adjacent leaves, and often oviposturing or stabbing behavior. Contact with a host-damaged leaf edge also elicits a host recognition response manifested by a significant increase in egg deposition (Table 1).

Specific stimuli associated with *B. pratensis* response to damaged leaves have not been identified. *B. pratensis*, as well as other tachinids (Dowden, 1934; Bess, 1936; Hassel, 1968), display oviposition behavior after contact with damaged leaves, whether eaten, torn or cut, so it is unlikely that a physical cue, such as a chewed edge, is stimulating. Sugars have been identified in leaf exudates associated with host location by the tachinid *C. albicans* (Embree and Sisojevic, 1965; Hassel, 1968), and they serve a general role in invertebrate arrestment (Dethier et al., 1960). Initial oviposition by *B. pratensis* is synchronized with the appearance of fourth-instar gypsy moth (Godwin and ODell, 1981). Since tarsal contact with damaged leaf edges elicits search and stabbing behavior during the preoviposition period (Godwin and ODell, 1981), contact chemicals, perhaps sugars in exudates

from leaves damaged by younger instars, could provide the initial stimulus for arrestment and search within the host habitat. Leaf damage also may provide gustatory stimulation for host habitat location, as reported for other tachinids (Monteith, 1964; Roth et al., 1978).

Once *B. pratensis* is fecund, host-chewed leaves influence where, as well as how many, eggs are laid. In laboratory cages, flies were attracted to areas on the side and top of the cage with the most light, and laid eggs there. They also were attracted to leaves. Our observations suggest that the apparent phototaxic response, the attraction to leaves, and the response to damaged leaves determined where eggs were laid. In field-cage tests, phototaxis, that is, flight to the top of cage, occurred only when flies were not preconditioned by exposure to host-browsed foliage. In the laboratory there was a great difference among individual females in the total number of eggs laid and in where they laid eggs (leaves vs bag); thus, total counts such as 8594 eggs laid on leaves vs. 2120 laid on bags are not significantly different (Table 1). The variability in number of eggs laid was further demonstrated in field cages by the significance of the contribution of the "total egg production" covariate to the "between cages" sum of squared errors (Table 2 ANOVA). Nevertheless, we believe the low proportion of eggs laid on undamaged leaves (20%), compared to those cut (38%), and host damaged (49%), is indicative of a host-selection response to damaged foliage.

Possible sources of host-produced stimuli, probably contact chemicals (kairomones), are the short strands of silk (Weseloh, 1977) we consistently found along leaf edges chewed by fourth-, fifth-, and sixth-instar gypsy moth; larval regurgitate (Nettles, 1982); and/or a combination of a host factor and leaf exudate (Sato, 1979).

In the field study of host density presented here, *B. pratensis* laid significantly more eggs in the areas with greater numbers of host-damaged leaf clusters (Table 2). We suggest that host apparency or susceptibility (see ODell and Godwin, 1979b), as determined primarily by the concentration of contact chemical (exudate, host chemical, or both) and perhaps the act of egg deposition (host acceptance response), effects a host-density response which arrests movement and maximizes the potential of eggs being ingested by concentrating them in crown areas with the greatest likelihood of being browsed.

B. pratensis's phototaxic and host-density response, coupled with a search behavior that affected daily perusal of all areas of simulated crown and the deposition of eggs on 299 of 300 leaf clusters, would seem to provide an efficient host-finding mechanism (as defined by Vinson, 1976) for locating a host whose attributes seem to limit apparency. For example, gypsy moth larvae usually are not available during the oviposition period; they generally feed at the top of the tree, and their feeding periodicity and locomotor activity often effect change in where freshly browsed leaves are found each day.

Identification of behavioral patterns and host-associated stimuli which mediate oviposition site selection will enhance the manipulation of parasite behavior. *B. pratensis*'s partial oviposition restraint (see Flanders, 1942) in the absence of host-derived chemical(s) provides a mechanism for bioassay of extracts to further identify components of the host-finding system. Extraction or synthesis of a kairomone that arrests migratory behavior and stimulates oviposition could be used to retain *B. pratensis* in target areas, and maintain effective activity. Kairomone might be incorporated into artificial media for inducing oviposition for mass rearing purposes.

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SYNTHESES OF UNCONJUGATED (*Z*, *Z*)-DIOLEFINIC INSECT PHEROMONES ON INSOLUBLE POLYMER SUPPORTS

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Abstract—A 2% cross-linked styrene–divinylbenzene copolymer containing pendant trityl chloride groups was used as the solid support in the synthesis of (*Z*, *Z*)-3,13-octadecadien-1-yl acetate, a component of the sex attractant of the lesser peachtree borer, *Synanthedon pictipes*, the peachtree borer, *Synanthedon exitiosa*, and the cherry tree borer, *Synanthedon hector*. This solid-phase synthesis is compared with a similar synthetic approach in solution. The solid-phase synthesis of (*Z*, *Z*)-7,11-hexadecadien-1-yl acetate, a component of the pheromone of the pink bollworm moth, *Pectinophora gossypiella* is described.

Key Words—(*Z*, *Z*)-3,13-Octadecadien-1-yl acetate, (*Z*, *Z*)-7,11-hexadecadien-1-yl acetate, solid phase synthesis, *Synanthedon pictipes*, lesser peachtree borer, *Synanthedon exitiosa*, peachtree borer, *Synanthedon hector*, cherry tree borer, *Pectinophora gossypiella*, pink bollworm moth, Lepidoptera, Sessiidae, Gelechiidae.

INTRODUCTION

As stated by Henrick (1977): "The structures of many lepidopterous sex pheromones are deceptively simple but their efficient synthesis in high stereochemical purity has offered considerable synthetic challenge." A wide variety of syntheses of insect pheromones has been described (Henrick, 1977; Rossi, 1977). In our own laboratories we have synthesized a large number of stereochemically pure monoolefinic insect pheromones (Svirskaya et al., 1979; Fyles et al., 1977; Leznoff et al., 1977) on insoluble polymer supports (Leznoff, 1978; Fréchet, 1981). One advantage of using the 1 or 2% styrene–divinylbenzene copolymer supports containing trityl chloride groups (Fyles

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and Leznoff, 1976; Fréchet et al., 1976) was the selective monoblocking by the polymer of the symmetrical diol starting materials by the "fishhook" principle (Leznoff, 1978). Other advantages include a stereoselective Wittig reaction simulating "salt-free" conditions (Leznoff et al., 1977) and a facile borane addition reaction in which borane impurities are removed by simple filtration of the polymer (Fyles et al., 1977). Finally, the whole procedure of pheromone synthesis on solid phases has the potential to be automated, as shown for polypeptide (Erickson and Merrifield, 1976) and now oligonucleotide synthesis (Alvarado-Urbina et al., 1981).

We now report the preparation of some diolefinic insect pheromones on solid phases using symmetrical difunctional intermediates. Although the synthesis of (*Z*, *Z*)-3,13-octadecadien-1-yl acetate **(1)**, a sex pheromone of the lesser peachtree borer, *Synanthedon pictipes* (Grote and Robinson), the peachtree borer, *Synanthedon exitiosa* (Say), and the cherry tree borer, *Synanthedon hector* (Butler), has been accomplished in solution (Doolittle et al., 1980; Uchida et al., 1978; Ebata and Mari, 1979) in several related ways, a retrosynthetic analysis of **1** reveals the presence of a symmetrical difunctional $-(CH_2)_8-$ unit in the middle of **1** and, since solid-phase methodology lends itself to the use of symmetrical difunctional intermediates, the advantageous use of polymer supports in the preparation of **1** is indicated.

METHODS AND MATERIALS

All melting and boiling points are uncorrected. Infrared spectra (IR) were recorded on a Unicam SP1000 IR spectrophotometer as neat films between NaCl disks or KBr plates. Nuclear magnetic resonance spectra (NMR) were recorded on a Varian EM360 spectrometer, with deuteriochloroform as solvent and tetramethylsilane as an internal standard. Mass spectra (MS) were recorded at 70 eV on a VG Micromass 16F mass spectrometer in the EI mode.

High-pressure liquid chromatographs were run with a Waters Associates model 440 instrument, with an R-400 refractive index detector and a 30 × 0.4-cm reverse-phase μBondapak C-18 column using water–acetonitrile (1 : 3) for the C_{16}–C_{18} diyne acetates, and a Zorbax ODS column (25 cm × 0.9 mm) using water–acetonitrile (1 : 9) for the C_{16} diene–acetate and acetonitrile eluant for the C_{18} diene acetate, at a flow rate of 1.0–1.5 ml/min. Experiments involving reactive organometallic reagents or boron hydrides extremely sensitive to air and moisture were carried out under an argon atmosphere, using dry and pure solvents.

Tetrahydrofuran (THF) was refluxed over $LiAlH_4$ for 4 hr and distilled under argon. Freshly distilled THF was always used. Hexamethylphosphoric

triamide (HMPT) (Caution: hazardous) and other solvents were dried and distilled according to standard methods.

Silica gel was used for all thin and preparative layer chromatography (TLC) unless otherwise specified. Preparative TLC fractions were extracted with ether in a Soxhlet extractor. Filtration was done under vacuum through sintered glass Buchner funnels. The phrase "polymer was washed below" refers to the following procedure: after a polymer-bound compound was filtered; it was washed at least two times with the same solvent in which the reaction was carried out. The polymer was washed 2–3 times with THF, ethanol, 3–5 times with water, and 2–3 times with ethanol. The polymer was then washed 2–3 times with the solvent in which the reaction was carried out and finally 2–3 times with ethanol and 3–4 times with ether. The polymers, after being washed and air-dried, were transferred to a thimble in a Soxhlet extractor in which molecular sieves (3 Å) had been placed in a second thimble, and the polymer was extracted with dry benzene (or ether) for 3–5 hr under reflux conditions. The polymer was washed with dry ether and dried in vacuo. The reaction schemes are shown in Figures 1 to 4.

Polymer-BoundDiol (3). The slurry of 15 g of **2,** containing 1.3 mmol Cl/g and 15 g of 1,2-ethanediol in 5 ml pyridine was heated at 70–80° (bath) under argon overnight. Polymer **3** was isolated as previously described (Fyles and Leznoff, 1976). Cleavage with acetyl chloride in glacial acetic acid gave 1 mmol/g of 1,2-ethanediyl diacetate.

Polymer-Bound Monomesylate (4). Into a three-necked flask fitted with a magnetic stirrer, a dropping funnel, a thermometer, and a $CaCl_2$ drying tube was placed 15 g of vacuum-dried polymer **3,** 150 ml of dry methylene chloride, and 20 g of dry triethylamine. To this suspension at −10 to 0° was added slowly 10 g of freshly distilled methanesulfonyl chloride. The reaction mixture was stirred at 0° for 12–16 hr, and the resin **4** was filtered, washed, extracted with benzene in a Soxhlet extractor, and vacuum dried. The slightly yellow polymer **(4)** formed showed very strong absorptions at 1170 and 1360 cm^{-1} in its IR spectrum. Standard acid cleavage of 1.0 g of **4** with 0.35 M HCl in dioxane (Fyles, 1976) after preparative TLC gave 0.61 mmol/g of **5.**

Polymer-Bound 3-Butyn-1-ol (6). A slurry of 3 g of the LiC≡CH·EDA complex and 10 g of **4** in 75 ml of a 2:1 mixture of THF and HMPT under argon was heated at 60° (bath) overnight. The reaction mixture was cooled, and the excess of lithium acetylide was quenched with THF–H_2O (1:1). The resin **(6)** was filtered, washed, extracted with ether in a Soxhlet extractor, and dried in vacuo.

Polymer-bound 3-butyn-1-ol **(6)** was also prepared from 5 g of **2** and 5 g of **7** in pyridine according to the procedure described above for **3.**

Polymer-Bound 12-Bromo-3-dodecyn-1-ol **(9).** Into 100 ml of THF in a 250-ml three-necked flask, was placed 8 g of polymer **6.** The flask was

equipped with a magnetic stirrer, thermometer, argon–air inlet, condenser (without water) connected with a large needle directly to the atmosphere, and kept at 60° (oil bath). To the stirred slurry was added, over a 15-min period, 10 ml of *t*-BuLi (2 M in pentane).

The reaction is very exothermic. The pentane was evaporated after the addition of *t*-BuLi was complete, water was passed through the condenser, and the resulting blood-red mixture was stirred for a further 0.75 hr at 60°. The reaction mixture was cooled to room temperature, and 13.5 g of 1,8-dibromooctane **(8)** in 40 ml of HMPT was added (at once). The reaction mixture was stirred overnight, treated with THF–H_2O, and filtered. The excess of **8** was recovered from the filtrate (10 g). The polymer **(9)** was washed, extracted with benzene in a Soxhlet extractor, and dried. Acid cleavage of 1.0 g of **9** and purification by preparative TLC on silica gel (eluant ether–C_6H_6 1:4) gave 87 mg (0.33 mmol/g) of **10.** IR (neat) ν cm^{-1}: 3350 (OH) and 1040 (C-O). NMR: δ 3.8–3.2 (4H, m, overlapping triplets); 2.62–1.10 (17H, broad); 1.4 (s). (Found: C 55.29; H 8.39. Calc. for $C_{12}H_{21}BrO$: C 55.17; H 8.10.) A similar experiment was carried out using **6,** prepared directly from commercial **7.** Acid cleavage gave 0.34 mmol/g of **10.**

*Polymer-Bound 3,13-Octadecadiyn-1-ol **(12).*** To a stirred solution of 4.1 g (50 mmol) of 1-hexyne in 20 ml of THF at −10 to 0° was added dropwise 35 ml (51 mmol) of *n*-BuLi in hexane under argon. The reaction mixture was stirred at 0° for 1 hr, and a slurry of 1-lithiohexyne **(11)** was added by syringe to the suspension of 7.5 g of **9,** containing 2.4 mmol of **10** in 50 ml of HMPT. The stirring was continued overnight, the reaction mixture was cooled and hydrolyzed with THF–H_2O. The product **12** was isolated by filtration, washed, and dried. Acid cleavage of 1 g of **12** and purification of the diynol **13** by preparative TLC on silica gel (eluant ether–C_6H_6, 1:4) gave 72 mg (0.27 mmol/g) of pure **13,** mp 27–28°. The IR and NMR spectra were identical with the published spectra (Doolittle et al., 1980; Uchida et al., 1978; Ebata and Mori, 1979). Analysis of the acetate by HPLC (mobile phase H_2O–CH_3CN, 1:3) showed one single peak of chemical purity greater than 99%.

*Polymer-Bound Dienol **(14).*** To a 10-mmol solution of $(Sia)_2BH$ or slurry of dicyclohexylborane in THF, prepared as described previously (Svirskaya et al., 1980), was added at 0–2° C 2–3 g of **12** containing 0.5–0.7 mmol of **13** under argon. The reaction mixture was stirred at 0–2° (cold plate) for 24 hr. To the cool suspension, 2 ml of AcOH was added dropwise. The cold plate was removed. The mixture was allowed to warm to room temperature and was stirred for an additional 0.5 hr. The polymer was filtered, washed, and dried.

(Z,Z)-*3,13-Octadecadien-1-ol **(15).*** Acid cleavage of **14** and purification by column chromatography gave product of >99% purity. The IR and NMR spectra of **15** were similar to those of an authentic sample.

(Z,Z)-*3,13-Octadecadien-1-yl Acetate **(1).*** Acetylation of **15,** as described

in the literature (Uchida eta l., 1978) gave acetate **1**, which was analyzed by HPLC. The μBondapak C_{18} column did not completely separate isomers (>99% purity), but isomers can be separated using a Zorbax ODS (Dupont) column. The analysis indicated ratios of *Z*, *Z* to (*Z*, *E* and *E*, *Z*) as 93:7 (on the polymer) and 95–96:5–4 in solution.

9-Tetradecyn-1-ol ***(22).*** Compound **21** was prepared from 8-chlorooctan-1-yl tetrahydropyranyl ether **(20)** (7.44 g, 30 mmol) and 1-lithiohexyne **(11)** (which was formed from 2.87 g, 35 mmol of hexyne and 23 ml, 33 mmol of *n*-BuLi) in a solution of THF–HMPT by the method of Schwartz and Waters (1972). The crude **21** (10 g) was treated with a methanolic solution of *m*-benzenedisulfonic acid to remove the THP-protecting group. The pure alcohol **22** was obtained after column chromatography on silica gel (eluant, ether–benzene, 15:85) and distillation in 84% yield (5.3 g), bp 118–120°/0.35 mm, n_D^{20} 1.4630 [lit. (Uchida eta l., 1978) bp 122–126/0.65 mm, n_D^{22} 1.4621].

1-Bromo-9-tetradecyne ***(23).*** The bromination of **22** (5.2 g, 24 mmol) with bromine (4.8 g, 30 mmol), triphenylphosphine (7.5 g, 30 mmol), and pyridine (5 ml) in dioxane (100 ml), as described by Disselnkotter et al. (1976), gave the crude product **23** in quantitative yield. Column purification on silica gel (eluant, hexane–benzene 1:1) and further distillation afforded pure product **23** (5.4 g) in 84% yield, bp 126–128°/0.1 mm. NMR δ: 3.6 (t, 2H, J = 7 Hz), 2.0 (m, 4H), 1.8–1.2 (m, 16H) 0.9 (t, 3H, J = 7 Hz).

3,13-Octadecadiyn-1-ol ***(13).*** To a stirred solution of the lithium salt of the tetrahydropyranyl ether of 3-butyn-1-ol **(16),** prepared from 3.1 g, 20 mmol of the tetrahydropyranyl ether of 3-butyn-1-ol in 20 ml THF, and 20 mmol *n*-BuLi in hexane at −10 to 0° (the mixture was kept 1 hr at 0°), was added dropwise 4.4 g (16 mmol) of 1-bromo-9-tetradecyne **(23)** in 20 ml HMPT. The deep blue solution was stirred for 0.5 hr at 0°, 2 hr at room temperature, and 1 hr at 40°. The mixture was poured into ice-water, the resulting colorless solution was separated, and the aqueous layer was thoroughly extracted with hexane. The combined organic extracts were washed with water and dried over K_2CO_3. Removal of the solvent gave 5 g (90%) of crude tetrahydropyranyl ether of **13.** The hydrolysis of the protecting group was effected by warming the crude product with *m*-benzenedisulfonic acid in aqueous methanol at 50° for 2 hr and at room temperature overnight to give the free alcohol **13.**

Diyne **13** was worked up in the usual manner and purified by column chromatography on alumina (Woelm, neutral, grade II, eluant ether–benzene, 15:85). The pure fraction (3.5 g, 85%) was distilled (Kugelrohr, bath temperature) at 140–145°/0.01 torr, mp 27–28°. Recrystallization from hexane gave **13** mp 29–30°. Analysis of the acetate of **13** on the HPLC showed that the product was >99% pure [lit. (Doolittle et al., 1980; Uchida et al., 1978) mp 26–27, 27–28°].

Borane Reduction of Diynol ***13*** *to* ***15*** *in Solution.* The diynol **13** was

reduced with dicyclohexylborane in THF at −5 to 0° for 4 hr and for an additional 2 hr at room temperature, or with disiamylborane for 24 hr at 0°. (Reduction of the diynol **13** with $(Sia)_2BH$ for 6 hr gave a mixture of mono and bis reduction product.)

Products were hydrolyzed with glacial acetic acid at 40–50° for 5 hr and oxidized with 30% H_2O_2 in 6 N NaOH. The dienol was worked up and purified by column chromatography on alumina (grade 2) to give the *Z, Z* isomer in 80–85% yield of >99% purity. HPLC analysis of the acetate (Zorbax ODS column) gave 95–93% of the *Z, Z* isomer.

Polymer-Bound Diol (27). A slurry of 20 g of **2** and 18 g of **26** in 125 ml of pyridine was stirred at room temperature for 48 hr to give polymer **27.** Cleavage of **27** with 0.3 N HCl in dioxane, as previously described (Fyles and Leznoff, 1976), gave 1.1 mmol of **26**/g of **27.**

Polymer-Bound Monomesylate (28). Compound **28** was prepared according to the procedure described for **4** above. Cleavage of **28** as before yielded 0.62 mmol of 1,6-hexanediol monomesylate **(29)**/g of **28.**

Polymer-Bound 7-Octyn-1-ol (30). Compound **30** was prepared according to the procedure described for **6** above. Acid cleavage of **30** gave 0.31 mmol of 7-octyn-1-ol **(31)**/g of **30.**

Reaction of Polymer-Bound 7-Octyn-1-ol (30) with t-BuLi and 3-Octyn-1-yl Mesylate (32). Cleavage of polymer, which formed in the usual coupling reaction described for **9,** from polymer **30,** *t*-BuLi and 3-octyn-1-ol mesylate **(32)** gave only 0.30 mmol/g of the starting alcohol 7-octyn-1-ol **(31).**

Reaction of Lithium Salt of Tetrahydropyranyl Ether of 7-Octyn-1-ol with 1-Bromo-3-octyne (32a). Conditions of the coupling reaction were analogous to those for compound **13.** After the organic layer was separated, washed, dried; the solvent was distilled (short path); and the residue was chromatographed on silica gel. The first component isolated from the column (eluant pentane) as a colorless oil was 1-octen-3-yne **(33).** The physical and spectroscopic data were identical with those described by Anzilotti (1939). The compound eluted with benzene was the starting tetrahydropyranyl ether of 7-octyn-1-ol.

Polymer-Bound 7,11-Dodecadiyn-1-ol (35). The coupling reaction was carried out as for **12,** but the reaction mixture was stirred for 1 hr at room temperature and 1 hr at 40°. The product after cleavage was isolated by preparative TLC (solvent ether–benzene, 3:7) to give 30 mg (0.17 mmol/g) of 7,11-dodecadiyn-1-ol **(36).**

Polymer-Bound 7,11-Hexadecadiyn-1-ol (37). Polymer **37** was prepared in a typical procedure described above from the lithium salt of polymer-bound 7,11-dodecadiyn-1-ol and butyl bromide and in a separate experiment from polymer-bound 1,6-hexanediol monomesylate **(28)** and 1-lithio-1,5-decadiyne **(38).** After cleavage and purification using flash chromatography (Still,

1978), 30 mg (0.12 mmol/g) of 7,11-hexadecadiyn-1-ol (**39**) was isolated in the first experiment, and 50 mg (0.21 mmol/g) of **39** in the second.

Polymer-Bound (Z,Z)-*7,11-Hexadecadien-1-ol (40).* Reduction of the polymer **37** as for **12** gave **40.**

(Z,Z)-*7,11-Hexadecadien-1-ol (41).* Acid cleavage of 1 g of **40** gave 39 mg or 0.16 mmol/g of (*Z,Z*)-7,11-hexadecadien-1-ol (**41**). HPLC analysis of the acetate, prepared as before, using the μBondapak C_{18} column showed only a single peak (>99% purity), but isomers were separated on a Zorbax ODS column. Analysis by HPLC showed that reduction of diynols **37** and **39** with dicyclohexylborane gave dienols of higher stereochemical purity (93–96%) than reduction with disiamylborane (90%).

RESULTS AND DISCUSSION

The solution-phase synthesis of **1** by Doolittle et al. (1980) used 1-chloro-8-iodooctane prepared in 41% yield from the symmetrical 1,8-dichlorooctane, while an alternate synthesis by Uchida et al. (1979) used the tetrahydropyranyl ether of 8-chlorooctan-1-ol, itself prepared from the symmetrical 1,8-octanediol. Polymer-bound trityl chloride (**2**) reacted with excess 1,2-ethanediol to give the monoreacted polymer-bound monotrityl ether of 1,2-ethanediol (**3**) by methods previously described (Fyles and Leznoff, 1976). Subsequent reaction of **3** with methanesulfonyl chloride (MsCl) in methylene chloride and triethylamine (Svirskaya et al., 1979) yielded the polymer-bound monotrityl ether of 1,2-ethanediol monomesylate (**4**). This modified mesylation procedure affords the monomesylate **4** in high yield as acid cleavage (Svirskaya et al., 1979; Leznoff et al., 1977) of **4** liberates 0.61 mmol of 1,2-ethanediol monomesylate (**5**) per g of polymer **4.** Coupling of **4** with the lithium acetylide-ethylenediamine (LiC≡CH·EDA) complex in 2:1 tetrahydrofuran (THF)–hexamethylphosphoric triamide (HMPT) yielded the polymer-bound trityl ether of 3-butyn-1-ol (**6**). Alternatively, **2** reacted directly with commercially available 3-butyn-1-ol (**7**) to give **6** in one step, but from the more expensive precursor **7.** Treatment of **6** with *n*-BuLi and a large excess of the symmetrical 1,8-dibromooctane (**8**) led to the polymer-bound bromoalkyne (**9**). Acid cleavage of **9** gave 12-bromo-3-dodecyn-1-ol (**10**) in 54% yield based on **4** and showed that **9** contained at least 0.33 mmol of **10** per gram of polymer **9.** Coupling of **9** with 1-lithiohexyne (**11**) in THF-HMPT gave the polymer-bound diyne (**12**). Acid cleavage of **12** gave 3,13-octadecadiyn-1-ol (**13**) in 82% yield based on polymer **9.** Reduction of **12** with dicyclohexylborane (Zweifel and Polston, 1970) or disiamylborane (Brown and Zweifel, 1961) yielded the polymer-bound *cis,cis*-diene **14.** Acid cleavage of **14** gave (*Z,Z*)-3,13-octadecadien-1-ol (**15**) in 67% yield based on polymer **12.** Acetylation of **15** gave **1** (Figure 1) in nearly quantitative yield.

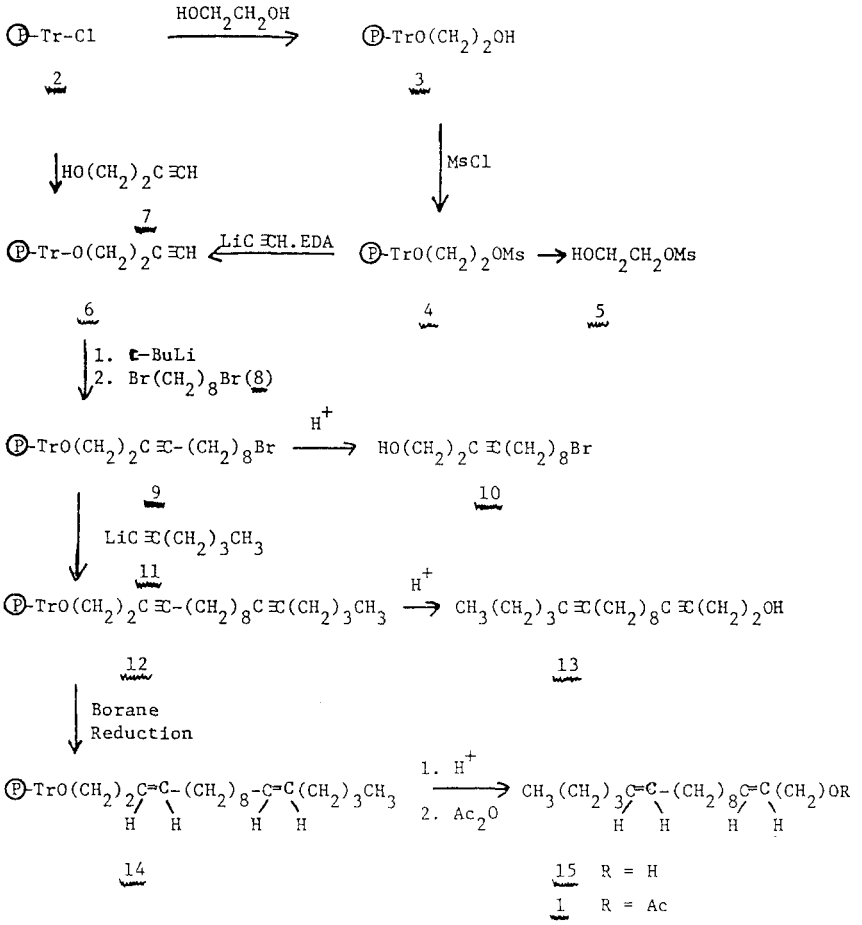

FIG. 1.

The seven step synthesis of **1** on insoluble polymer supports in an overall yield of 30% represents a synthesis long enough to set up for the automated procedures possible in solid-phase methodology. In addition, the use of the solid phase to monoprotect 1,2-ethanediol to form **3**, to monoreact with 1,8-dibromooctane **(8)**, and to facilitate the work-up of the borane reduction step leading to **14** (hydrogen peroxide does not have to be used to make borane impurities water soluble) illustrates some of the advantages of solid-phase synthesis of diolefinic pheromones.

As a control to the solid phase synthesis of **1**, we prepared **1** by solution methods related to, but not identical with, those previously described (Doolittle et al., 1980; Uchida et al., 1978) and to our solid-phase method

described above. Treatment of the lithium salt of the tetrahydropyranyl ether of 3-butyn-1-ol **(16)** with a large excess of the symmetrical 1,8-dibromooctane **(8)** in THF-HMPT gave the tetrahydropyranyl ether of 12-bromo-3-dodecyn-1-ol **(17)** in only 23% yield accompanied by many by-products, despite the fact that a similar sequence of reactions gave a monobromoalkyne in high yield from a symmetrical dibromo compound using the dihalide in excess (Burgstahler et al., 1977). This similar reaction was accomplished on solid phases in 54% yield with the additional advantage that the product was purified by simple filtration, the by-products and excess dihalide remaining in the filtrate. In a second synthetic approach to **1** in solution, 1,8-octanediol **(18)** was converted to 8-chloro-1-octanol **(19)** and hence to 8-chloro-1-octanol tetrahydropyranyl ether **(20)**. Coupling of **20** with 1-lithio-1-hexyne **(11)**, as before, yielded the tetrahydropyranyl ether of 9-tetradecyn-1-ol **(21)**. Acid hydrolysis of **21** gave 9-tetradecyn-1-ol **(22)** in 84% yield from **20** as shown in Figure 2. Conversion of **22** into 1-bromo-9-tetradecyne **(23)** with bromine and triphenylphosphine in pyridine (Disselnkötter et al., 1976) was achieved in 84% yield. This procedure represents an improvement over previous methods (Doolittle, 1980; Uchida, 1978). Coupling of **23** with **16**, as before, followed by acid hydrolysis, gave 3,13-octadecadiyn-1-ol **(13)** in 85% yield. Reduction of **13** with dicyclohexylborane or disiamylborane, as above, yielded (*Z*, *Z*)-3,13-octadecadien-1-ol **(15)** in 85% yield. Acetylation of **15** yielded **1** in nearly quantitative yield.

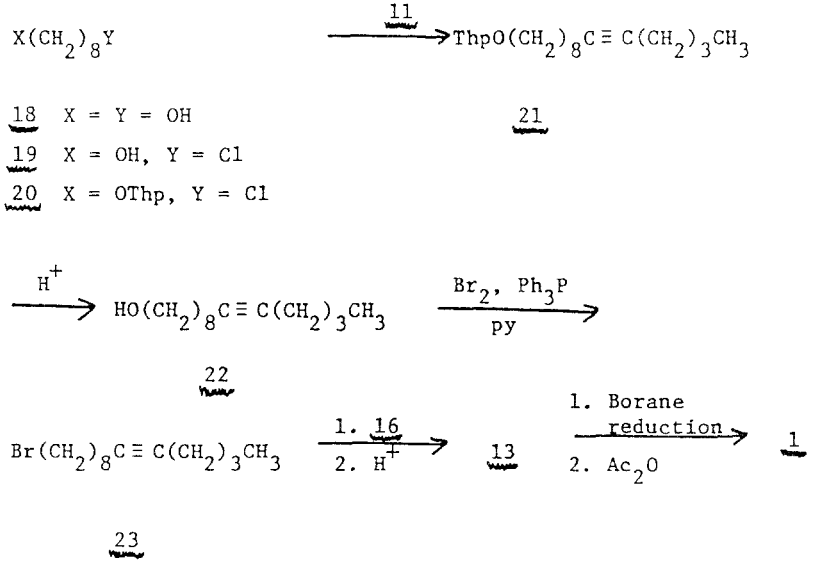

FIG. 2.

The almost identical approach to the synthesis of **15** on polymer supports and in solution via the symmetrical **8** favors the solid-phase approach. The much better solution-phase synthesis of **1** (Figure 2) went in 32% yield based on the symmetrical **18** and **16** in eight steps. The polymer-based synthesis gave **15** in 30% overall yield from **8** and **16** in only five steps, or from **8** and the more simple 1,2-ethanediol in seven steps with the advantages outlined before. The main disadvantage of polymer-based synthesis arises from the fact that the solid-phase syntheses work more efficiently using polymers containing only 10–25% of the phenyl groups of **2** functionalized and hence reaction volumes tend to be five to ten times larger than comparable reactions in solution. One long standing problem in organic synthesis on polymer supports has been recently alleviated using [^{13}C]NMR spectroscopy (Jones et al., 1982). In fact, we have recently characterized polymer-bound trityl alcohol (**2**, Cl=OH), **3**, **4**, **6**, and **9** by this method. Cleavage of a polymer-bound substrate from the polymer still remains the major quantitative method of evaluating the yield of each reaction step.

The solid-phase synthesis of (*Z*, *Z*)-7,11-hexadecadien-1-yl acetate **(24)**, a sex pheromone component of the pink bollworm moth, *Pectinophora gossypiella*, has been reported by many synthetic routes (Henrick, 1977; Rossi, 1977; Disselnkötter, 1976; Su, 1974; Sonnet, 1979; Mori, 1975; Anderson, 1975) in solution. In the same retrosynthetic analysis of **24** as that described above for **1**, the symmetrical synthon in the middle of the molecule is simply 1,2-dibromoethane **(25)**. We felt, however, that the coupling of an acetylene to **25** would give a β-bromoacetylenic intermediate, which on subsequent reaction would lead to elimination instead of coupling. This prediction was in fact borne out, as shown in Figure 3.

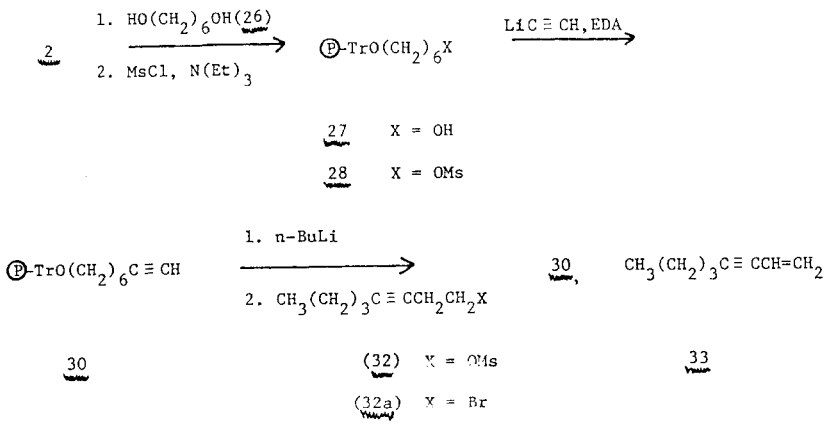

FIG. 3.

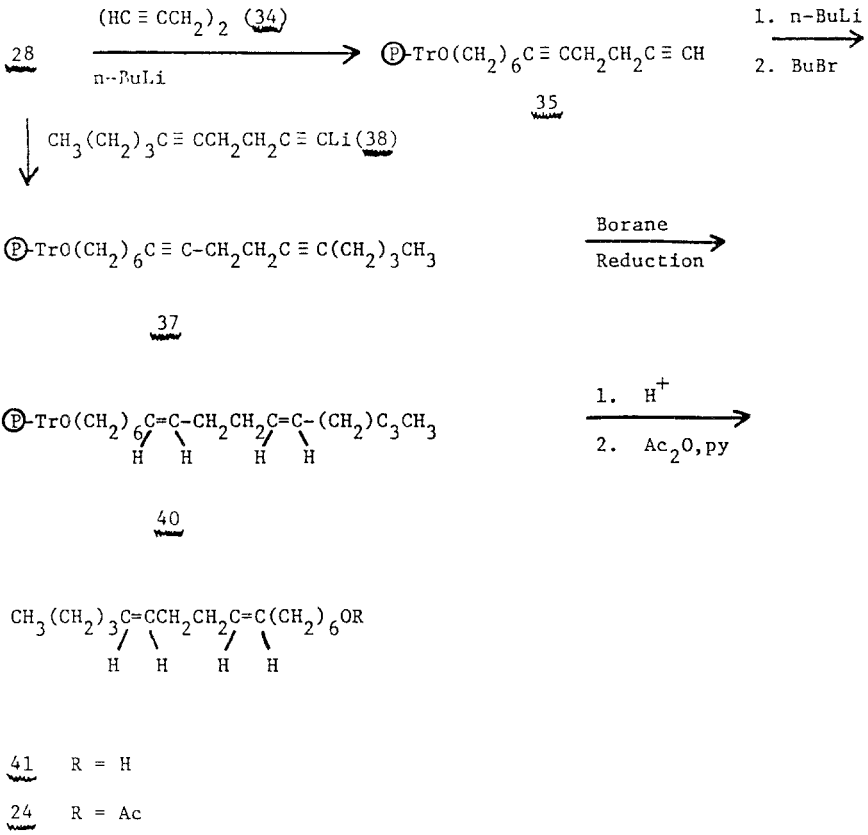

FIG. 4.

Thus polymer **2** reacted with 1,6-hexanediol **(26)** at one end only to give the polymer-bound monotrityl ether of 1,6-hexanediol **(27)** (Svirskaya et al., 1979; Fréchet, 1981) which was mesylated, as for **4**, to give the polymer-bound monotrityl ether of 1,6-hexanediol monomesylate **(28)** (Leznoff et al., 1977). Acid cleavage of **28** liberated 0.62 mmol of 1,6-hexanediol monomesylate **(29)**. Treatment of **28** with the lithium acetylide complex, as before, gave the polymer-bound monotrityl ether of 7-octyn-1-ol **(30)**. Acid cleavage of **30** gave 7-octyn-1-ol **(31)** (Ames and Goodburn, 1967) in 50% yield based on **28**. When **30** reacted with *t*-BuLi and 3-octyn-1-yl mesylate **(32)**, only **30** was recovered. In a model reaction, 1-bromo-3-octyne **(32a)** (Disselnkötter et al., 1976) reacted with *n*-BuLi to give the known elimination product 1-octen-3-yne **(33)** (Anzilotti and Vogt, 1939). Thus, 1,2-dibromoethane would not be a suitable symmetrical intermediate for the synthesis of 1,4-diynes. Another symmetrical synthon derived from **24** can be envisioned, namely, 1,5-

hexadiyne **(34).** Thus, treatment of **28** with the lithium salt of **34** led to the polymer-bound trityl ether of 7,11-dodecadiyn-1-ol **(35).** Acid cleavage of **35** gave 7,11-dodecadiyn-1-ol **(36)** in 27% yield based on **28.** Subsequent coupling of **35** with *n*-BuLi and butyl bromide gave polymer-bound trityl ether of 7,11-hexadecadiyn-1-ol **(37).** Alternatively **37** was prepared by direct coupling of **28** with *n*-BuLi and 1,5-decadiyne **(38).** Acid cleavage of **37** liberated 0.21 mmol of 7,11-hexadecadiyn-1-ol **(39)** per g showing that **37** was formed in 37% yield using **38** but in only 20% yield using **34.** Borane reduction of **37** via **38,** as before, gave the polymer-bound trityl ether of (*Z*,*Z*)-7,11-hexadecadien-1-ol **(40).** Acid cleavage of **40** afforded (*Z*,*Z*)-7,11-hexadecadien-1-ol **(41)** in 67% yield based on polymer **37.** Acetylation of **41** gave the desired pheromone **24** in high yield (Figure 4).

The overall yields of **24** synthesized via **38** on solid phases, as shown in Figure 4, are similar to the yields obtained by similar solution methods. The advantages and disadvantages of solid-phase synthesis were similar to those described above for the synthesis of **1,** except that for 1,4-diynes, one cannot use the inexpensive symmetrical dihalide. Unfortunately the symmetrical synthon **34** gives low yields, probably due to allenic impurities (Mori et al., 1975). Analysis of **1** and **24** by high-pressure liquid chromatography (HPLC) showed that all the products exhibited not less than 90% and more commonly 93–98% of the *cis,cis* isomer. The stereochemical purity of **1** was identical when **1** was synthesized by solid phase or solution methods.

The syntheses of **1** and **24** demonstrate a practical synthesis of unconjugated diolefinic pheromones using solid-phase methodology. Furthermore, one can see that unconjugated tri-, tetra-, and oligoacetylenic compounds can be prepared by the repetitive additions of symmetrical difunctionalized synthons to a growing polymer-bound hydrocarbon chain.

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APPARENCY OF PULSED AND CONTINUOUS PHEROMONE TO MALE GYPSY MOTHS[1]

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Abstract—Quiescent male gypsy moths (*Lymantria dispar*) exposed in a wind tunnel to either pulsed (0.5-, 2- or 5-sec on, followed by a two-fold time interval off) or continuous streams of synthetic pheromone responded similarly in the proportions and latencies of wing fanning. Similarly, upwind anemotactic flight tracks in pulsed (1-sec on and 1-sec off) and continuous plumes of pheromone were indistinguishable. These data suggest that in the gypsy moth (1) pulsed pheromone stimuli would not lower the threshold, despite the improvement in the signal-to-noise ratio; and (2) temporal modulation of the pheromone plume at 1-sec intervals does not alter the "preprogrammed" upwind flight pattern.

Key Words—Pheromone, attractant, gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae, threshold, anemotaxis.

INTRODUCTION

The threshold for eliciting a behavioral response to a pheromone generally is characterized as the minimum concentration of pheromone necessary to evoke that behavior. The threshold of the wing-fanning response in male *L. dispar* to female-produced pheromone can be described by the increasing proportion responding and the decreasing latency of response when the concentration is increased (Cardé and Hagaman, 1979; Hagaman and Cardé, 1984). In a wind tunnel assay the threshold for a time-averaged concentration

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of pheromone eliciting a response by ca. 50% of the males within 20 sec was 2×10^{-17} g/cm^3 of (+)-disparlure (Hagaman and Cardé, 1984).

However, a time-averaged concentration of pheromone is unrepresentative of the complex temporal patterning of the stimulus encountered and potentially perceived in the field by either a quiescent or a flying male. As originally emphasized by Wright (1958), turbulent dispersion causes a pheromone plume to writhe and twist as it is transported downwind, thereby creating, particularly close to the source, a train of pheromone pulses relative to a fixed sampling point. Also, the zigzag flight path characteristic of upwind flight to pheromone can carry a moth in and out of a pheromone plume, creating an on–off pattern of contact with the stimulus (Cardé and Hagaman, 1979). Further, females of many species of moths, including the gypsy moth, rhythmically pulse (protrude and retract) their pheromone-emitting gland at ca. 1-sec or longer intervals (e.g., Cardé and Roelofs, 1973; Conner, et al., 1980). The interval between pulses in 1-day-old female gypsy moths is more variable than in the arctiids examined to date. The mean pulse frequency was 3.2 sec (6.5 SD) with a range of 0.5–50 sec (in 43 one-day-old females at 25° during the 9th to 10th hours of a 16-hr photophase, Charlton and Cardé, unpublished).

Both of these mechanisms impart a temporal pattern to the pheromone plume. The pulses generated by turbulent dispersion could serve as orientation cues, signifying, for example, distance to the source as proposed by Wright (1958). Or the pulses from females could be important in species recognition by elevating the information content of the signal and imparting to it a temporal pattern (Bossert, 1968).

The temporal pattern of a signal alters the number of signal-to-noise comparisons transmitted by the sensory receptors, if the interpulse interval is sufficient to allow recovery of the receptor from stimulation. By providing additional comparisons, a pulsed pheromone message (whether achieved by a pulsing gland, turbulent dispersion, or both) could diminish the threshold and thus render a pulsed plume more "apparent" than a continuous one. In the following tests with the male gypsy moth we consider (1) the effects of a temporal modulation of the pheromone stimulus upon threshold, as measured by the proportion responding and the response latency; and (2) the path of the zigzag upwind flight in discrete and pulsed pheromone plumes.

METHODS AND MATERIALS

Pulse Generator. A system for delivering discrete, predictable, and consistent units of pheromone-laden or pheromone-free air to the moths over variable time intervals was developed. The device provided an isokinetic and periodic injection of pheromone-laden air into a coflowing air stream. The periodic introduction of the pheromone can be characterized in terms

of the period (T) of a complete cycle and the fraction (τ / T) of the time that the pheromone-laden air enters the streaming flow. Schematic representations of the pulse generator and the quantities τ and T are presented in Figures 1–3. The pneumatic device and the control circuitry met the design conditions of $1.5 \leqslant T \leqslant 180$ sec and $0.5 \leqslant \tau \leqslant 5$ sec.

An important design constraint was that the concentration of the pheromone-laden air stream should be constant over many cycles of an experimental trial. This was achieved by maintaining a constant air flow over the pheromone dispenser and internally directing the pheromone laden air to either the delivery tube ($0 \leqslant t < \tau$) or to the exhaust ($\tau \leqslant t < T$). Also, leakage of pheromone-laden air into the coflowing stream during the exhaust portion of the cycle should be prevented. Thus it was necessary to ensure that the flow past the pheromone dispenser and into the pulse generator experiences a constant pressure difference between the pulse generator and the pheromone dispenser chamber.

A schematic diagram of the internal geometry of the pulse generator is presented in Figure 1. The features of this design, and the developmental efforts that produced it, are detailed in the Appendix. The significant features of the device and its performance are presented below.

The geometric configuration of the internal passages were evolved using

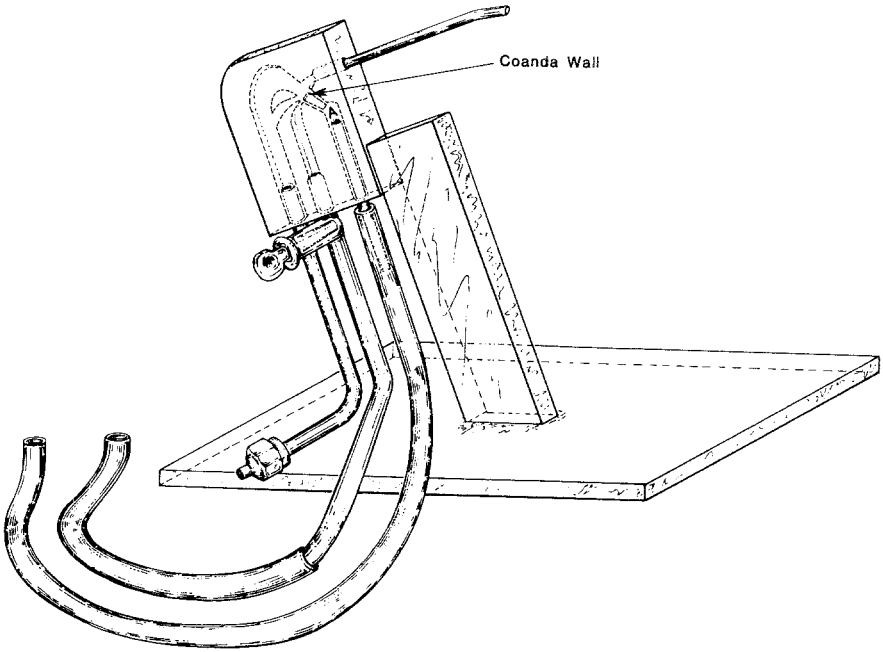

FIG. 1. Pulse generator.

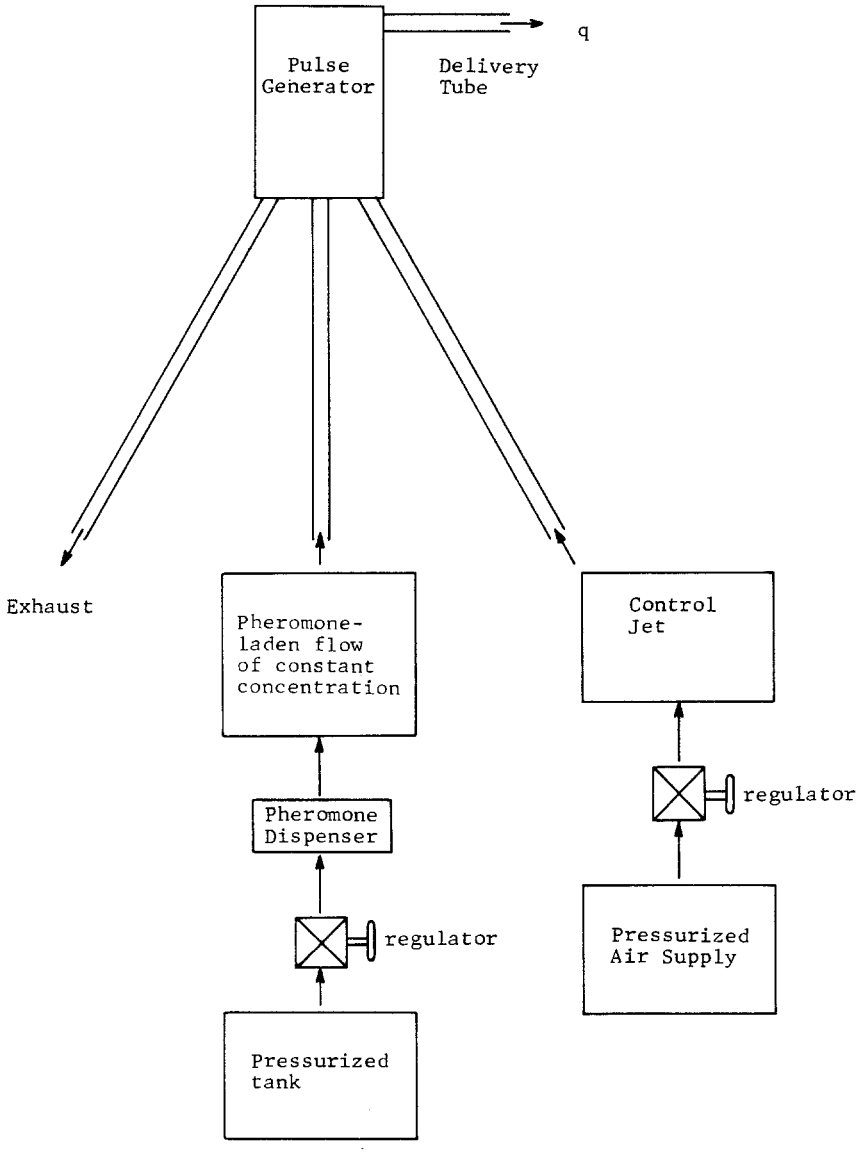

FIG. 2. Schematic of the pulse generator.

a 15 × scale model. The performance of a smaller scale prototype device was also evaluated; for example, it was confirmed visually that none of the fluid in the source flow was emitted from the delivery tube during the exhaust portion of the cycle. These observations also revealed that a delay time was an intrinsic feature of the pulse generator. Specifically, if the source portion of the cycle is initiated at t_c, the pheromone-laden air enters the coflowing stream

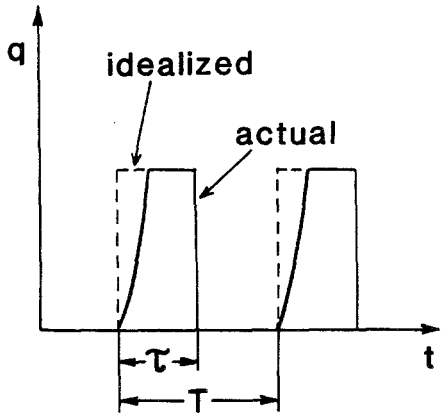

FIG. 3. Flow characteristics of the pulse generator, where q = flow velocity and t = time. τ is the duration of the pheromone-on cycle and T is the duration of a complete on–off cycle.

at $t_c + 0.3$ sec (Figure 3). The test system that was used to arrive at this result is shown in Figure 4. The attainment of the maximum flow was identified by the magnitude of the stagnation pressure in the jet from the delivery tube.

The pulse generator was placed 10 cm from the upwind end of a 2.8 × 0.8 high × 1.5 m wide clear acrylic plastic (Plexiglas) wind tunnel (Cardé and Hagaman, 1979), and it was angled 40° above the floor of the wind tunnel so that turbulence generated by the pulse apparatus would not affect the continuous pheromone stream or the pulses. The valves, regulators, etc. controlling the pulse generator were outside the wind tunnel and connected to the pulsator by glass or Tygon plastic tubes. The wind tunnel was housed in an environmental chamber maintained at 20° and 45–55% relative humidity. The tunnel airflow was 42 m/min and pheromone-laden air was exhausted at the downwind end of the wind tunnel.

Moths. The moths shipped as pupae by the USDA Gypsy Moth Methods Development Laboratory, Otis, Massachusetts. The pupae and adults were

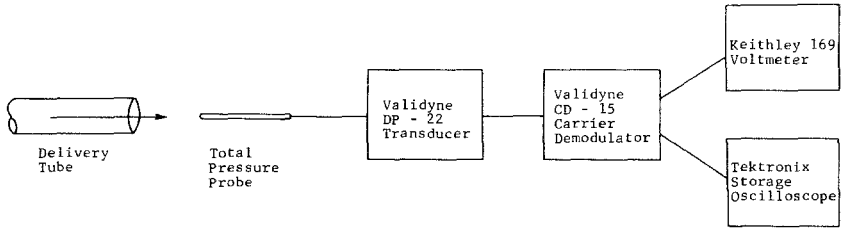

FIG. 4. A schematic of the test system for measurement of the flow of pheromone-laden air from the delivery tube.

held in screen emergence cages (30 × 30 × 36 cm) within an environmental chamber at 24° C and 50–60% relative humidity on a 16L:8D photoperiod. All moths used for experiments were 42–54 hr old and tested between the 6th and 10th hours of photophase.

Pheromone. The synthetic (+)-disparlure used was 97% pure with <0.5% *trans* isomer as determined by GLC analysis on a 50 m × 0.5 mm Carbowax 20 M capillary column at 180° (Cardé and Hagaman, 1979). Pheromone sources were dispensed in 2 μl of hexane on 1 × 0.3-cm filter paper triangles supported on 3-cm-long wire. Prepared sources were aged in an exhaust hood at 30 m/min airspeed for 2 hr. The sources were prepared in a separate room, sealed in glass tubes, and held until use in an exhausted vent in the bioassay room; all were used within 2 hr after aging.

Ultrasound-Elicited Behavior. Ultrasound is known to elicit predator-avoiding behavior in the male gypsy moth. A moth wing fanning in response to a pheromone stimulus will often cease fanning when exposed to high frequency sound (Baker and Cardé, 1978). Consequently, the apparatus was tested for ultrasound using a Holgate ultrasonic detector. Both the solenoid valve and the air passing through the pulsator produced some ultrasound. To eliminate the possibility of sound affecting the behavioral response, both tympanic membranes of the moths were ruptured according to the technique described by Baker and Cardé (1978). An experiment comparing wing-fanning responses of moths with ruptured tympana to moths handled similarly but without ruptured membranes showed no differences between the two groups. The 30 2-day-old moths were tested in response to 100 ng disparlure employing procedures described by Cardé and Hagaman (1979). Moths with ruptured membranes had a mean wing-fanning latency of 0.18 sec (SD = 0.14) with a similar mean of 0.14 sec (SD = 0.05) for moths with intact membranes.

Testing Procedure. Moths with tympanic membranes punctured on day 1 were selected randomly from the holding cages, transferred into individual 30-ml clear plastic cups resting on 6-cm-diam. screen disks, and brought into the wind tunnel chamber for a 1–2 hr acclimatization period.

Shortly before (2–4 min) a block of trials was to begin, the disparlure source was placed in the pheromone-holding chamber with the filter paper triangle sitting directly in the path of the continuous airstream (Figure 1). All air velocities and pressures had been adjusted previously to the desired settings. At the start of each trail a moth was placed facing directly upwind on a 14-cm-high glass stand 1 m downwind of the pulsator. The cup was removed and the trial started. The moth was observed for 1 min prior to the release of a designated frequency of pheromone emission. Those moths which wing fanned during this time were not included in the data. After the background run (during which time the pheromone was shunted out the exhaust) the pheromone was presented either continuously or pulsed according to the frequency set, based on a randomly selected treatment. Latency of wing fan-

ning was recorded. If no response occurred within 3 min, the nonresponding moth was then exposed to a continuous flow of pheromone. Moths not responding to the continuous stream of pheromone were not included in the data summary. After a trial, while pheromone was shunted out through the exhaust, the moth was removed and the holding stand was replaced with an acetone-rinsed one. Six treatments were run in each block of trials. Between blocks, the pheromone stimulus was removed and the pulse generator rinsed with ethanol to ensure against accumulation of pheromone within the Plexiglas chamber. The six treatments tested were: (1) disparlure pulsed at a frequency of 0.5 sec on and 1 sec off for 180 sec; (2) disparlure pulsed at 2 sec on and 4 sec off for 180 sec; (3) disparlure pulsed at 5 sec on and 10 sec off for 180 sec; (4) disparlure for 5 sec on and off for 175 sec; (5) a continuous flow of disparlure for 180 sec; and (6) no disparlure for 180 sec.

Flight Track. The procedures for handling the moths and setting the pulse generator were identical to those used in the previous assay. After the moths initiated flight, their track was recorded on videotape (Cardé and Hagaman, 1979). The flight track was viewed at 1/60-sec intervals on a videomonitor, and the position and time at the end of each leg of the zigzag path (Marsh et al., 1978) were recorded. These coordinates were transferred to a computer program which determined the distance and rate of movement upwind, crosswind, and along the leg, the interleg angle (Figure 5).

Moths were exposed to pheromone from a 10-ng source either as a continuous stream followed by a pulsed regime of 1 sec on and 1 sec off or the

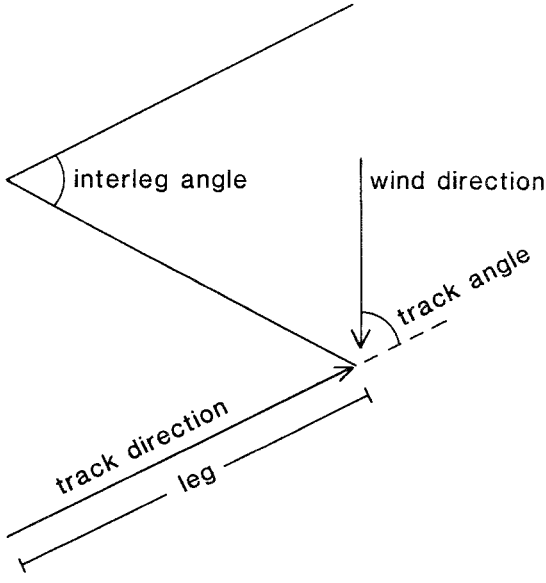

FIG. 5. Flight track of males viewed from above showing terminology used.

reverse of this sequence. Moths were allowed to fly upwind under one treatment until they had traversed the 2-m center section of the wind tunnel. The pheromone treatment then was switched, and the males were guided downwind by moving the striped floor pattern of the wind tunnel rapidly toward the downwind end of the tunnel (Cardé and Hagaman, 1979). Movement of the floor pattern was stopped when the moth reached the downwind end of the 2-m observation section, and the moth was allowed to proceed upwind again. Treatments were alternated with the same individual until the male no longer flew upwind to a given source.

RESULTS AND DISCUSSION

The mean wing-fanning latency of males exposed to a continuous stream of pheromone was 0.6 min, and 100% of the males responded (Figure 6). None

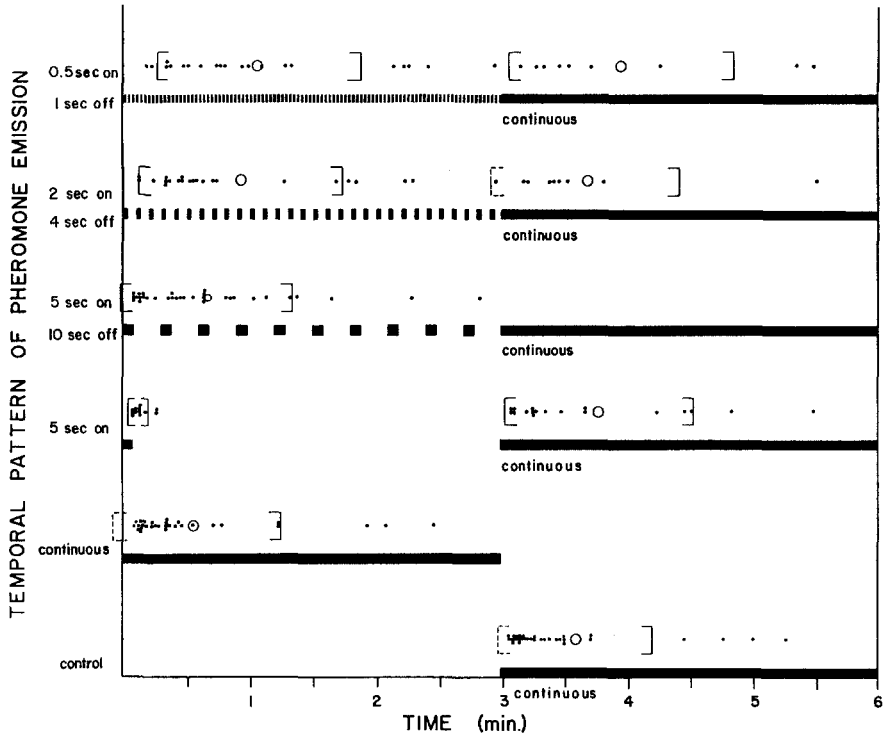

FIG. 6. Effect of pulsed and continuous streams of pheromone upon the latency male wing-fanning response to (+)-disparlure in the gypsy moth, *Lymantria dispar*. The dots represent the time at which wing fanning was initiated by individual; the open circles the mean time of wing-fanning initiation; and the brackets the standard deviations. The black bars are periods of pheromone-on.

of the control group of males responded during the 180 sec without pheromone. The three pulsed pheromone treatments produced a total of 1 min of pheromone exposure during 3 min (a one-third reduction in total pheromone delivered, relative to a continuous stream). The regime of 0.5 sec on and 1 sec off produced wing fanning within 3 min in 75% of the males; the mean latency was 1.2 min. Similarly, the 2-sec-on/4-sec-off and the 5-sec-on/10-sec-off patterns produced wing fanning in 75 and 100% of the males, respectively, with mean latencies of 0.8 and 0.7 min. A single 5-sec exposure followed by 175 sec without pheromone elicited wing fanning in 38% of the males with a mean latency of 0.2 min. The comparatively short reaction time for males in this treatment is related to the low proportion responding; a similar proportion of males responded rapidly during the first 15 sec following the first of sequential 5-sec pulses of pheromone in the previous treatment (Figure 6). The longer mean latency of the latter pulsed treatment is caused by the additional males responding during subsequent pulses.

Thus, our hypothesis that a pulsed pheromone message would be more "apparent" than a continuous stream of pheromone because of the added number of comparisons of signal to background is not supported in our studies of the threshold of the male gypsy moth with the pulsed regimes tested.

Flight tracks of males proceeding upwind along a pulsed train of pheromone and a continuous stream were similar in all respects: in their ground speeds along the plume axis and each leg of the zigzag, in the frequencies of turns, and in the interleg and track angles (Table 1). These data and the generally similar upwind flight behavior of male gypsy moths in discrete narrow plumes and wide homogeneous clouds (Cardé and Crankshaw, unpublished) strongly suggest that the zigzag flight path of the gypsy moth in the wind is largely a "preprogramed" reaction to the presence of pheromone, as recently suggested by Kennedy et al. (1980) for a tortricid moth. The form of the zigzag in the wind would thus appear to be set to a major extent by the average flux of pheromone impinging upon the male's antennae rather than by the spatial and temporal structure of the plume.

The principal questions to be considered in defining the potential importance of discontinuities in the pheromone plume are: (1) Does a pulsed train of pheromone exist either at a fixed sampling point downwind (corresponding to a quiescent male) or along the centerline of a plume (roughly corresponding to the position of male flying upwind along the plume)? (2) Is a perceiving organism in a pulsed plume capable of distinguishing its temporal pattern (a function of limitations of the sensory input or possibly its processing)? (3) If the foregoing requirements are satisfied, is this information used to discern the species identity of the emitter, to define its proximity, or to aid in the location process? These questions will be considered first as they apply to airborne communication in the wind.

The dynamics of pheromone dispersal in nature has received scant experimental study. The Sutton equation has been used widely (Wright, 1958;

TABLE 1. FLIGHT CHARACTERISTICS OF MALE GYPSY MOTHS FLOWN IN CONTINUOUS FOLLOWED BY PULSED (1.0 SEC ON AND 1.0 SEC OFF) STREAMS OF PHEROMONE OR THE REVERSE OF THIS SEQUENCE[a]

Treatment	Legs/moth	Turns/sec	Distance (cm/leg)			Speed (cm/sec)			Track angle	Interleg angle
			x	*y*	*z*	*x*	*y*	*z*		
Continuous										
1st flight ($N = 8$)	41.0	0.5 ± 0.2	3.3 ± 1.4	6.7 ± 2.2	9.0 ± 2.3	12.1 ± 5.9	19.8 ± 9.9	25.0 ± 11.4	36.2 ± 7.6	107.9 ± 15.2
2nd & 3rd flight ($N = 5$)	23.2	0.5 ± 0.2	4.2 ± 1.4	7.4 ± 2.7	10.3 ± 2.6	13.9 ± 5.7	19.8 ± 7.6	26.7 ± 9.8	37.9 ± 6.7	105.0 ± 14.2
Pulsed										
1st flight ($N = 7$)	39.0	0.5 ± 0.1	3.1 ± 2.1	8.5 ± 4.9	10.9 ± 4.9	12.6 ± 5.0	23.0 ± 12.2	28.4 ± 12.6	32.6 ± 9.8	115.6 ± 19.1
2nd & 3rd flight ($N = 4$)	35.0	0.4 ± 0.2	3.0 ± 2.3	6.0 ± 1.3	7.8 ± 1.9	10.3 ± 3.1	18.1 ± 2.2	22.1 ± 2.6	31.5 ± 8.7	116.7 ± 16.6

[a]Each value is the mean ± SD for each individual's mean for all legs in a treatment. The following terminology is used: *x* is the distance traveled upwind, *y* is the distance crosswind, and *z* is the distance along the leg; the track angle is the angle between the leg and upwind; and the interleg angle is the angle between legs.

Bossert and Wilson, 1963) to depict how pheromone from a point source would disperse in the wind, but this model as well as the more recent Gaussian plume model predict downwind concentrations at a fixed sample point over a minimum sampling interval of several minutes. In field trials using the male gypsy moth as an indicator of the threshold, these equations did not offer an accurate prediction of concentration (Elkinton et al., 1984), because the behavioral response of the male occurs within a fraction of a minute over which time the concentration must be defined. Aylor et al. (1976) have emphasized that it is peak rather than average concentrations which are likely to determine the threshold of response, but their method of estimating peak vs. average concentrations for a continuously emitting source are relative to the plume centerline; the temporal pattern impinging on a fixed sample point as the plume meanders along the swirling eddies was not considered.

Murlis and Jones (1981) reported from their experimental analysis that ions emanating continuously from a point source in the field arrive at a fixed sampling point up to 15 m downwind as discrete "bursts," typically of 0.1 sec duration 0.5 sec apart. Murlis and Jones thus contend that an odor plume, even from a continuously emitting source, would arrive downstream not as a continuous stimulus but instead as bursts that vary in strength and temporal patterning. The fluctuations in the ion signal were attributed to the process of turbulent dispersion as the ions are transported by small-scale eddies. Although the evidence of Murlis and Jones suggests that a pheromone source constantly emitting into wind would arrive at a fixed sample point downwind as a discontinuous signal, the precise temporal pattern and intensity of the signal currently cannot be predicted.

Whether a responding organism could decipher these temporal patterns at the olfactory receptor level and whether these patterns influence behavior at a more central integrative level can only be inferred. Electroantennagrams (EAG) from the male gypsy moth to 1000-ng sources of synthetic pheromone showed complete decay of the signal within ca. 1 sec (unpublished observations), a time sufficient to allow perception of the signals used in our tests. Conner et al. (1980) demonstrated that gland pulses of ca. 1.5 sec duration in the arctiid moth *Utethesia ornatrix* could be detected by the male as monitored by EAG. The pattern of intermittent pheromone stimulation over ca. 0.1- to 0.5-sec intervals suggested by Murlis and Jones (1981) is somewhat more rapid than evaluated by EAG in these species.

As noted earlier, a gypsy moth flying upwind follows a zigzag path centered along the plume's centerline. Airspeed is not regulated directly; rather, apparent groundspeed is modulated by the velocity of the ground pattern as perceived by the eyes. (Marsh et al., 1978; Cardé and Hagaman, 1979). The form of the zigzag path (angle between legs, speed up the legs, etc.) is controlled largely by pheromone concentration (Cardé and Crankshaw, unpublished). Because males flying along the plume centerline can have wide lateral excursions that carry them well beyond the time averaged plume's

boundary over durations of up to 0.5 sec (Cardé and Hagaman, 1979; Cardé and Crankshaw, unpublished), the actual temporal pattern of pheromone impinging upon the male's antennae from a continuous flow of pheromone could be pulsed.

Our observations with the gypsy moth were designed to compare the effects of pulsed vs. continuous pheromone upon the initiation of wing fanning and upon sustained upwind flight. A remaining, unexplored role for pulsing is to signify the proximity of the pheromone source. The frequency of pulses (generated by turbulent dispersion) encountered by a flying moth could increase as the moth drew closer to the source (Wright, 1958). These cues could evoke specific behaviors such as landing.

A final consideration is the effect pulsing the pheromone gland has upon the rate of pheromone emission. Extrusion and retraction of the abdominal tip could spread the pheromone over considerably larger surface area than the gland surface alone, thereby elevating the emission rate, and increasing the communication distance. Possibly, pulsing could also elevate the emission rate by raising the temperature of the pheromone-producing gland or enhancing transport of the pheromone to the gland surface.

The biological significance of gland pulsing and discontinuous plumes generated by either pulsing or turbulent dispersion ought to be explored with other Lepidoptera, especially those such as the arctiids *Utethesia* and *Holomelina*, in which the calling individuals pulse their pheromone gland rhythmically.

APPENDIX: DEVELOPMENT AND DESIGN OF PULSE GENERATOR

Figure 1 presents a scaled, planview, drawing of the pulse generator. The essential operating requirements were to provide a variable duration: $1.5 \leqslant T \leqslant 180$ sec (note that $\tau \leqslant T$).

The following characteristics define the operating principles for the pulse generator:

1. The source flow was directed to the delivery tube by the curved wall. This flow pattern was stable since the outer presence is greater than that at the surface. This phenomenon is the Coanda effect; it represents a specific aspect of the Euler "n" equation: $\partial p/\partial n = \rho V^2 R$ where n is the outward drawn normal for the streamlines with radius or curvature (R), and p = pressure, V = velocity and ρ = density [see Potter and Foss (1975), p 357].

2. The "exhaust portion" of the cycle was initiated when the plenum at A in Figure I was pressurized (with high-pressure air) and the source flow was directed toward the receiver.

3. Because the source flow was controlled by the pressure difference between the pheromone dispenser and the ambient pressure within the pulse generator, and because the latter pressure was maintained at a value estab-

lished by the exhaust fan, the source flow was not affected by this switching process.

4. The control jet (Figure 2) induced a reverse flow in the delivery tube. This ensured that pheromone-laden air did not enter the streaming flow during the off phase.

The final configuration of the pulse generator was defined using a 15× scale model.This large model was used to examine the influence of alternative geometric configurations, and its use was an essential part of the development program. However, it was not possible to execute the model study at the Reynolds number of the prototype because the velocity in the model would have been too small. Since the separation of the source flow from the Coanda wall was recognized to be potentially Reynolds number dependent, it was necessary to make direct observations using a prototype. These studies were executed in the 0.5 × 1.2 × 7.3-m wind tunnel facility designed and operated by Prof. R.E. Falco (Falco, 1980). The prototype was placed in a 1 m/sec air flow and an oil fog was used to mark the source flow. A vertical light sheet, formed by placing a cylindrical lens in an argon laser light beam, was used to illuminate the source flow from the delivery tube. This confirmed that the pulse generator provided a sharp cut-off of the source flow and that there was no leakage from the source flow during the exhaust portion of the cycle.

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FIELD EVALUATION OF CHEMICAL ATTRACTANTS AGAINST THE FLY *Fannia femoralis* (DIPTERA: MUSCIDAE)

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Abstract—Four chemical compounds previously shown to be attractants to *Hippelates* eye gnats and houseflies were evaluated against *Fannia femoralis* (Stein). Two of the compounds, trimethylamine hydrochloride and *n*-butyric acid, were determined to be the principal attractants against this fly. A composition containing trimethylamine hydrochloride (yielding trimethylamine in presence of water), *n*-butyric acid, and an anchovy meal inert carrier proved highly attractive. To evaluate the practical use of the synthetic fly attractant (SFA) formulation, a large-scale trial over an entire poultry ranch was implemented using SFA formulation and a sugar toxicant (as a killing agent) in specially designed traps. Within 2 weeks after initiation of SFA–toxicant treatments, significant reduction in *F. femoralis* population from the pretreatment level occurred. This high level of reduction prevailed during the remainder of the treatment period.

Key Words—*Fannia femoralis*, Diptera, Muscidae, attractants, synthetic fly attractants, trimethylamine hydrochloride, *n*-butyric acid, fly control.

INTRODUCTION

Synanthropic flies, such as *Musca domestica* L., *Fannia canicularis* (L.), *Fannia femoralis* (Stein), and *Muscina stabulans* (Fallen) are often found in large numbers on many poultry ranches in southern California. Heavy breeding of these pestiferous flies in accumulated chicken manure under cage rows of laying hens can occur for 9–10 months of the year. When high adult populations of the flies are encountered, control measures, such as space or residual chemical sprays (Loomis and Deal, 1977) are generally employed. In

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some situations, use of sugar baits complement other control measures. Naturally produced lures have also been employed, as Rooney and McKeen (1974) have reported that aqueous solutions of molasses fermented with yeast were attractive to *F. canicularis* and other filth-breeding flies. Hwang et al. (1978), working with fermented molasses solutions, isolated and identified ethanol as the major attractive substance for a number of synanthropic flies. It was further shown that *F. canicularis* was highly attracted to ethanol, but *M. domestica* and *M. stabulans* manifested a lesser attraction.

In search of chemical attractants for *Hippelates* eye gnats, Hwang et al. (1975, 1976) studied an attractive fermented suspension of whole chicken egg from which they isolated and identified various compounds, and when combined in proper proportions, were highly attractive to the eye gnat *Hippelates collusor* (Townsend). Mulla et al. (1976) developed suitable attractive formulations from these compounds for eye gnats, which are highly pestiferous in some of the agricultural valleys of California. During the course of these studies, it was also noted that several other species of synanthropic flies were attracted. Subsequently, the positive response of *M. domestica* to formulations of these attractants was documented by Mulla et al. (1977).

In southern California, some poultry ranches experience heavy populations of *F. femoralis* from February to July. Since little information on attractants or baits for this species is available, the chemical attractants which induced positive responses in *H. collusor* (Mulla et al., 1976) and *M. domestica* (Mulla et al., 1977) were studied against field populations of *F. femoralis*. As a result of these studies, the authentic compounds needed for attracting this fly were identified and field-tested against heavy populations of this insect.

METHODS AND MATERIALS

All experiments were carried out on a commercial egg-producing poultry ranch in the San Gabriel Valley, Los Angeles County, California. During the period of testing (February to May), large numbers of *F. femoralis* adults were observed actively hovering around the ranch and neighboring establishments. The ranch, with a capacity of 160,500 laying hens, consisted of 13 houses of different sizes. Wire cages were arranged above ground in double back-to-back rows with each individual cage containing 2–3 birds. All houses had corrugated metal roofs and were open on the sides. This ranch is isolated from other poultry ranches in southern California by urbanization, the next closest ranch being more than 10 miles away. All adult flies present on the ranch were deemed to be breeding there, as no other major breeding sources were noted within miles.

Experiments were designed to assess the attractancy of various chemicals

against adult *F. femoralis* present in high numbers on the ranch. The composition of the synthetic fly attractant (SFA) tested contained the test chemicals in a proportion which had been found to attract other synanthropic flies (Mulla et al., 1976, 1977). A composition containing the attractant chemicals, trimethylamine hydrochloride (2.5%), indole (0.25%), ammonium sulfate (40%), and *n*-butyric acid (2.0%) were combined with a behaviorally inert anchovy meal carrier (55.25%). Trimethylamine hydrochloride and ammonium sulfate yield trimethylamine and ammonia in the presence of water. This composition was used as a standard and compared with other preparations. In some experiments the amount of the carrier as well as the chemical attractants were varied depending on the composition to be tested.

A number of methods were employed in evaluating the attractants. In the first series of experiments, plastic 3.8-liter jar traps were used (Hwang et al., 1978). Each jar was provided with two rows of six holes (3.5 cm diameter) which permitted fly entry and exit. A 120-ml cup (Sweetheart No. S-304) containing damp substrate (wet sand or chicken manure), essential for the elaboration of some of the attractants, was placed inside the jar. On the surface of the damp substrate was placed 5 g of an SFA formulation and 5 g of the toxicant, Improved Golden Malrin®, a sugar bait containing 1% methomyl and 0.025% muscalure. Improved Golden Malrin killed the flies quickly before they could exit the jars. When liquid attractants were tested, 25 ml was placed in the cups (without damp substrate), which were then covered with cheesecloth to exclude flies, and 5 g of the toxicant bait was sprinkled around the base of the cup at the bottom of the jar. The jars were set on the ground for 24 hr in the poultry ranch in matched pair sets, 1.2 m apart. One jar had the test composition while the other contained the standard formulation. After the exposure period, the flies were collected and the traps reset and reversed for another 24 hr to account for positional effects. In each test, four replicates were set, and the pairs had a distance of 2–3 m between any two sets. After exposure, the jars containing the dead flies were brought to the laboratory where the flies were counted and identified as to species.

In further testing, a faster method of evaluation was employed, using a CRC rotary olfactometer developed for *Hippelates* eye gnat attractant research (Mulla et al., 1960). The CRC olfactometer is a circular table (119 cm diam.) which revolves at 1/4 rpm to reduce positional effects. Pans 20 × 20 cm were used in place of the glass funnels normally used in tests against eye gnats. The pans were set at the outer edge of the table. Pans contained damp substrate (manure) covered by a damp paper towel, on top of which was placed a mixture of 5 g of solid attractant and 5 g of Improved Golden Malrin. When liquid attractant such as ethyl alcohol was tested, 25-ml samples were placed in the 120-ml cups and covered with cheesecloth. The cups were placed in the center of the pans over paper towels where 5 g of Improved Golden Malrin was sprinkled around the cups. The paper towels facilitated collection

and counting of dead flies. The pans were set in matched pairs with three sets randomly replicated on each table. The materials were exposed for 1–2 hr, allowing for a large number of flies (100–200) to be killed in at least one of the attractant formulations. Dead flies accumulated on the paper towels in each pan were brought into the laboratory for counting and identification.

Another modification of the CRC olfactometer was used in a third series of tests. In place of the 400-cm^2 pans, 240-ml squat cups (Dixie Cup No. 2168) were used. This modification allowed for an increase from three matched-pair pan sets (six pans) to 10 matched-pair cup sets (20 cups per table). Each cup contained 120 g sand dampened with 25 ml water, on top of which a mixture of 0.5 g of the attractant and/or 0.5 g Improved Golden Malrin was placed. The cups were placed at the edge of the revolving turntable in matched-pair replicated groups. Flies attracted to SFA were killed in the cups, and the cups containing dead flies were covered with lids and brought into laboratory for counting.

A fourth series of tests was conducted using a rotary rod olfactometer (Mulla et al., 1973). This modification of the olfactometer allowed for an increase to 40 Dixie No. 2168 cups. The same evaluation procedures as in the above CRC olfactometer cup test were followed. Here the separate chemicals constituting SFA were evaluated. The attractant chemicals (0.5 g) and 0.5 g Improved Golden Malrin were placed in each cup on top of damp sand.

After determination of the attractive chemicals, an experiment was conducted to demonstrate the efficacy of the SFA against *F. femoralis* by treating the entire ranch. Standard SFA formulation was used for the treatment because other muscoid flies besides *F. femoralis* were also present, and assessment of their response to the attractant would provide additional information. However, the results relating to these species are not presented, as the numbers captured were so low that definite conclusions could not be ascertained.

The experiment on the efficacy of SFA on the ranch was conducted by setting 29 bait traps. The traps were made by placing a screen hood or cover over a 20-liter plastic bucket, containing the attractant (Figure 1). Each bucket contained a red clay brick in water with 1 cm of the brick above the water. The SFA composition (5 g) was placed on top of the damp brick. The screen hoods were moistened with water by an atomizer and then sprinkled with Improved Golden Malrin which adhered to the screen. Flies attracted to SFA in the bucket would land on the screen hoods, feed on Improved Golden Malrin, and die. The traps were recharged with attractant weekly, and toxicant or water 1–3 times per week, as needed. The traps were located throughout the poultry ranch, 4–5 traps per acre. To determine efficacy of SFA for a long period, the test continued for 11 weeks. Pretreatment counts were taken during the first 3 weeks. The treatment was begun on the 4th week and continued for the remaining period.

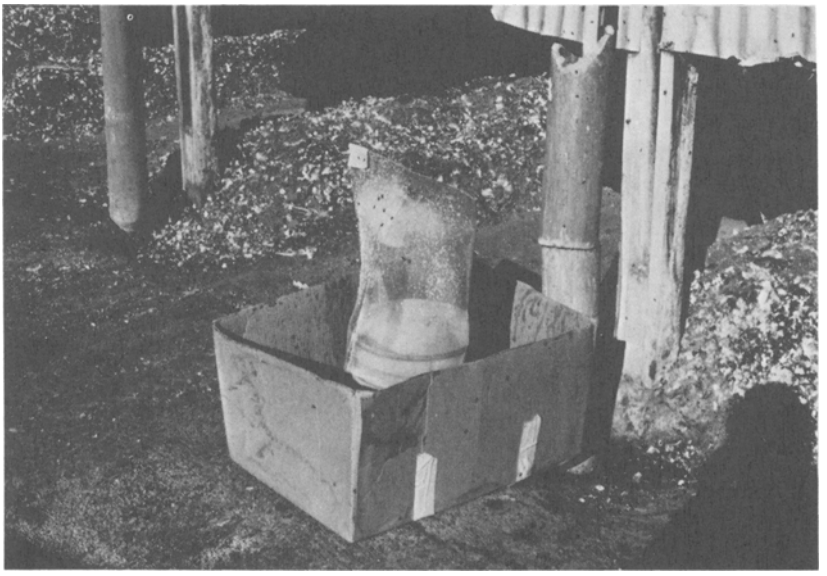

FIG. 1. Screen covered trap employed in ranch-wide control of *F. femoralis*.

The isolation of this ranch from other ranches prevented its comparison with any nearby control ranches. Effectiveness of treatment was determined by assessing population trends prior to and during the treatment period. Adults of *F. femoralis* were sampled with sticky fly tapes (Fly-Catcher, Aeroxon Products, Inc., New Rochelle, New York). Each week, 12 sticky fly tapes were set throughout the ranch for a 24 h period. *F. femoralis* on the tapes were counted under a dissecting stereomicroscope in the laboratory. The data are presented as average numbers of flies per tape per 24 h.

Statistical analyses were conducted on all data [transformed to log $(n + 1)$] to determine significant differences among means of treatments. Each test was subjected to either matched-pair t-test analysis (Schefler 1969) or analysis of variance followed by Duncan's multiple-range test, and significant differences among means of each individual test were determined at the 1, 5, or 10% probability levels (Little and Hills, 1962).

RESULTS AND DISCUSSION

In the first series of tests using jar traps, potency of attractants on sand and manure was studied. SFA on damp sand was compared with damp chicken manure which had been observed to have some degree of attractancy to pestiferous flies. SFA on damp sand was significantly more attractive (at

TABLE 1. EVALUATION OF SYNTHETIC FLY ATTRACTANT COMPOSITION AGAINST *Fannis femoralis*, USING MATCHED-PAIR TESTING METHOD ON A POULTRY RANCH[a]

Substrate	Attractant	Trap location	Mean No. flies/trap[b]	
			Male	Female
Test 1A				
Damp sand	Synthetic fly attractant	X	0	125***
Damp manure	None	Y	2	0
Test 1B				
Damp manure	None	X	7	14
Damp sand	Synthetic fly attractant	Y	6	198***
Test 2A				
Damp manure	Synthetic fly attractant	X	0	544***
Damp manure	None	Y	6**	6
Test 2B				
Damp manure	None	X	1	2
Damp manure	Synthetic fly attractant	Y	0	876***
Test 3A				
Damp sand	Synthetic fly attractant	X	0	367***
None	25% EtOH solution	Y	2	3
Test 3B				
None	25% EtOH solution	X	1	2
Damp sand	Synthetic fly attractant	Y	0	320***

[a]Using 3.8-liter plastic jar traps. Synthetic fly attractant contained: 2.5% trimethylamine hydrochloride, 0.25% indole, 40% ammonium sulfate, 2% *n*-butyric acid, and 55.25% anchovy meal. Each treatment was provided with the toxicant bait Improved Golden Malrin.
[b]Mean number based on four matched-pair sets. Significant differences between means of each pair in a column are determined: ** at 0.05 and *** at 0.01 probability level.

least 14×) to *F. femoralis* than damp manure alone (Table 1, test 1A and 1B) and attracted females, almost exclusively.

In the next test, the attractancy of SFA on damp manure was compared to the damp manure alone. Again, regardless of the position of traps, significantly more *F. femoralis* were attracted to SFA on manure than to manure alone (Table 1, test 2A and 2B). The ratio of flies in SFA–manure and manure-alone traps was >100:1, and all flies attracted to SFA–manure were females. Tests 1 and 2 thus indicate that spot treatments on damp manure with attractant–toxicant baits could possibly reduce numbers of *F. femoralis*.

In the third test of this series, the SFA on damp sand was compared with 25% ethyl alcohol, a good attractant for *F. canicularis* (Hwang et al., 1978). Significantly more *F. femoralis* were attracted to SFA on damp sand than to ethyl alcohol (Table 1, test 3A and 3B). All flies attracted were females. Results indicated ethyl alcohol to be a poor attractant for *F. femoralis*. Damp

sand alone provides no stimulus as an attractant to *F. femoralis* (personal observation).

Since ethyl alcohol proved to be unattractive in the above test, it was decided to test a combination of the SFA–manure against an ethyl alcohol–manure combination. Damp manure was placed in 400-cm^2 pans and covered with a paper towel. On the top of the towels were placed 120-ml cups, each containing 25 ml of ethyl alcohol. The pans were arranged on the CRC olfactometer. This test clearly established that SFA–manure combination was significantly more attractive (attracting 3135 flies) than the ethyl alcohol–manure combination (attracting 1469 flies). About 99% of the flies attracted were females. This test showed that the combination of manure–ethyl alcohol may have some enhanced attractancy, as neither the manure nor ethyl alcohol alone shown any high degree of attractancy to *F. femoralis* in the jar trap tests (see Table 1). When both *Fannia canicularis* and *F. femoralis* are present, a combination of ethyl alcohol and SFA could be used to lure both species to traps, as ethyl alcohol is an attractant for the former species (Hwang et al., 1978) and not the latter species.

It should be pointed out that in all tests conducted, Improved Golden Malrin was used for toxicant effects. One of the components of Improved Golden Malrin is muscalure, a house fly attractant which elicits contact response (Carlson et al., 1971). We decided to test the role of this attractant against *F. femoralis*. Tests 2A and 2B of Table 1 indicate that Improved Golden Malrin on damp manure had little or no attractancy to *F. femoralis*. In another test, when SFA with Improved Golden Malrin and Improved Golden Malrin alone were compared in squat cups, significantly more *F. femoralis* (282) were attracted to SFA cups than to cups with Improved Golden Malrin alone (44 flies). These tests indicated that Improved Golden Malrin has a much lower attractancy than SFA to *F. femoralis*.

After establishing attractancy of SFA to *F. femoralis*, the attractancy of each constituent or combination of constituents was investigated and compared to the entire SFA mixture. In test A (Table 2), attractancy of individual components as well as the anchovy meal carrier was evaluated. Anchovy meal showed a low level of attractancy which was increased significantly by the addition of either trimethylamine hyrochloride (2.5%) or *n*-butyric acid (2%). On the other hand, the attractancy was not significantly increased by incorporating indole (0.25%) or ammonium sulfate (40%) into the anchovy meal. The attractancy of each individual component (with anchovy meal) was significantly lower than that of SFA.

The next test (Table 2, test B) in this series involved addition of two ingredients to the carrier. Combinations of TMA·HCl–indole, TMA·HCl–ammonium sulfate, or indole–*n*-butyric acid as pairs yielded similar levels of attractancy, which in each case was significantly lower than that of the complete mixture (SFA). Indole–ammonium sulfate or ammonium sulfate–*n*-

TABLE 2. EVALUATION OF VARIOUS COMPONENTS OF SYNTHETIC FLY ATTRACTANT AGAINST *F. femoralis*, ON A POULTRY RANCH[a]

Test	Composition by constituents (%)					Mean No. flies/cup[b]
	Anchovy meal	TMA·HCl	Indole	Ammonium sulfate	*n*-Butyric acid	
A	100					22 c
	97.5	2.5				70 b
	99.75		0.25			45 bc
	60			40		22 c
	98				2	69 b
	55.25	2.5	0.25	40	2	204 a
B	97.25	2.5	0.25			101 b
	57.5	2.5		40		113 b
	95.5	2.5			2	167 a
	59.75		0.25	40		53 c
	97.75		0.25		2	121 b
	58.0			40	2	70 c
	55.25	2.5	0.25	40	2	215 a
C	57.25	2.5	0.25	40		48 b
	95.25	2.5	0.25		2	94 a
	55.5	2.5		40	2	85 a
	57.75		0.25	40	2	51 b
	55.25	2.5	0.25	40	2	83 a

[a]Using rotary rod olfactometer and open cup method of evaluation.
[b]Mean based upon 5 replicates for each test. Each test analyzed independently of each other. Means followed by same letters are not significantly different from each other at 0.05 level.

butyric acid showed lowest level of attractancy. The combination of trimethylamine hydrochloride–*n*-butyric acid showed the highest attractancy of all pairs of compounds tested and was not significantly different from SFA.

In the third test (Table 2, test C) of this series, compositions lacking only one of the components (combinations of three) were evaluated and compared with SFA. Deletion of *n*-butyric acid from SFA resulted in significant reduction of attractancy. Likewise, deletion of trimethylamine hydrochloride from SFA resulted in significant reduction of attractancy. From these studies, it is evident that trimethylamine hydrochloride and *n*-butyric acid are the main attractants in SFA for *F. femoralis*.

As trimethylamine hydrochloride and *n*-butyric acid were proven to be the principal attractants for *F. femoralis* in SFA, varying concentrations of each of these components were tested in the whole composition. The concentration of trimethylamine hydrochloride was varied from 0 to 5%. No significant difference among the 1–5% concentrations was noted (Table 3, test

TABLE 3. EFFECT OF VARIOUS CONCENTRATIONS OF TRIMETHYLAMINE HYDROCHLORIDE AND *n*-BUTYRIC ACID IN SFA FORMULATION IN LURING *F. femoralis*[a]

Component	Concentration in formulation (%)	Mean No. flies/cup[b]
Test A		
Trimethylamine hydrochloride[c]	0.0	24 b
	1.0	61 a
	2.0	77 a
	2.5	81 a
	5.0	88 a
Anchovy meal carrier	100	4 c
Improved Golden Malrin	0.5	2 c
Test B		
n-Butyric acid[d]	0.0	13 b
	0.25	55 a
	0.5	60 a
	1.0	67 a
	2.0	46 a
Anchovy meal carrier	100	5 c
Improved Golden Malrin	0.5	3 c

[a]Tested using rotary rod olfactometer with cups containing damp sand as substrate.
[b]Mean based upon 5 replicates. Tests analyzed independently of each other. Means followed by same letter in each test not significantly different from each other at 0.05 level.
[c]Composition also contained 0.25% indole, 40% ammonium sulfate, 2% *n*-butyric acid, and 54.25–59.25% anchovy meal.
[d]Composition also contained 0.25% indole, 40% ammonium sulfate, 2.5% trimethylamine hydrochloride, and 55.25–57.25% anchovy meal.

A). Anchovy meal and Improved Golden Malrin by themselves (as indicated in earlier tests) were unattractive. The composition lacking trimethylamine hydrochloride attracted significantly lower numbers of flies than any of the other compositions containing this compound.

Various concentrations of *n*-butyric acid from 0.25 to 2.0% in the SFA compositions lured variable but insignificantly different, numbers of flies (Table 3, test B). Anchovy meal and Improved Golden Malrin were again not attractive to the flies. The composition lacking *n*-butyric acid attracted significantly lower numbers of flies as compared to compositions with this acid. Attractant compositions for *F. femoralis* can be prepared containing 1–5% trimethylamine hydrochloride and 0.25–2.0% *n*-butyric acid, without increasing or decreasing initial attractancy. No studies were conducted on longevity of action with the various concentrations examined. As *n*-butyric acid has an unpleasant odor, its inclusion in attractant formulations could be at the lower concentrations when used in areas close to human habitation.

After establishing attractancy of SFA to *F. femoralis*, a large-scale trial

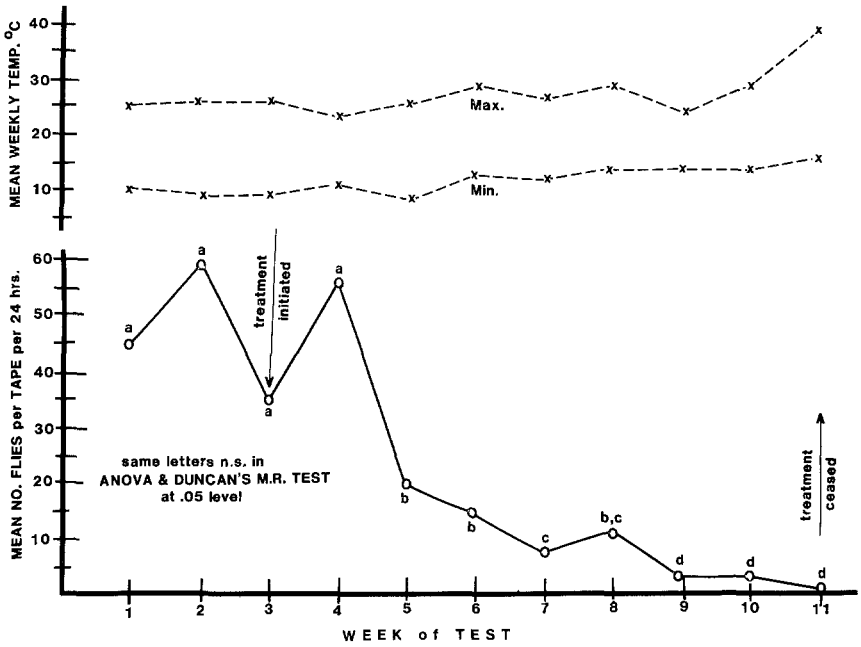

FIG. 2. Population trends of *F. femoralis* as measured by sticky tapes prior to and during treatment with synthetic fly attractant-toxicant bait.

was then initiated. For 3 weeks prior to initiation of the area-wide control test, a high number of *F. femoralis* were captured on sticky tapes (Figure 2). Although the actual numbers fluctuated the first 3 weeks, no significant differences were indicated by statistical comparisons. One week after initiation of treatment (week 4 of test), the fly population activity was still relatively high and was not statistically different from the three pretreatment counting dates. Due to the high density of flies and emergence of new adults, it was not expected that the treatment would provide immediate control of flies.

However, within two weeks after initiation of the treatment (week 5 of test), and through the next 6 weeks of this study, significantly fewer *F. femoralis* were collected. There was a large drop in numbers at week 5 of the test and a weekly decline thereafter. The isolated location of the ranch probably precluded incursion of flies from outside, and thus the locally produced *F. femoralis* population declined rapidly. Table 1 indicates that SFA exclusively attracted females. As females were killed, their ability to repopulate the ranch ceased as evidenced by the results obtained in the latter part of the study.

A glance at the air temperatures indicates little or no rise in maximums during the day and little or no drop in minimum temperatures at night. Slight

increases in the daytime temperatures occured during the last two weeks of the test. The 11th and final week of the test had a marked increase in daytime temperature. The sharp decline in populations soon after initiation of treatment could not be attributed to warming of the weather, as this did not occur during the test. *F. femoralis* has been observed to thrive under the range of temperatures noted here. Optimum mean developmental temperatures for this species are 21–27° C, while 32° C and above or 10° C and below induce heavy mortality in these flies (Deal, 1967). No such high or low temperatures were experienced during this test.

From results obtained, it appears that stations baited with synthetic fly attractant had a marked and significant role in reducing adult *F. femoralis* populations on this poultry ranch. The use of these specific attractants in combination with toxicants provides a safe and practical measure for the control of *F. femoralis* on poultry ranches. An important advantage of this method would be little or no risk to beneficial fauna in poultry manure. This type of attractant–toxicant combination could be employed in traps (as in this test) or applied as spot treatments on damp manure and/or damp ground, which are always present on poultry ranches yielding high fly densities.

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ZINC-MEDIATED HATCHING OF EGGS OF SOYBEAN CYST NEMATODE, *Heterodera glycines*

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Abstract—Egg hatching of the soybean cyst nematode, *Heterodera glycines*, was not affected by millimolar concentrations of calcium sulfate or calcium chloride. However, zinc chloride and zinc sulfate caused strong and moderate increases in hatching, respectively. The inhibitors of calcium transport, ruthenium red and lanthanum chloride, and calcium ionophore A23187 had no effect on hatching in the presence or absence of 3 mM zinc chloride. Selected chelators decreased the zinc-induced hatching of *H. glycines* eggs. Eggs exhibited a formation constant with zinc between 5.5 and 11.2. The addition of zinc chloride after chelation with EDTA and rinsing caused expected hatching rates. Concentrations of calcium chloride, manganese chloride, and magnesium chloride had no effect on hatching of eggs in zinc chloride, but reduced hatching at higher concentrations, possibly by osmotic influences. Hatching of eggs was increased as the time of exposure to zinc chloride was increased and was maximal at 28° C and a pH of 5.3. Picrolonic acid, a known hatching stimulant, increased *H. glycines* hatching, while sodium metavanadate had no effect. Analysis of seasonal hatching during 1981–1982 in untreated control eggs indicated that hatching was most pronounced in May.

Key Words—Nematode egg hatching, soybean cyst nematode, *Heterodera glycines*, nematode, hatching stimuli, zinc chloride, zinc sulfate.

INTRODUCTION

Hatching of cyst nematode eggs is incompletely understood. Calcium-mediated changes in egg permeability are implicated in hatching of *Globodera*

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rostochiensis. Calcium may bind to the eggshell and cause a synergistic effect with the hatching factor from the roots of host plants (Atkinson and Ballantyne, 1979). However, other evidence contradicts the role of calcium. Clarke and Hennessy (1981) reported that free calcium was not essential for hatching of nematode eggs. A calcium-binding site has been found on the eggshell of *G. rostochiensis* (Taylor and Atkinson, 1980). Our knowledge of the hatching process in cyst nematodes has been reviewed by Perry and Clarke (1981).

In contrast, calcium salts have little or no effect on hatching of eggs of *H. glycines* (Lehman et al., 1971; Clarke and Shepherd, 1966; Tefft et al., 1982), although zinc salts are an effective stimulant for hatching at concentrations that approximate soil levels (Krauskopf, 1972). Zinc salts were reported as a stimulant of egg hatching in the soybean cyst nematode, *Heterodera glycines,* although other ions were less effective (Clarke and Shepherd, 1966). Generalized activity was found also in other species of nematodes. Additionally, hatching of eggs and exsheathment of larvae of the animal parasite *Haemonchus contortus* were caused partially by the metalloenzyme, leucine aminopeptidase, which utilizes zinc as a cofactor (Rogers and Brooks, 1976, 1977). Okada (1971a,b; 1972a,b) investigated the role of plant and cyst components on hatching of *H. glycines.*

Since extensive knowledge of hatching of cyst nematode eggs is limited mostly to those species that are probably calcium-mediated, study of *H. glycines,* which hatches independently of calcium, seems warranted. Additionally, an understanding of the regulation of egg hatching in *H. glycines* may allow prediction of economic thresholds of host damage based on environmental data or synchronization of hatching as a method of agricultural control.

METHODS AND MATERIALS

Heterodera glycines was maintained in greenhouse-reared soybean plants as previously reported (Rende et al., 1982). Groups of 40 mature cysts were collected and manually opened. After straining through a 325-mesh screen to remove cyst fragments, eggs were washed three times in 20–30 ml of reagent-grade water (18 mΩ). Reagent-grade water was used throughout this study.

After recovery of eggs on a 0.45-μm filter, various test compounds were added to the egg suspension with dilution to the desired concentration. Then, 200 μl aliquots were placed in the wells of microtiter plates. At least 12 wells were used for any treatment with 10–20 eggs per well, and several concentrations were used for each treatment.

Eggs and larvae were counted five times during a two-week period to

determine the hatch rate in the treatments versus reagent-grade water or 3 mM zinc chloride as controls. The hatch rate was expressed as percent change in the number of treated larvae divided by percent change in the number of control larvae. Thus, a hatch rate of one signifies no difference between treatment and control. Comparisons were made based on the 14-day count of hatching. Some experiments did not allow the calculation of results versus an internal control of zinc or water. Accordingly, the percent change in the number of hatched larvae was determined in these trials. Studies were conducted at 25° C unless otherwise indicated.

Initially, the absence of any effect of calcium (Tefft et al., 1982) was reexamined with present procedures. Thus, eggs were incubated in calcium chloride (0–10 mM) and calcium sulfate (0–13.5 mM) to determine any change in hatch rate v. the water control. Likewise, the stimulatory role of zinc was determined by exposure of eggs to zinc sulfate (0–200 mM) and zinc chloride (0–7 mM). Zinc chloride (3 mM) was used as a hatching stimulus for subsequent study, based on these results.

Additional studies examined the possible interaction of zinc and calcium on hatching of *H. glycines*. Eggs were incubated in ruthenium red (0–100 μM) and lathanum chloride (0–300 μM) to inhibit calcium uptake with and without 3 mM zinc chloride. Eggs were maintained also in the calcium ionophore A23187 (U.S. Biochemical Corp.) and 6 mM calcium chloride to determine any effect of calcium transport on the hatch rate. Ionophore was prepared as a saturated solution in 1% DMSO for testing over a 0–40% final concentration.

Several chelators of zinc and other ions were tested also for influences on zinc-mediated hatching. The chelators, ethylene diamine tetraacetic acid (EDTA), diamino cyclohexane tetraacetic acid (CDTA), hydroxyethylene diamine triacetic acid (HEDTA), nitrilotriacetic acid (NADT), and sodium citrate, were tested over a 0.5–8 mM range to determine the hatch rate of *H. glycines* eggs in 3 mM zinc chloride during the two-week period of incubation. The pH was maintained beween 5 and 6 in these studies, based on the affinity of the chelator. In another experiment, eggs were exposed to 10 mM EDTA for one or seven days. After rinsing with water, eggs were held in 3 mM zinc chloride with and without calcium chloride (0–80 mM) to observe subsequent hatching.

Ionic competition was studied also. Eggs were held in 3 mM zinc chloride (0–100 mM) with calcium chloride (0–100 mM), magnesium chloride (0–100 mM), and manganese chloride (0–100 mM). Hatching in these mixed solutions of divalent ions was compared to the 3 mM zinc-only control. Additional experiments were performed on zinc-induced hatching of *H. glycines* eggs. Eggs were exposed to 3 mM zinc chloride for 0–96 hr. After rinsing, eggs were held in water to determine hatching v. nonexposed eggs in

reagent-grade water. Temperature influences on zinc-hatching were studied by exposure of eggs to 3 mM zinc chloride at 5, 10, 16, 25, 28 and 37° C for two weeks. Effects of pH were studied by determination of percent hatching of larvae in 3 mM zinc chloride from pH 3.6 to 7.6.

The effects of other reported hatching agents were tested also. Thus, eggs were incubated in picrolonic acid (0–0.9 mM) and sodium metavanadate (0–18 mM) to determine the hatch rate. Additionally, seasonal hatching of untreated *H. glycines* eggs was tabulated from egg counts that were taken on day zero as water controls during the past year of study.

Data were evaluated by linear regression and the 0.05 probability level was considered significant. Regression coefficients have been used to provide estimates of hatching after two weeks for comparison of various treatments.

RESULTS

Increased levels of zinc chloride significantly increased the hatch of *H. glycines* eggs ($r = 0.89$) (Figure 1). The hatch rate was elevated 12-fold as the concentration of zinc chloride was raised from zero to 1.77 mM. Further

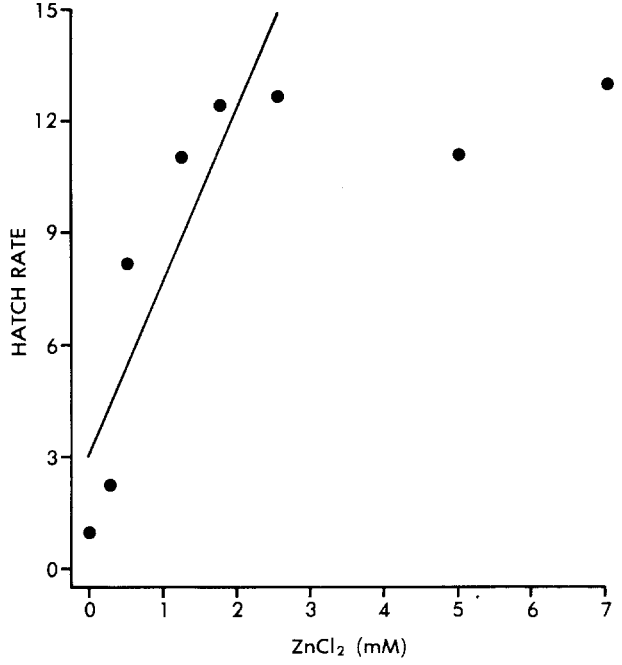

FIG. 1. Hatch rate of *H. glycines* eggs after two weeks in the indicated concentrations of zinc chloride (mean = 7.89 ± 1.90, $r = 0.89$).

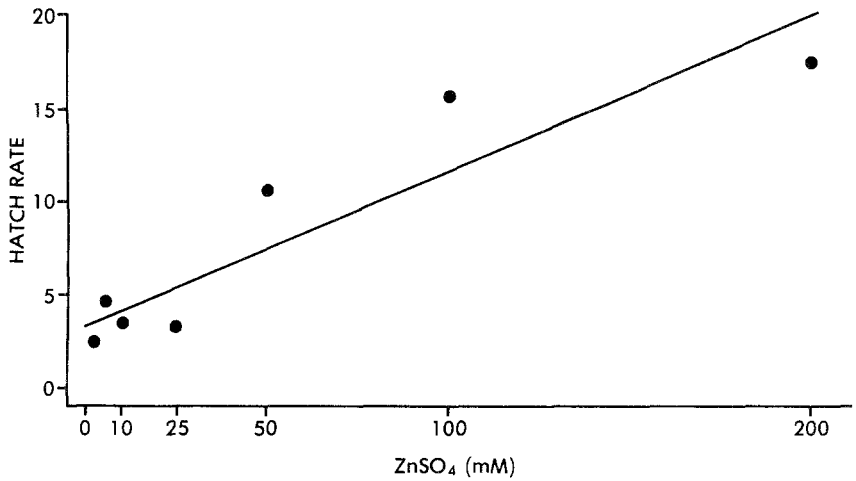

FIG. 2. Hatch rate of *H. glycines* eggs after two weeks in the indicated concentrations of zinc sulfate (mean = 7.34 ± 2.27, $r = 0.92$).

increases to 7 mM caused no additional hatching. Zinc sulfate also elevated the hatch rate significantly ($R = 0.92$) (Figure 2). However, the activity of zinc sulfate was less than that of zinc chloride at comparable concentrations, according to regression analysis. No osmotic effect was evident even with 200 mM concentrations.

In contrast, incubation of *H. glycines* eggs in calcium sulfate and calcium chloride showed no alteration of hatching vs. the water control. The means of the hatch rates were 0.84 (MSE = 0.15) and 0.37 (MSE = 0.23) for calcium sulfate and calcium chloride, respectively. The coefficient of correlation for both substances was negative, but insignificant. Thus, these calcium salts have no apparent influence on hatching at the tested range.

The effects of the calcium-transport inhibitors, ruthenium red and lathanum chloride, were tested also with and without the addition of 3 mM zinc chloride to the eggs. Incubation in only ruthenium red gave a mean hatch rate of 1.12 (MSE = 0.17) which was not different from the water control. Addition of zinc chloride to this inhibitor yielded a mean hatch rate of 0.98 (MSE = 0.15) that was also similar to the control. Variation between the controls was insignificant. Exposure of *H. glycines* eggs to the inhibitor lathanum chloride with or without zinc chloride gave mean hatch rates of 1.88 (MSE = 0.37) and 1.48 (MSE = 0.25), respectively. No dosage dependency was evident for either inhibitor of calcium transport. Thus, inhibition of calcium transport has little effect on egg hatching or hatching that is induced by zinc chloride.

In contrast, treatment of eggs with various chelators of zinc reduced or

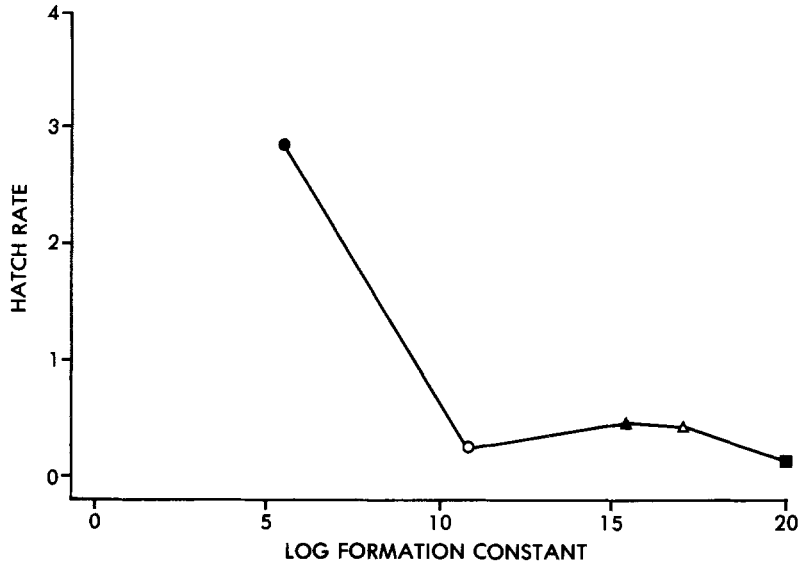

FIG. 3. Hatch rate of *H. glycines* eggs after two weeks in 3 mM zinc chloride and various chelators ● = sodium citrate, ○ = NADT, ▲ = HEDTA, △ = EDTA, ■ = CDTA) with the indicated formation constants for zinc, as prediceted by regression analysis.

eliminated the hatching of *H. glycines* when compared to the water controls (Figure 3). Hatch rates of eggs were decreased below the control by chelators with formation constants for zinc that were over 5.5. Since zinc-stimulated hatching occurred in sodium citrate, the formation constant of zinc with eggs is greater than that of the chelator (5.5). However, reduced hatching in NADT indicated that the formation constant of this chelator (11.2) for zinc is greater than that of the eggs. Thus, the formation constant of eggs of *H. glycines* for zinc is between 5.5 and 11.2.

Eggs were chelated also for one or seven days with 10 mM EDTA with the subsequent addition of zinc chloride alone or with various concentrations of calcium chloride. After removal of the chelator by rinsing, the addition of 3 mM zinc chloride caused hatching that was identical to the untreated zinc control. Increased levels of calcium chloride did not effect the hatch rate. Similar results were observed after one or seven days of chelation. The activity of the chelators is apparently related to the binding of zinc rather than calcium. Thus, calcium is not required for egg hatching of *H. glycines.*

In the above experiments, pH was adjusted between 5 and 6 with sodium hydroxide and hydrochloric acid for solubility of the various chelators. Exposure of eggs to comparable concentrations of sodium chloride as a control caused no alteration in hatching.

Competition between 3 mM zinc chloride and other ions was studied also. The addition of calcium chloride up to 100 mM gave a mean hatch rate of 1.03 (MSE = 0.22, $r = -0.25$). However, little hatching was seen over 80 mM, which suggests an osmotic influence. No significant change in hatching occurred after the addition of magnesium chloride or manganese chloride ($r = -0.72, -0.62$, respectively) to the solution of 3 mM zinc chloride. The mean hatch rates in the chlorides of magnesium and manganese were 1.1 (MSE = 0.17) and 0.75 (MSE = 0.21), respectively. However, the hatch rate was reduced over 40–50 mM concentrations, which again suggests an osmotic effect.

The hatch rate of *H. glycines* eggs increased as the time of exposure to 3 mM zince chloride was raised (Figure 4). Little difference was seen after 30 min of exposure, but a 2-hr exposure caused a 95% increase in hatching. Further increases in exposure time resulted in even greater hatching. Thus, the induction of egg hatching by zinc chloride (3 mM) occurred within 0.5–2 hr.

Zinc-mediated hatching was more pronounced at 28° C than higher or lower temperatures (Figure 5). Insignificant changes in larval numbers were seen at 10° C while hatching increased over 16° C. Reduced hatching was

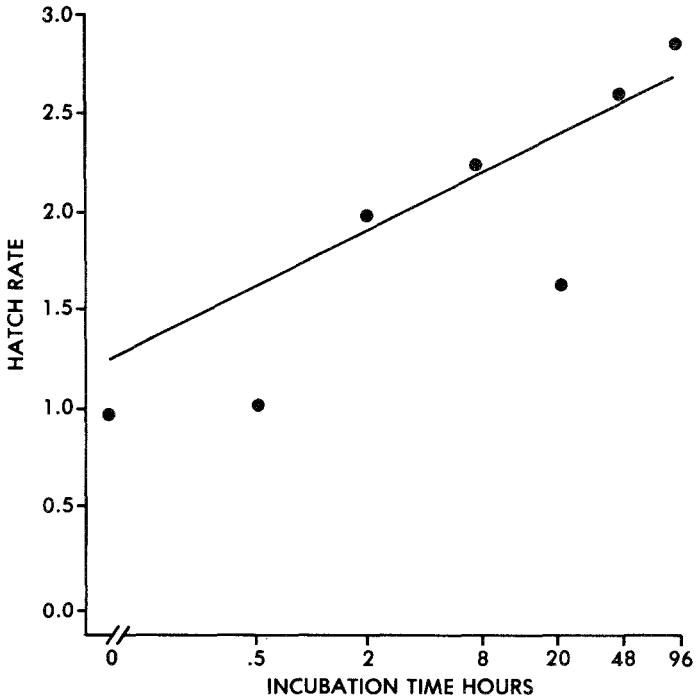

FIG. 4. Hatch rate of *H. glycines* eggs in water after the indicated timed exposures to 3 mM zinc chloride ($r = 0.83$, MSE = 0.26).

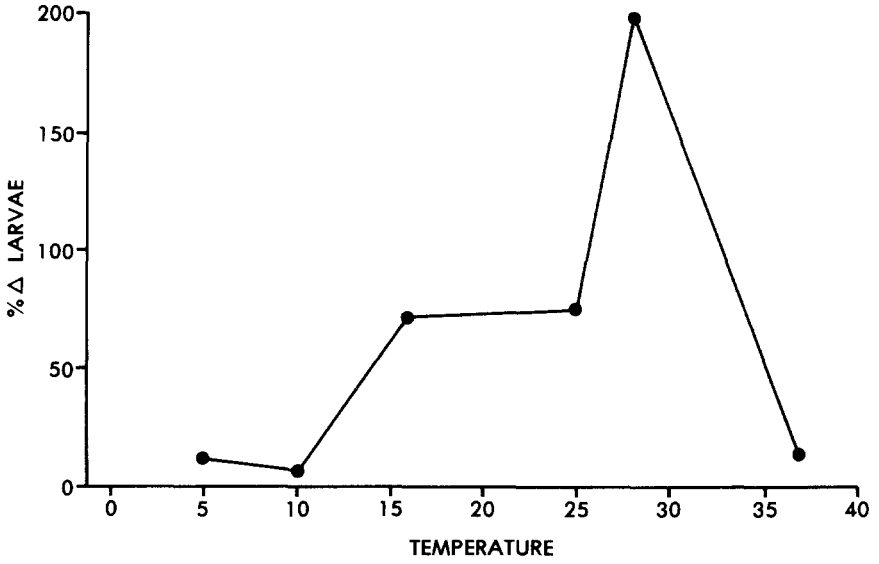

FIG. 5. Percent change (%Δ) in hatching of *H. glycines* eggs in 3 mM zinc chloride after two weeks at the indicated temperatures, as predicted by regression analaysis.

found at 37°C. Therefore, hatching mediated by zinc chloride appears temperature dependent.

The pH of the 3 mM solution of zinc chloride altered hatching of *H. glycines* eggs (Figure 6). A sevenfold increase in the number of larvae occurred around pH 5.3 to 6.1, but hatching declined at 7.1. The hatching stimulation from zinc chloride appears optimal under slightly acidic conditions.

Other hatching stimuli gave varied results. Picrolonic acid increased the hatching of *H. glycines* over various concentrations with a fivefold elevation at 0.9 mM. In contrast, concentrations of sodium metavanadate up to 18 mM failed to alter the hatching. Other known compounds that cause hatching in cyst nematodes may also differ in the various species.

Hatching of *H. glycines* eggs was tabulated also from the water controls that were used throughout experimentation to evaluate any seasonality of hatching (Figure 7). Compilation of data from January to December 1981 revealed the highest hatching occurred in May under untreated laboratory conditions. An approximate twofold increase was found at this time when compared to other months. Thus, all treatments require an internal control as designed in this study to account for seasonal fluctuations of hatching.

DISCUSSION

Hatching of *H. glycines* eggs is apparently not mediated by calcium as are other cyst nematode species, based on the results of chelation, inhibition, and

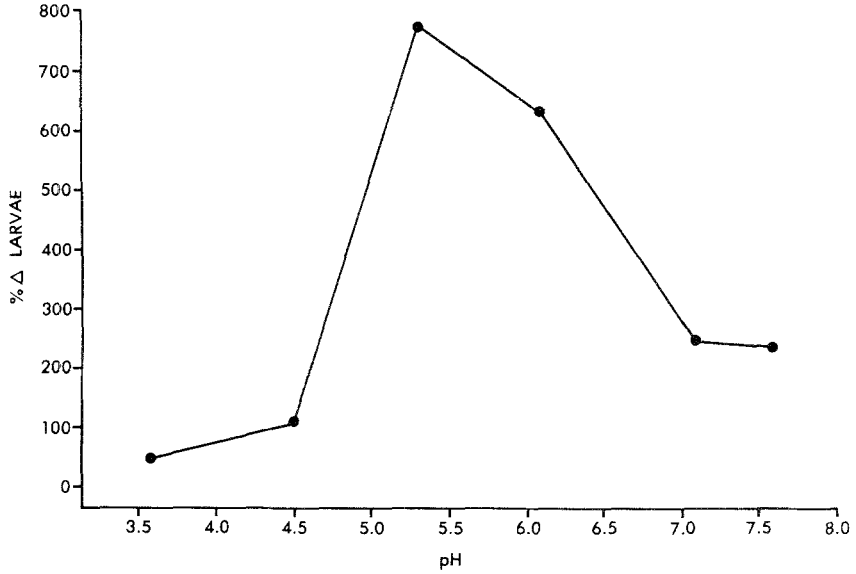

FIG. 6. Percent change (%Δ) in hatching of *H. glycines* eggs in 3 mM zinc chloride after two weeks at the indicated pH values, as predicted by regression analaysis.

transport in this study. However, zinc may be responsible for modulation of hatching, at least in vitro. The stimulation of egg hatching by zinc is quite specific when compared to other ions. Lack of competition from other ions, such as calcium, magnesium, and manganese supports its specificity. However, the function of zinc remains unclear, although a role within a metalloenzyme is possible, based on the reports of Rogers and Brooks (1976, 1977) for another nematode.

The in vitro effects of zinc are dependent on concentration, time of exposure, temperature, and pH. The influence of temperature on hatching revealed a Q_{10} of 3.64 over the interval of 5–28° C, which is indicative of physiological function. However, temperature may affect directly the activity of the larvae. The maximal stimulation of hatching by zinc at pH 5.3 is probably related to its greater solubility in acidic conditions. Thus, acidic soils may enhance hatching of the soybean cyst nematode which increases with elevated temperatures. Norton et al. (1971) have correlated soil conditions in soybean fields to nematode populations. Alternatively, potential interactions with hatching factors from plants may be involved also.

Clarke and Shepherd (1966) examined the role of a number of inorganic ions in hatching of cyst nematodes and found hatching effects for various salts of zinc. Zinc sulfate was more active at 3 mM than zinc chloride which showed 20% less activity for *H. glycines*. Zinc chloride was more active in this study than zinc sulfate. No resolution of this difference is apparent other than the

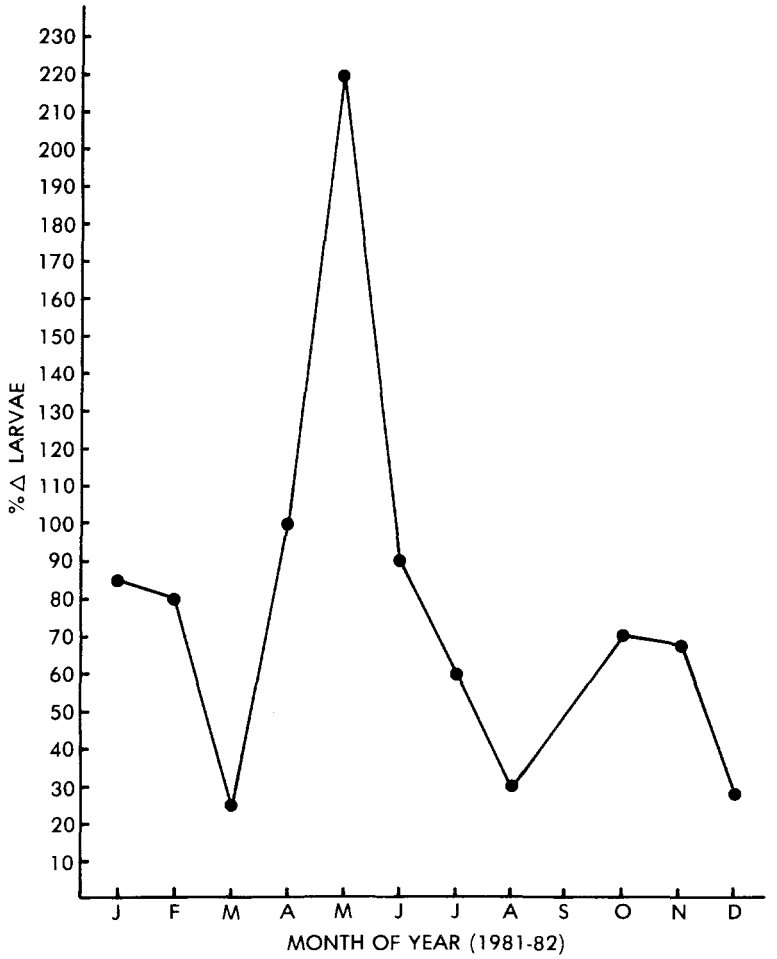

FIG. 7. Percent change (%Δ) in hatching of *H. glycines* eggs in the untreated water controls in 1981–1982.

responsiveness of geographic strains of the nematode. However, other compounds, such as picrolonic acid and sodium metavanadate, exhibit vast variation in their activity for hatching of species of cyst nematodes. Thus, generalization of any hatching stimulus to diverse species, or even strains, of cyst nematodes may prove difficult until a greater understanding of the hatching mechanism and its ecological regulation is attained.

Ecological regulation of the hatching process could optimize the level of larval nematodes to coincide with the time of host availability or density. Ross (1963) found a seasonal elevation of the numbers of larvae in the nematode

cyst with increased population occurring until 16–18 weeks after planting of the host. Comparable results were evident from tabulation of our data.

Chemical factors from the host plants are involved also in hatching of *H. glycines* eggs (Tsutsumi and Sakurai, 1966; Tefft et al., 1982). Mansamune et al. (1982) reported that glycinoeclepin from the kidney bean was a natural hatching stimulus for *H. glycines*. Active compounds from the host plant are possibly synergistic with calcium (Perry and Clarke, 1981) and thus lead to hatching of nematode eggs. Since the egg hatching of the soybean cyst nematode is apparently refractory to any influences of calcium, an intriguing area of future research should investigate any kairomone influences of host roots on zinc-mediated hatching.

In addition to its probable involvement as a hatching stimulus for *H. glycines*, larvae of the nematode were attracted to zinc chloride and other ions by in vitro assay. Compounds from the host roots were also attractive to larvae (Papademetriou and Bone, 1983). Thus, the interaction of the plant stimulant and zinc may cause not only hatching, but subsequent orientation to the host for invasion with resultant pathology.

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EFFECT OF MULTILURE AND ITS COMPONENTS ON PARASITES OF *Scolytus multistriatus* (COLEOPTERA: SCOLYTIDAE)

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Abstract—Several hymenopterous parasites of *Scolytus multistriatus* are attracted to components of its aggregation pheromone, multilure. *Cheiropachus colon*, *Entedon leucogramma*, *Dendrosoter protuberans*, *Spathius benefactor*, and *Cerocephala eccoptogastri* are attracted in various degrees to multilure, its components (multistriatin, 4-methyl-3-heptanol, and cubebene), and component combinations. *C. colon* was trapped in greatest numbers, yet was usually less numerous than *E. leucogramma* and *D. protuberans* in the study area. Impact of traps on *C. colon* may conceivably be reduced by multistriatin content in baits and/or by withholding traps until *S. multistriatus* flight begins.

Key Words—Aggregation pheromone, *Scolytus multistriatus*, Coleoptera, Scolytidae, *Cheiropachus colon*, *Entedon leucogramma*, *Spathius benefactor*, *Dendrosoter protuberans*, *Cerocephala eccoptogastri*, Hymenoptera, Pteromalidae, Braconidae, Eulophidae, European elm bark beetle, parasites, kairomone, multilure.

INTRODUCTION

Multilure (Pearce et al., 1975), the aggregation pheromone for the smaller European elm bark beetle, *Scolytus multistriatus* (Marsham), is being used to bait traps for elm bark beetle surveys and elm bark beetle suppression studies (Lanier et al., 1976). A study of nontarget insects captured at multilure-baited and nonbaited traps has shown that several hymenopterous parasites of *S. multistriatus* are apparently attracted to multilure-baited traps placed on both elms and non-elms (Kennedy, 1979). The parasites are: *Spathius benefactor*

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Matthews, Braconidae; *Entedon leucogramma* (Ratzeburg), Eulophidae; *Cheiropachus colon* (L.), Pteromalidae; *Dendrosoter protuberans* (Nees), Braconidae; and a possible hyperparasite of *Dendrosoter*, *Cerocephala eccoptogastri* Masi, Pteromalidae (Grissell, 1981), previously reported as *C. rufa* (Walker) by Kennedy (1979). All but *S. benefactor* are European in origin, and all are now established in the United States.

To define the attractant activity of multilure to these parasites, we investigated parasite response to the three components of multilure: 4-methyl-3-heptanol and multistriatin (beetle-produced pheromones) and cubebene [a host (elm)-produced synergist]—singly, in doublets, and as the 3-component blend (Pearce et al., 1975).

METHODS AND MATERIALS

The study was conducted in 1976 and 1977 in an urban residential area in Detroit, Michigan, and in an elm nursery on US Department of Agriculture property at Delaware, Ohio.

The following treatments (individual multilure components and combinations) were evaluated: (1) synthetic 4-methyl-3-heptanol (99%) (H); (2) synthetic multistriatin, mixed isomers (37% α-multistriatin) (M); (3) distilled cubeb oil (containing 70% α-cubebene) (C); (4) the three doublet combinations of these compounds; and (5) multilure (H:M:C), the 3-component blend. The three chemical components (H, M, and C) were supplied by Albany International, Chemicals Division, Columbus, Ohio.[1]

In Detroit, Stikem Special®-coated paper traps (45.7 × 67.3 cm) were fastened at a height of about 3 m to the boles of 80 mature non-elm trees located at the curbside; the same trees were used each year. There were 10 replicates of each of the seven treatments and 10 unbaited traps—a total of 80 traps. The 1976 trapping period was May 5 through October 12, and the 1977 trapping period was April 21 through October 20. Usually, the traps were changed at 2-week intervals, but in 1976, several periods were longer. The treatments were assigned at random to the trap positions.

At Delaware, the paper traps were placed on living, healthy (disease- and beetle-free) elms (10–20 cm dbh) in the nursery and on utility poles surrounding the nursery. There were six replicates (three on elms and three on poles) of each of the seven treatments and six unbaited traps—a total of 48 traps. In 1976, the trapping period was April 19 to June 10. Traps were changed at approximately 2-week intervals, but one period lasted 27 days (April 28–May 25). In 1977, the trapping period was April 18 to May 16, and the traps were changed weekly.

[1]Mention of a proprietary product in this paper does not constitute an endorsement of this product by the USDA Forest Service.

In both study areas, multilure and its components were dispensed from Conrel® hollow fiber baits (Albany International, Controlled Release Division, Needham Heights, Massachusetts) (Lanier et al., 1976) attached to the center of the traps. The controlled-release dispensers were designed to release the pheromone components at the rate of 400:100:800 μg/day (H:M:C). Each component was released from a separate cluster of hollow fibers; the rates of release and ratios of the components as used in multilure were determined to be the most efficient for attracting *S. multistriatus* (Cuthbert and Peacock, 1978).

When the traps were removed from the field, they were taken to the laboratory where the *S. multistriatus* were counted and the beetle parasites were removed and placed in vials of kerosene. The parasites were then washed in acetone, placed in alcohol, identified, and counted.

Index of attraction (Lanier et al., 1976)[2] was used to compare the response of the parasites to the components and component combinations of multilure. The statistical analysis of the Detroit data was run on the sums of the captures for the entire trapping season each year because data from the 2-week trapping periods were variable and sparse. Preliminary testing indicated nonnormal distribution of parasite responses, so the Kruskal-Wallis nonparametric test was used. Because of the number of tests done, simultaneous testing procedures were used at the significant level of 0.05. Delaware data were not analyzed statistically.

RESULTS AND DISCUSSION

In Detroit about 5000 parasites (including *C. eccoptogastri*) were captured in 1976 and nearly 8500 parasites were captured in 1977. A summary of 1977 parasite and beetle catches by trapping period is given in Table 1. At Delaware, a little over 5000 parasites were captured during 1976. Because the experiment was of short duration at Delaware in 1977, only *C. colon* and *S. multistriatus* were captured in numbers sufficient to be included in the tables (530 *C. colon* were captured, but only 17 *S. benefactor*, 5 *E. leucogramma, and 3 D. protuberans*).

Response of C. colon. Greater numbers of *C. colon* were captured in Detroit (4000 in 1976, 5149 in 1977) and at Delaware (4478 in 1976, 530 in 1977) than any other parasite species. Significantly more *C. colon* responded to those component combinations that included multistriatin than to those that did not; there were no significant differences among the combinations containing M. The order of the combined index of attraction (IA) of the

[2]Index of attraction(IA) = number responding to treatment/number responding to standard multilure mixture. IA for multilure = 100.

TABLE 1. ELM BARK BEETLE AND PARASITE CATCHES BY TRAPPING PERIOD FOR DETROIT, MICHIGAN, 1977, IN PERCENT OF TOTAL CAPTURED

Period	*S. multistriatus*	*C. colon*	*E. leucogramma*	*D. protuberans*	*C. eccoptogastri*
4/21–5/5	0.006	19.5	0.5	2.6	0.0
5/5–5/19	14.0	54.1	3.6	6.9	0.09
5/19–6/2	10.6	5.8	46.8	5.2	42.7
6/2–6/16	2.5	1.6	33.4	2.7	43.4
6/16–6/30	0.8	0.5	0.9	1.1	4.9
6/30–7/14	2.8	1.8	0.2	1.4	1.6
7/14–7/28	16.8	7.5	5.0	12.6	1.2
7/28–8/11	15.3	1.9	1.8	14.8	2.5
8/11–8/25	6.6	1.7	1.6	17.4	2.7
8/25–9/8	24.7	1.6	5.7	15.0	0.6
9/8–9/22	5.4	0.8	0.0	11.9	0.2
9/22–10/6	0.4	1.9	0.2	6.7	0.1
10/6–10/20	0.07	1.1	0.2	1.6	0.0
Total individuals	174,592	5149	440	705	2135

component combinations containing multistriatin from highest to lowest response of *C. colon* are: M + H (IA = 124); M (IA = 120); M + C + H (IA = 100), and M + C (IA = 89) (Table 2). Percentages of the total *C. colon* captured that were caught at traps baited with M, alone or in some combination, are: Detroit, 1976 and 1977, 89 and 90, respectively; and Delaware, 1976 and 1977, 77 and 88, respectively. The response of *C. colon* to H (IA = 17), C (IA = 13) and C + H (IA = 21) was weakly positive as compared to the unbaited traps (IA = 1).

At Delaware, H attracted more *C. colon* on traps attached to both elms and poles (IA = 115 and 107, respectively) in 1976 than in 1977, when response to H was considerably lower (IA = 43 and 40 on elms and poles, respectively, Table 3). Contamination of the Delaware baits may have occurred. That is, it is possible that baits that supposedly contained only H, did in fact contain some M. The response of *C. colon* to H at Detroit (IA = 27 and 11 for 1976 and 1977, respectively) was considerably reduced relative to Delaware both years. At Detroit the IA for C + H was 28 (1976) or less and 74 or less at Delaware (1976). The disparity in response by *C. colon* to H at Delaware and Detroit may have been due to the different trap locations at each locality.

None of the combinations tested had any repellent effect because all baited traps caught more parasites than unbaited check traps. The unbaited traps captured a total of only 19 *C. colon* in Detroit and Delaware during the 2-year study. At Delaware, 10 *C. colon* were trapped on the unbaited traps on elm, perhaps indicating a weak attraction of *C. colon* to the host tree.

Peak collections of *C. colon* occurred before the peak flight of the

TABLE 2. INDEXES OF ATTRACTION OF *C. colon* TO MULTILURE AND ITS COMPONENTS AT DETROIT, MICHIGAN[a]

Component(s) in lure	1976	1977	Combined index[b]
M + C + H[c]	100[d]	100	100
M + C	103	81	89
M + H	160	103	124
C + H	28	17	21
M	148	104	120
H	27	11	17
C	8	16	13
Unbaited traps	<1	<1	<1

[a]4000 and 5149 were captured in 1976 and 1977, respectively.
[b]Combined index based on sum of numbers trapped for 1976 and 1977.
[c]Multilure standard; M = multistriatin, C = cubebene, H = 4-methyl-3-heptanol.
[d]Index of attraction (IA): Number of parasites responding to treatment/number responding to standard multilure mixture. IA for multilure (HMC) is 100.

TABLE 3. INDEXES OF ATTRACTION OF *C. colon* TO MULTILURE AND ITS COMPONENTS AT DELAWARE, OHIO

Component(s) in lure	Traps on elms[a]		Traps on utility poles[b]	
	1976	1977	1976	1977
M + C + H[c]	100[d]	100	100	100
M + C	178	93	204	266
M + H	159	94	140	180
C + H	67	17	74	33
M	199	145	162	233
H	115	43	107	40
C	5	1	14	0
Unbaited traps	2	2	0	0

[a]3208 and 402 were captured in 1976 and 1977, respectively.
[b]1270 and 128 were captured in 1976 and 1977, respectively.
[c]Multilure standard; M = multistriatin, C = cubebene, H = 4-methyl-3-heptanol.
[d]Index of attraction (IA): Number of parasites responding to treatment/number responding to standard multilure mixture. IA for multilure (HMC) is 100.

overwintering beetle generation each year at both Detroit and Delaware. In 1977 at Detroit, 80% of the total catch of *C. colon* had occurred by June 2 when only 25% of the *S. multistriatus* had been trapped (Table 1). The same pattern is evident in catches at multilure-baited traps in Delaware during 1976 (Figure 1).

In the laboratory, *C. colon* oviposits on late-instar larvae of *S. multistriatus* (Kennedy, 1974). The pattern of early emergence of *C. colon* suggests that *C. colon* may oviposit on late-instar overwintering larvae of *S. multistriatus* during April and early May. There is no early summer emergence of *C. colon* to take advantage of the late-instar *S. multistriatus* larvae that are present in July and early August.

Multistriatin is a possible kairomone for *C. colon*. However, this parasite emerges in the spring before the beginning of beetle emergence—and well before active production of M by beetles. (It also emerges several weeks before any beetle larvae from spring bark beetle emergence are suitably mature for parasite oviposition.) Thus, there appears to be a lack of synchronization in the beetle-parasite relationship. Several conjectures may explain this apparent anomaly: (1) the parasite is responding in the spring to minute quantities of residual M remaining from adult beetle activity the preceding year; (2) larvae of the beetle produce M, which is attractive to the parasites; (3) M is not normally present in the environment when the majority of *C. colon* are active, so its behavior in these tests can be considered artificial; and (4) M has no

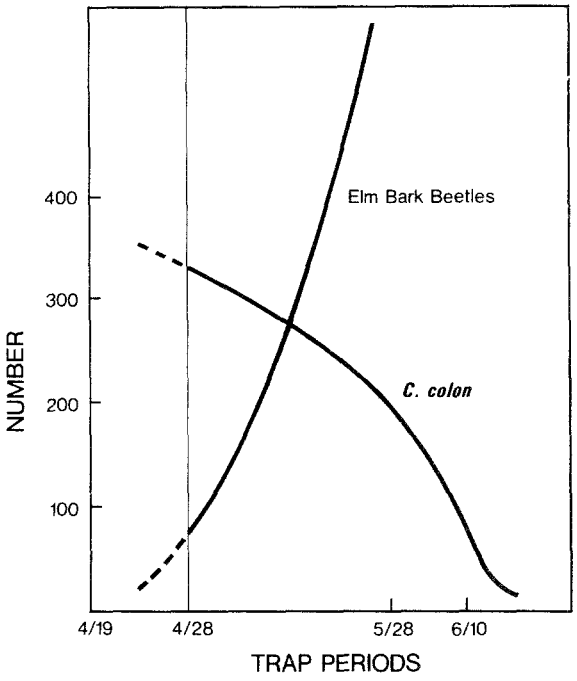

FIG. 1. Numbers of elm bark beetles and *C. colon* trapped on multilure-baited traps at beginning of trapping season, Delaware, Ohio, 1976.

natural role in parasite behavior, and other chemicals serve as kairomones that mediate parasite behavior. It is also possible that the biological relationship of the parasite to the bark beetle has been misinterpreted.

Response of E. leucogramma. For both 1976 and 1977, *E. leucogramma* was the second most numerous parasite found on the traps (however, in 1977 there were more *D. protuberans* caught in Detroit than *E. leucogramma*). The number of *E. leucogramma* trapped was only one tenth the number of *C. colon* trapped.

Statistical analysis of the response of *E. leucogramma* showed that at both Detroit and Delaware the response to M + C + H and M + C were significantly different from the other treatments (Table 4).

E. leucogramma response to M + C + H and M + C was about twice that of the third-ranking component or combination (Table 4). The IA of C + H and M at Delaware was about twice the response at Detroit.

In nature, *E. leucogramma* is attracted to the entrance of the elm bark beetle maternal gallery, where it enters and oviposits into bark beetle eggs that have been deposited along the sides of the gallery. This is done even as the bark

TABLE 4. INDEXES OF ATTRACTION OF *E. leucogramma* TO MULTILURE AND ITS COMPONENTS

Component(s) in lure	Detroit, Michigan[a]			Delaware, Ohio[b]
	1976	1977	Combined index	1976
M + C + H[c]	100[c]	100	100	100
M + C	81	111	96	99
M + H	32	24	28	13
C + H	26	12	19	54
M	23	21	22	41
H	18	4	11	14
C	8	12	10	None
No bait	1	1	1	None

[a]421 and 440 were captured in 1976 and 1977, respectively.
[b]307 were captured on traps placed on elms; 5 were captured on traps placed on utility poles baited with M + C + H, M + C, C + H.
[c]Index of attraction (IA): Number of parasites responding to treatment/number responding to standard multilure mixture. IA for multilure (HMC) is 100.

beetle moves in and out of the gallery. The coincident peak captures for the beetle and *E. leucogramma* on multilure-baited traps are consistent with their biological interaction (Table 1).

E. leucogramma is caught in far fewer numbers than *C. colon*, but its biology seems to be in closer synchrony with the beetle life cycle. It is reasonable to conclude that *E. leucogramma* is attracted in nature to a combination of the elm-produced attractant, C, and the beetle-produced kairomone, M. Cubebene could function as the chemical that attracts the parasite to elm trees, and then M would lead the female parasite to the precise oviposition site, the entrance to the beetle gallery. H probably does not attract this parasite, even though it is contained in multilure (HMC), which is slightly more attractive than the M + C mixture. H is produced only by virgin beetles (Pearce et al., 1975; Gore et al., 1977; Lanier et al., 1977) and thus is probably not present at the site of the beetle egg gallery.

Response of D. protuberans *and* S. benefactor. *D. protuberans* was trapped in fewer numbers than *C. colon* or *E. leucogramma* in both Detroit and Delaware, except that the number caught in Detroit in 1977 was greater than the number of *E. leucogramma* caught (Table 1).

For the 1976 data in Detroit, there were no significant differences found in response of *D. protuberans* to any of the components of multilure or combinations. This was probably due to the small number (158) of *D. protuberans* trapped. In 1976, C attracted the most *D. protuberans*, but the

M-containing combinations were closely grouped and not greatly different (Table 5). For 1977, 705 *D. protuberans* were trapped. A significant response was found when testing the entire array of treatments, although no significance was indicated when testing only M and the combinations containing M. The components and combinations of M + C, M, and M + C + H attracted the greater numbers, but M + H ranked high also. Comparison of *D. protuberans* catches at Delaware in 1976 showed the response of this parasite was similar to that found in Detroit (Table 5).

Spathius benefactor oviposits on late-stage elm bark beetle larvae, but only one individual was trapped in Detroit (in 1977). However, 114 were trapped at Delaware in 1976, and the greater responses were to M + C and M + C + H (Table 5). It is clear that H alone is unattractive to either of these two parasites, because H-baited traps captured less parasites than unbaited traps in three instances. In Detroit, M + H-baited traps captured a moderate number of *D. protuberans*, but at Delaware few *D. protuberans* and *S. benefactor* were captured. The combined IA for M + H was 78 for *D. protuberans* at Detroit, and 15 at Delaware. The IA for M + H was 35 for *S. benefactor* at Delaware. H may even be a deterrent to these two parasites, depressing the more positive effects of multilure and M.

The response of *D. protuberans* to C was variable. Except for the Detroit

TABLE 5. INDEXES OF ATTRACTION OF *D. protuberans* AND *S. benefactor* TO MULTILURE AND ITS COMPONENTS

	Detroit, Michigan[a]— *D. protuberans*			Delaware, Ohio[b], 1976	
Component(s) in lure	1976	1977	Combined index	*D. protuberans*	*S. benefactor*
M + C + H	100[c]	100	100	100	100
M + C	116	131	128	66	260
M + H	104	73	78	15	35
C + H	56	60	59	52	55
M	88	108	105	63	40
H	8	16	14	0	15
C	132	28	45	15	30
No bait	28	10	13	11	25

[a]158 and 705 *D. protuberans* were captured in 1976 and 1977, respectively.
[b]92 *D. protuberans* captured; 87 on traps on elms; 5 on utility pole traps (on M, H, M + C) not included in IAs. 114 *S. benefactor* captured; 112 on traps on elms, 2 on utility poles (M, H) not included in IAs.
[c]Index of attraction (IA): Number of parasites responding to treatment/number responding to standard multilure mixture. IA for multilure (HMC) is 100.

response in 1976, when C attracted a great number of *D. protuberans* (IA = 132), C usually ranked slightly above H in effectiveness. Cubebene had a combined IA of 45 at Detroit and 15 at Delaware; an IA of 30 was recorded for *S. benefactor* at Delaware. The C + H combination at both locations was usually a stronger attractant than either component separately. And at Detroit the IA for cubebene for the combined years was less than the C + H combination. The emergence of *D. protuberans* in 1977 peaked in late summer around August to September (Table 1). The same pattern was recorded in 1976.

The braconid parasites, *D. protuberans* and *S. benefactor*, are apparently much less attracted to multilure and its components than *E. leucogramma* and *C. colon*. This may be because these braconids attack late-stage bark beetle larvae, ovipositing through the bark several weeks after the pheromone has been produced by the boring activity of the female bark beetles. *C. colon* seems to be exceptionally attracted to M but it also attacks late-stage bark beetle larvae, and this may indicate that *C. colon* has a response mechanism that differs from that of the braconids.

Response of C. eccoptogastri. *C. eccoptogastri* is associated with Scolytidae and may be a primary parasite; it may also be a secondary parasite associated with Braconidae (Grissell, 1981). This parasite was trapped in relatively high numbers (Table 1), and its response to multilure and its components were very similar to that of *C. colon*, particularly in Detroit (Table 6). At Delaware only 53 individuals were trapped and M + C + H and M + C had IAs of 100 or more; the IA for M + H was 50.

TABLE 6. INDEXES OF ATTRACTION OF *C. eccoptogastri* TO MULTILURE AND ITS COMPONENTS

	Detroit, Michigan[a]			
Component(s) in lure	1976	1977	Combined index	Delaware, Ohio[b], 1976
M + C + H[c]	100[c]	100	100	100
M + C	85	91	90	111
M + H	258	124	145	50
C + H	2	5	5	6
M	152	143	145	17
H	4	11	9	11
C	1	5	4	None
No bait	None	<1	<1	None

[a]495 and 2135 were captured in 1976 and 1977, respectively.
[b]53 were captured, 37 on elms, 16 on utility poles.
[c]Index of attraction (IA): Number of parasites responding to treatment/number responding to standard multilure mixture. IA for multilure (HMC) is 100.

Sex of Captured Parasites. Most of the parasites captured were females, regardless of species or the attractant tested: 90–98% for *C. colon*, 98% for *E. leucogramma*, 86–100% for *D. protuberans*, and 75% for *S. benefactor*. *C. eccoptogastri* was not sexed. The male–female parasite ratio of the emerging population of the five parasite species during this study is unknown. However, a previous study (Kennedy, unpublished) in the Detroit area yielded 61% females of 2664 *D. protuberans* tabulated from infested bolt emergence. Catches of males on attractant-baited traps in our study may be low because males remain at the parasite emergence site and mate with the females as they emerge. But females apparently respond to elm- and bark beetle-produced attractants in the host-seeking process.

Catches of Parasites: Elms vs. Utility Poles. The numbers of parasites caught at traps placed on elms and utility poles in the Delaware study are shown in Table 7. Trap catches of all parasites and for all component combinations were much higher at traps on elms than traps on utility poles. Only catches on traps baited with cubebene alone were low and comparable for both elms and utility poles; this result is especially true for *C. colon*. Cubebene was probably not a strong attractant for any of the parasites except *D. protuberans* at Detroit in 1976. Other odorants or physical factors may be responsible for the increased catches at traps on elms compared to those on poles, regardless of the beetle-produced attractant involved.

Parasite Ratios: Trapped and Naturally Occurring. Parasites reared from naturally infested wood usually show a different numerical relationship from that exhibited by catches on traps. Of the four parasites, only *C. colon* is attracted in large numbers, and perhaps disproportionately relative to its actual ratio in beetle populations. Usually, *C. colon* is reared from infested elm wood in fewer numbers relative to the other parasites than was shown by the results of the trapping. *C. colon* accounted for 67% of the total number of parasites (including *C. eccoptogastri*) trapped at Detroit and 89% of those trapped at Delaware. Relative to these figures, *C. colon* has a rate of parasitism (based on numbers of emerged parasites and beetles) that is usually less than 5% and may be 1% or less (Kennedy, unpublished data).

The braconids, *D. protuberans* (where established) and *S. benefactor*,

TABLE 7. PARASITES CAUGHT ON ELMS AND UTILITY POLES

Parasite	Elm	Utility Pole
C. colon	3208	1270
E. leucogramma	307	5
S. benefactor	112	2
D. protuberans	87	5
C. eccoptogastri	37	16

may account in nature for a rate of parasitism of up to 15% or more, and *E. leucogramma* for a rate of 10–20% (Kennedy, unpublished data). These species accounted for 11–33% of the parasites trapped in this study, so the parasite catches at traps may represent the actual relative ratios of the parasites in Detroit and Delaware during the trapping period.

CONCLUSIONS

This study was subject to uncontrolled field conditions such as variable temperatures affecting elution rates of the bait components. Also, the sources of beetle and parasite populations were not evenly distributed throughout the study area and, therefore, not equally available at each trap site. Thus, random distribution of bait components might allow a weak attracting component to outperform a stronger component when the stronger component is in competition with adjacent or nearby strong components. However, overall performance allows some conclusions to be made about attraction of the parasites to individual multilure components and component combinations.

C. colon is attracted to M, but is not strongly attracted by the other multilure components. H and C did, however, seem to enhance attraction to M to some degree. The combination M + C attracts *E. leucogramma*, but *E. leucogramma* catches were much lower than those recorded for *C. colon*. *D. protuberans* and *S. benefactor* may be attracted in some degree to M, but the results on this study were highly variable. In any case, far fewer of these two parasites were captured than were *C. colon* or *E. leucogramma*. The hyperparasite, *C. eccoptogastri*, like the other parasites, was attracted to M-baited traps.

It is possible that, in this study, the parasites responded to other than the beetle-active isomers or enantiomers, since in the case of all three components, the test materials were racemic mixtures.

Multilure baits, which by necessity contain M, could conceivably reduce populations of these beneficial parasites. Alternatives that might reduce parasite losses would be: (1) deploy beetle traps in the spring after peak flight by *C. colon* (and before beetle flight); (2) develop a bait that contains amounts of M that reduce attraction of the parasites, while still maintaining potency for *S. multistriatus*; or (3) use a combination of both (1) and (2).

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FACTORS AFFECTING LEVELS OF SOME PHENOLIC COMPOUNDS, DIGESTIBILITY, AND NITROGEN CONTENT OF THE MATURE LEAVES OF *Barteria fistulosa* (PASSIFLORACEAE)

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Abstract—Levels of total phenolics, condensed tannins, acid detergent fiber, pepsin/cellulase digestibility, and nitrogen in mature leaves of 26 individuals of the ant-plant, *Barteria fistulosa*, have been determined. Analysis of the results in terms of the presence or absence of ants and the position of the branch from which the leaves were collected showed no relationship with concentrations of phenolics or fiber and only a weak relationship with digestibility and nitrogen. By contrast, light intensity strongly influenced levels of phenolics, notably condensed tannins, so that mature leaves of individuals growing in direct sunlight were less digestible and appeared to be of lower quality as food for herbivores than did mature leaves of individuals in shaded positions. Possible reasons for the variation in condensed tannin levels are discussed.

Key Words—Phenolics, tannins, condensed tannins, fiber, digestibility, nitrogen content, *Barteria fistulosa*, ant-plant, mature leaves, chemical variation, herbivory, *Pachysima aethiops*, Hymenotera, Formicidae.

INTRODUCTION

Barteria fistulosa Mast. (Passifloraceae) is a small tree, up to 15 m tall, found in the rain forest of western and central Africa, from Nigeria to Zaire (Keay, 1954). It appears to be most common in gaps in the forest canopy but is found in a range of sites with differing light intensities, varying from extensive clearings to beneath closed canopy. *B. fistulosa* is an obligate ant-plant, saplings being colonized when 1–1.5 m tall by *Pachysima aethiops*, a large

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pseudomyrmicine ant with a very painful sting. Colonization of *B. fistulosa* coincides with the development of the first horizontal, hollow branches, within which the colony becomes established. The natural history of the mutualistic association between ant and tree has been documented by Janzen (1972).

In the Douala-Edea Forest Reserve in western Cameroon *Barteria fistulosa* is quite common in disturbed natural sites and is abundant in large man-made clearings, such as abandoned farms in the vicinity of Lac Tissongo. Many trees in these artificial clearings are occupied by an unidentified species of *Crematogaster* and not by *Pachysima aethiops*. Young saplings are sometimes heavily defoliated by insect herbivores, notably the larvae of acraeid butterflies and noctuid moths, but these are unable to survive if confronted with an established *Pachysima* colony. In fact, trees with a thriving *Pachysima* ant colony are notable for their almost complete freedom from attack by insect herbivores (Janzen, 1972) and the absence of epiphyllae from the leaves, and are only attacked by large herbivores (e.g. black colobus monkeys, *Colobus satanas*) when leaves can be obtained without direct confrontation with the defending ants. Black colobus monkeys have been observed to virtually destroy unoccupied *Barteria* (McKey, 1974) and to pluck individual leaves from occupied trees while sitting in safety in adjacent trees (D.B. McKey, unpublished observations).

Chemical studies carried out in the field in 1976 and 1979 (P.G. Waterman, unpublished observations) demonstrated that the mature leaves of saplings not yet colonized by *Pachysima* were rich in cyanogenic glycosides which readily liberated hydrocyanic acid on mechanical damage of the leaf. As the tree grows, the ability to produce hydrocyanic acid diminishes rapidly with no trace of this compound detectable in the mature leaves of large trees under field conditions. Other chemical investigations performed in the laboratory, using a single sample of mature leaves originating from a tree growing in an extensive clearing, indicated the presence of quite high concentrations of procyanidin- and prodelphinidin-based condensed tannins and of fiber, with consequent low digestibility (Choo et al., 1981). Although the nitrogen content of this sample was above the average of those measured for mature leaves from Douala-Edea (Waterman et al., 1980), the high concentrations of both condensed tannins and fiber make this food type appear far less suitable for a ruminant-like herbivore than those normally selected by the black colobus (McKey et al., 1981). However, in the Douala-Edea Reserve these monkeys do feed on *Barteria* leaves, an observation that appears to be at odds with our understanding of factors influencing their selection of food items.

Janzen (1981) has postulated that the chemical defenses of ant-plants have been reduced along some axes in the course of ant and plant coevolution, and Rehr et al. (1973) presented evidence of such reduction in chemical

defenses of Central American ant acacias. In view of these findings, we decided to investigate the chemical composition of mature leaves of this species in greater detail. In this paper, we report the results of a comparison of levels of some digestion inhibitors (polyphenols, measured as total phenolics and condensed tannins, acid detergent fiber), cellúlase digestibility, and nitrogen content in 26 samples of mature leaves of *B. fistulosa*. Collections were made in order to examine the following factors that might influence leaf chemical composition:

1. Vertical branches against horizontal branches. Young *B. fistulosa* saplings have only vertical stems with no horizontal branches available for colonization by *Pachysima*. We considered it possible that vertical stems might be more heavily defended because of the absence of the ant mutualist.

2. Branches occupied by ants against those unoccupied. We considered it possible that a direct or indirect (through enhancement of the condition of the plant) effect of colonization might be a reduction in levels of digestion-inhibitory compounds. In this comparison the *Pachysima*- and *Crematogaster*-occupied plants (five and three, respectively) were treated together, which had no effect on the outcome in practice.

3. Plants growing in extensive, fully insolated clearings against plants growing in shaded situations at the edge of light gaps and under the canopy. Observations of black colobus feeding all involved plants of the latter category.

METHODS AND MATERIALS

Plant Material. All samples were collected within the confines of the Douala-Edea Forest Reserve, Cameroon, in July 1979. For a description of the study site see Gartlan et al. (1980). A voucher specimen of *B. fistulosa* (Waterman and McKey-805) has been deposited at the herbarium of the Royal Botanic Gardens, Kew, England. Leaf samples were oven dried at 50° C within 4 hr of collection and were then stored in sealed bags in the dark until transported to Glasgow for analysis. In the laboratory, samples were ground to give a maximum particle size of 1 mm.

Analysis of Polyphenols. Samples were assayed for both total phenolics (TP) and condensed tannins (CT). Hydrolyzable tannins were absent. Total phenols were estimated using the Folin-Denis method (760 nm) on extracts prepared in 50% aqueous ethanol under reflux with results expressed in terms of tannic acid. Condensed tannins were estimated on cold methanol extracts using the *n*-butanol–hydrochloric acid method (550 nm) with results expressed in terms of quebracho tannin. The detailed assay procedures have been described elsewhere (Gartlan et al., 1980). The drying process is likely to have caused some decrease in amounts of extractable polyphenols, but

there is no reason to expect that rank order will have changed, particularly as only a single species is involved in the study (Gartlan et al., 1980; Martin and Martin, 1982). It should be noted that the two assays performed are calibrated with respect to different standards and that estimates of TP and CT are not absolute with respect to one another.

Acid Detergent Fiber (ADF). ADF was measured exactly as described by van Soest (1963) using ground leaf samples of about 500 mg. This assay is considered to measure the cutin and lignin of the leaf, together with that part of the cell wall polysaccharide most intimately bound to the lignin and therefore least attainable by herbivores.

Cellulase Digestibility (CDIG). Samples of ground leaf (400 mg) were predigested with pepsin in 0.1 M hydrochloric acid for 24 hr and then incubated with fungal cellulases at pH 4.6 for 48 hr. The exact techniques and reagents employed have been described previously (Choo et al., 1981).

Nitrogen content of ground samples (about 300 mg) was measured by the Kjeldahl method. Crude protein content of the samples (PROT) was estimated as N (%) $\times$ 6.25.

RESULTS

The results of the analysis of 26 samples of *Barteria fistulosa* mature leaves for TP, CT, ADF, CDIG and PROT are given in Table 1 together with information on the three variables as they relate to each sample. An initial observation concerning these data is that there are considerable variations in the values obtained for different samples, particularly for the two polyphenol assays, compared with previous studies we have carried out on multiple collections of other species from this forest (Gartlan et al., 1980; P.G. Waterman, unpublished observations). One specimen gave particularly low values for both total phenols (1.36%) and condensed tannins (1.49%), of which the latter was shown, on the basis of acid hydrolysis and thin-layer chromatography, to be primarily made up of procyanidin. The occurrence of individuals with low condensed tannin content in a species that is generally condensed tannin-rich has not previously been noted among trees of the Douala-Edea flora. Similar investigation of the other 25 samples showed condensed tannins that appeared to be made up of about equal quantities of procyanidin and prodelphinidin. However, condensed tannin content among these did vary considerably, from 5.39% to 29.48% when estimated as quebracho tannin. Total phenolics were also highly variable, between 3.81% and 10.69% when estimated as tannic acid.

Levels of acid detergent fiber were generally between 40% and 50%, but on a few occasions levels approaching 60% were found. Likewise, cellulase digestibility was generally between 25% and 35% but with some specimens

TABLE 1. TOTAL PHENOLS (TP), CONDENSED TANNINS (CT), ACID DETERGENT FIBER (ADF), CELLULASE DIGESTIBILITY (CDIG), AND CRUDE PROTEIN[a] IN MATURE LEAVES OF *Barteria fistulosa*[b] GROWING UNDER DIFFERING BRANCH ORIENTATION AND LIGHT INTENSITY AND IN PRESENCE OR ABSENCE OF ANT COLONIES

Position of leaves	TP	CT	ADF	CDIG	PROT
Vertical branches					
In extensive light gaps					
Unoccupied by ants	8.35	17.33	43.24	30.35	9.95
	7.30	16.19	45.67	29.44	10.63
	8.88	19.24	41.86	36.39	10.25
	7.67	15.32	46.90	26.37	11.28
	7.32	15.70	45.91	27.87	9.18
	7.68	17.39	48.89	28.84	9.23
	10.69	29.48	45.10	25.44	10.44
	10.69	29.09	41.15	33.79	9.69
	9.01	18.26	41.11	30.79	10.34
In semishaded positions					
Unoccupied by ants	5.62	10.23	53.28	44.70	10.25
	6.69	13.82	44.68	32.23	12.02
	4.24	10.30	48.79	28.64	10.95
	4.79	8.47	39.24	42.87	11.75
	8.35	16.76	40.20	43.16	12.23
	1.36	1.49	49.67	48.52	12.86
Horizontal branches					
In extensive light gaps					
Unoccupied by ants	4.98	7.41	59.80	23.48	11.39
	9.07	23.16	45.13	31.52	9.37
Occupied by ants	9.48	20.23	39.74	25.49	10.54
	9.54	22.43	48.79	25.25	11.50
	6.50	11.85	54.06	22.49	12.35
	9.68	23.43	41.16	26.07	11.85
In semishaded positions					
Unoccupied by ants	6.10	19.45	43.95	28.65	12.46
Occupied by ants	4.11	5.39	41.03	35.93	12.32
	5.72	16.36	44.84	23.64	14.31
	3.81	6.73	50.90	30.18	12.74
	8.45	22.55	44.86	26.89	11.78

[a] $6.25 \times N$.
[b] All estimates as % dry weight.

showing markedly higher levels, approaching 50%. Levels of crude protein showed least fluctuation, between a low of 9.18% and a high of 14.31%.

Sample sizes were not adequate for a three-way analysis of variance with regard to the variables under study. Table 2 gives means and standard deviations for subsets of the 26 samples divided according to each of the three variables in turn. The differences between the means in each comparison

TABLE 2. MEANS, STANDARD DEVIATIONS (IN PARENTHESES), AND COMPARISON OF MEANS (*t* TEST)[a] FOR TOTAL PHENOLICS, CONDENSED TANNINS, ACID DETERGENT FIBER, CELLULASE DIGESTIBILITY, AND CRUDE PROTEIN LEVELS, AND COMPOSITE MEASURES OF THESE, FOR MATURE LEAVES OF *Barteria fistulosa* GROWING UNDER DIFFERING BRANCH ORIENTATION AND LIGHT INTENSITY AND IN PRESENCE OR ABSENCE OF ANT COLONIES

Variable	TP	CT	ADF	CDIG	PROT	PROT (ADF + CT)	PROT × CDIG (ADF + CT)
Vertical branches ($N = 15$)	7.24(2.46)	15.94(7.13)	45.05(4.00)	33.96(7.40)	10.74(1.10)	0.179(.04)	6.26(2.54)
vs.							
horizontal branches ($N = 11$)	7.04(2.29)	16.27(7.14)	46.75(6.13)	27.23(4.03)	11.87(1.26)	0.191(.06)	5.27(1.63)
t ratio	0.22	0.12	0.81	2.97**	2.40*	0.85	1.21
Unoccupied by ants ($N = 18$)	7.16(2.40)	16.06(7.06)	45.81(5.04)	32.95(7.24)	10.79(1.14)	0.177(.03)	5.99(2.40)
vs.							
occupied by ants ($N = 8$)	7.16(2.45)	16.12(7.30)	45.67(5.15)	26.99(4.27)	12.17(1.09)	0.200(.03)	5.50(1.83)
t ratio	0.01	0.02	0.06	2.61*	2.93**	1.52	0.57
Semishaded positions ($N = 11$)	5.39(2.06)	11.96(6.42)	45.59(4.57)	35.04(8.44)	12.15(1.05)	0.214(.03)	7.57(2.45)
vs.							
insolated positions ($N = 15$)	8.46(1.57)	19.10(5.89)	45.90(5.40)	28.24(3.87)	10.53(.98)	0.163(.02)	4.58(.64)
t ratio	4.14***	2.90**	0.16	2.49*	4.00***	4.68***	3.95***

[a] *t* test of the differences between two means (Sokal and Rohlf, 1969); ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

were assessed by a simple *t*-test (Sokal and Rohlf, 1969). This statistical analysis was also performed on combinations of measures [PROT/(ADF + CT)] and [CDIG × PROT/(ADF + CT)] that have previously been found to relate to leaf selection by some colobine monkeys (McKey et al., 1981; Waterman and Choo, 1981).

Of the parameters we examined, light intensity was the most strongly correlated with variation in leaf chemical composition. Trees in shaded locations produced foliage that contained much lower concentrations of total phenolics and averaged only about 60% of the condensed tannin content that was found in the leaves of fully insolated individuals. Perhaps as a consequence of their higher levels of polyphenolics, leaves of insolated plants were on average 20% less digestible than those of semishaded and shaded plants. Leaves of the latter group also contained about 15% more nitrogen than did those of insolated plants which could simply reflect the dilution of other components by the higher concentrations of polyphenolics in insolated leaves. Whatever the causal relationship between the individual differences, leaves from shaded plants offer greater concentrations of nitrogen relative to polyphenolics, and are more digestible, than are the leaves of insolated plants. This leads to highly significant differences in the two composite measures of leaf suitability to herbivores between shaded and insolated leaves.

The other two variables we examined, branch orientation and occupancy by ants, could not, in practice, be separated; all of the vertical branches were from unoccupied plants, and all but three of the horizontal branches were occupied by ants. Thus the two variables divided the plants in the same way and gave parallel results in tests for relationships within the chemical measures employed. Only a few strong relationships were evident for these comparisons. Leaves from vertical branches were more digestible and contained less nitrogen than leaves from horizontal branches. Likewise, leaves from unoccupied plants were more digestible and contained less nitrogen than leaves from occupied plants. No reasons can be suggested for these differences.

Table 3 presents the results of a correlation analysis between each of the five measures for all 26 samples and each of the six categories investigated. The most striking feature was the very strong positive correlation between total phenolics and condensed tannins for all comparisons, which indicated that condensed tannins made a consistent high contribution to the total phenolics of the leaf that was not influenced by any of these parameters. There were few other significant correlations to be noted. Surprisingly there was no overall correlation between fiber and digestibility (c.f. Choo et al., 1981), but there was a weak correlation (negative) between condensed tannins and digestibility. Once again the comparison between shaded and insolated individuals showed some interesting variation. In the latter, fiber did show a negative correlation with digestibility, the only subgroup where this was

TABLE 3. CORRELATION AMONG TOTAL PHENOLICS, CONDENSED TANNINS, ACID DETERGENT FIBER, CELLULASE DIGESTIBILITY, AND PROTEIN IN MATURE LEAVES OF *Barteria fistulosa* GROWING UNDER DIFFERING BRANCH ORIENTATION AND LIGHT INTENSITY AND IN PRESENCE OR ABSENCE OF ANT COLONIES

		Branch		Ants		Insolation	
Correlation between $N =$	All samples 26	Vertical 15	Horizontal 11	Present 8	Absent 18	Semishaded 11	Light gap 15
TP vs. CT	+0.93***[a]	+0.96***	+0.91***	+0.94**	+0.93***	+0.89***	+0.96***
TP vs. ADF	−0.43	−0.49	−0.40	−0.30	−0.49*	−0.40	−0.79***
TP vs. CDIG	−0.38	−0.55	−0.28	−0.55	0.40	−0.32	+0.37
TP vs PROT	−0.53**	−0.57	−0.58	−0.65	−0.60**	−0.14	−0.23
CT vs. ADF	−0.43*	−0.41	−0.49	−0.30	−0.49	−0.30	−0.67**
CT vs. CDIG	−0.41*	−0.57*	−0.22	−0.60	−0.42	−0.56	+0.35
CT vs. PROT	−0.42*	−0.56*	−0.38	−0.41	−0.52*	+0.05	−0.29
ADF vs. CDIG	−0.20	+0.03	−0.44	−0.34	−0.20	+0.03	−0.60*
ADF vs. PROT	+0.08	−0.07	+0.06	+0.29	+0.02	−0.27	+0.39
CDIG vs. PROT	+0.03	+0.56*	−0.19	−0.03	+0.38	−0.33	−0.65**

[a]***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

noted, and also showed an equally strong negative relationship to condensed tannins. By contrast, for the shaded individuals, the strongest negative correlation for digestibility was with condensed tannins, and there was no observable relationship at all between digestibility and fiber.

DISCUSSION

The clear inference to be drawn from these observations is that individuals of *Barteria fistulosa* growing in high light intensity respond by producing greater amounts of phenolics, specifically condensed tannins, than do individuals in more shaded positions. Syntheses of many classes of phenolics is known to increase with increased light intensity, and a variety of mechanisms have been proposed to account for this phenomenon (Smith et al., 1977). A positive relationship between light intensity and production of phenolics has recently been observed in the relatively condensed tannin-rich species *Sorghum bicolor* (Woodhead, 1981), but the phenolics involved were not confirmed as condensed tannins. Our study appears to be the first to specifically implicate condensed tannins.

We assume that the observed differences reflect environmentally induced variation rather than genetic variation in the production of polyphenolics. There are two ways in which such environmentally induced variation may be interpreted.

1. In highly insolated sites, the photosynthetic activity of a leaf may be so great in relation to the quantity of available nitrogen and minerals that, once the limited supply of these has been used in the production of primary metabolites, the remaining carbohydrates can only be used to produce nitrogen- and mineral-free molecules such as tannins and other phenolics. Variations of this hypothesis, which is part of the conventional wisdom of plant physiologists but to our knowledge has never been published, range from the notion that the plant must do something with the impinging energy and increased synthesis of phenolics acts as a safety valve for the conversion of otherwise potentially harmful radiant energy, to the suggestion that phenolics may simply be one of the few useful products that can be synthesized under conditions of superabundant energy and limiting nitrogen and mineral supply.

A similar kind of hypothesis can be developed from the mechanism of condensed tannin biosynthesis envisaged by Haslam (1977). He has proposed that condensed tannins arise from flav-3-en-3-ol precursors via the formation of a carbocation (Figure 1). The carbocation is an enzyme-bound intermediate in the formation of the flavan-3-ols, and polymerization to condensed tannins comes about by leakage of the carbocation from the enzyme and its coupling with the normally formed flavan-3-ol. Production of the carbocation is probably limited by the availability of the biological reductant

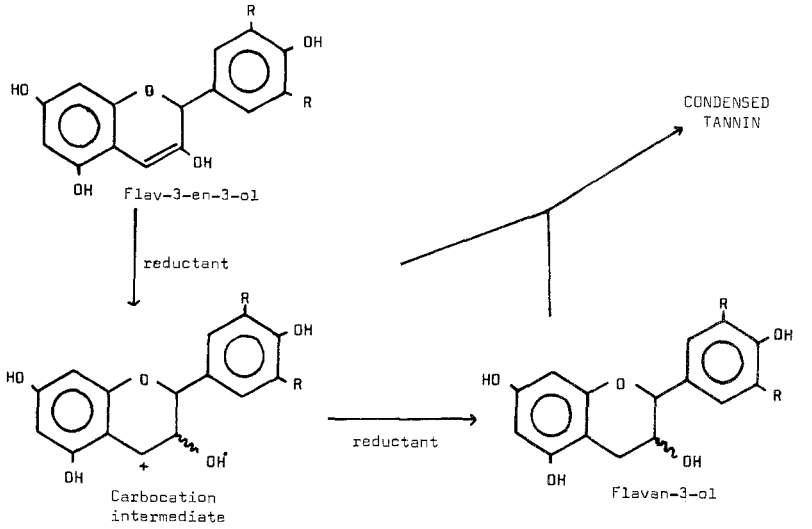

FIG. 1. Potential products of the flav-3-en-3-ol monomer with biological reductant (R = H or OH).

(perhaps NADPH), and this is likely to be increased when light intensity is high. Thus increased incident light initiates the production of more carbocation which is followed by greater leakage of the carbocation from the enzyme and, as a consequence, more polymerization. If this is so, then it is likely that condensed tannin levels in leaves will characteristically show a relationship to incident light intensity. An interesting prediction arising from this hypothesis is that levels of hydrolyzable tannins, which derive from a different biogenetic sequence not involving carbocation formation, may not show such a relationship with light intensity.

2. Such hypotheses tend to view the plant as passively responding to environmental variation in a necessary and mechanical fashion. However, it is to be expected that plants will have evolved substantial physiological control over processes so important to their fitness as the allocation of large amounts of fixed energy. The second type of interpretation assumes that plants have evolved mechanisms to control their responses to environmental variation and that any increase in production of phenolics in highly insolated sites occurs because it is in some way adaptive.

Reports that synthesis of phenolics increases with light intensity are widespread, and several hypotheses as to the adaptive significance of this response can be put forward.

i. Some workers have proposed that phenolic compounds are produced as some kind of protective response to combat the harmful effects of excess incident light. One potential role, that of screening out harmful UV radiation,

has been attributed to a number of phenolics including simple flavonoids (McClure, 1975), the phenylpropene-derived resin of the creosote bush, *Larrea dentata* (Rhoades, 1977), and, most convincingly, the young leaf anthocyanins found in many tropical rain forest species (Lee and Lowry, 1980). The ability to increase production of phenolics in response to increased light intensity could be interpreted as an adaptive feature of such a radiation screening system. Little is known regarding the potential of condensed tannins to intercept UV radiation but the relative absence of absorptive ability in the 290- to 400-nm range of the spectrum does not suggest that they will be very effective in this role.

ii. The phenolic resins of *Larrea dentata*, which occur primarily on the leaf surface, have been suggested to have an antidesiccant function (Rhoades, 1977), but there is no evidence to support this as a general role for phenolics. The few data that do exist tend to indicate that water stress, often an accompanying feature of light-rich environments, does not in itself generally lead to an enhancement of phenolic levels (Parker and Patton, 1975; Newman, 1978).

iii. A third possibility is that *Barteria fistulosa* has evolved to increase production of phenolic antiherbivore defenses when growing in light-rich clearings. Such increased production might be expected for two reasons: (1) in energy-rich sites additional defenses are likely to be metabolically cheap; (2) the number of potential herbivores is probably greater in light-gap sites than in semishaded sites in the forest. Certainly the composition of the herbivore fauna will be different in these two kinds of sites. Thus between-site variation in phenolic production may be an evolutionary response to quantitative and/or qualitative differences in the plant's herbivore fauna.

One consideration affecting interpretation of the variation of phenolic production by *B. fistulosa* is the question whether highly insolated sites have been a significant habitat for *B. fistulosa* over evolutionary time. In natural vegetation the tree is found most often in small forest light-gaps or in semishaded sites. If *B. fistulosa* has had little evolutionary experience with the highly insolated sites typical of man-made clearings, then the increased phenolic production might well be an uncontrolled response to novel conditions. If, on the other hand, *Barteria* has often been faced with such conditions over evolutionary time, it is more likely to have evolved a controlled response. Certainly some of the trees in large forest light gaps are as heavily insolated as trees in man-made clearings, but it is difficult to know what proportion of a plant population must occur in a habitat type for this to exert significant selective pressure on its physiological tolerances and phenoplastic responses.

Whatever the causal factors leading to it, the higher production of condensed tannins in sites with high light intensity could well have consequences for the quality of mature leaves of *Barteria fistulosa* as food for folivores. The acceptability of mature leaves to black colobus monkeys at

Douala-Edea has been shown to be related to the ratio of digestion inhibitors (ADF + CT) to the PROT content of the leaf or to the rate of PROT availability as measured by PROT × CDIG (McKey et al., 1981). Figure 2 represents the plot of PROT × CDIG against ADF + CT for all 26 samples of *B. fistulosa* mature leaf and for samples of the mature leaves of 20 other species from Douala-Edea, five of which are acceptable to black colobus. This plot clearly illustrates how the 15 samples of *B. fistulosa* mature leaves from insolated plants cluster and form part of a larger grouping that also contains many of the species that are unacceptable to black colobus, but only one that is acceptable. While some of the shaded individuals of *B. fistulosa* also produce foliage that fits into this grouping, others are found in the plot in close proximity to species that are utilized by black colobus and would be classified from the plot as potential food for this herbivore if the problem of circumventing the protective ants can be overcome.

The differences in levels of phenolic compounds, and particularly condensed tannins, between shaded and insolated plants are clearly the most striking finding of this study. Other differences are observed, but it is unclear how much significance they are to be accorded. Why nitrogen levels, for example, should be greater in the leaves of horizontal, particularly occupied branches is problematical, although the possibility that it is due to some extent to the assimilation of nitrogenous wastes from the ant colonies must not be overlooked.

Correlates of Cellulase Digestibility. Previous studies of the foliage of a large number of rain-forest tree species have implicated acid detergent fiber as a major factor influencing variation in cellulase digestibility (Choo et al., 1981). This study, on the other hand, fails to show this relationship, and condensed tannin content appears, overall, to exert a greater effect on digestibility. This seeming paradox may be an outcome of the fact that the degree of variation of a parameter varies with the level of comparison. In the between-species comparison (34 species) reported by Choo et al. (1981), the coefficient of variation for acid detergent fiber was 33%. In the present study, the coefficient of variation (for 26 samples of the same species) was only 10.9%. Regardless of how important fiber may be as a causal factor in determining digestibility, it cannot explain variability among samples unless it is itself variable. On the other hand, condensed tannins show variation at both levels (coefficient of variation 43.5% in this study). Their high variability at the between-individual level, where variability of fiber is low, raises their importance as a factor determining variation in digestibility at this level.

Chemical Defenses of Ant-Plants. Janzen (1981) has postulated that ant-plants have undergone a reduction in the overall levels of chemical defenses in the course of coevolution with their protective ants. In the case of Central American ant-acacia species there are data supporting this hypothesis (Rehr et al., 1973). The interindividual variation in condensed tannin content we

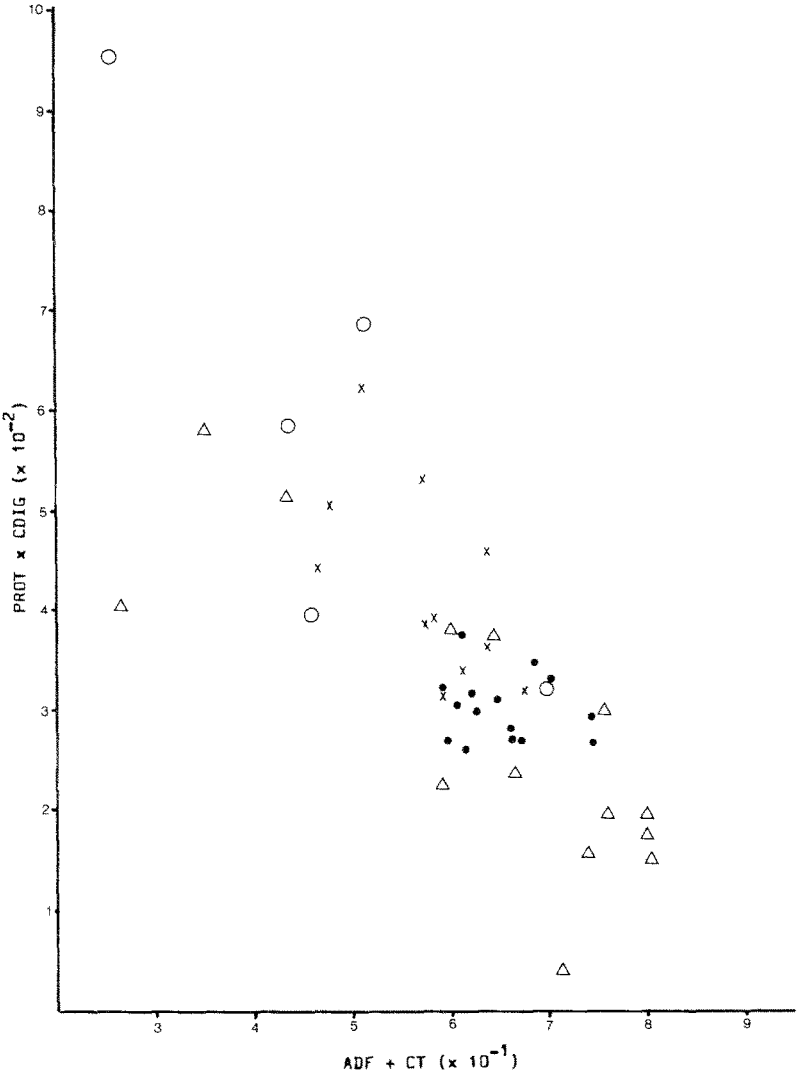

FIG. 2. Relationship between positive nutritive factors (PROT × CDIG) and total digestion-inhibitor content (ADF + CT) for the mature leaves of *Barteria fistulosa* growing in sites of high light intensity (small closed circles) and in shaded positions (crosses) and for the mature leaves of 20 sympatric species that are either acceptable to the black colobus monkey as food (open circles) or unacceptable (triangles).

have found in *B. fistulosa* illustrates one of the complications in testing this hypothesis with respect to this species. Identification of interspecific differences in the face of intraspecific genetic or phenoplastic variation will require very large sample sizes. The situation regarding any reduction in chemical defenses in *B. fistulosa* concomitant with the evolution of its protective association with *Pachysima* ants could be very complex, as may also be the case with ant-acacias (Seigler et al., 1978; Janzen, 1981).

The fiber and polyphenol digestion-inhibitor content of *Barteria fistulosa* mature leaves is certainly not exceptionally low, when compared to that of other Douala-Edea tree species, nor does it seem, on present evidence, to be lower than that of the related *Barteria nigritiana* Hook. *f.* (29% condensed tannin of similar type—one sample of mature leaf), a species not known to associate with *Pachysima*. Investigation of the entire suite of chemical defenses in these species may be required to determine why *B. fistulosa*, in contrast to *B. nigritiana* and virtually every other tannin- and fiber-rich species in the reserve, is heavily attacked by herbivores in the absence of its protective ants.

CONCLUSION

In this study we have been unable to show any relationship between the production of polyphenols and fiber in the mature leaves of *Barteria fistulosa* and the advent of nonchemical protection by *Pachysima* ants. On the other hand, there is strong evidence that levels of polyphenols, specifically condensed tannins, are positively influenced by increased light intensity while levels of nitrogen and digestibility both decrease under these conditions; the overall result is that the foliage has a markedly lower nutrient-to-digestion inhibitor ratio when compared to that of the same species growing in lower light intensity. Why condensed tannin levels should increase with increasing light intensity remains unclear, although a number of possible explanations have been put forward and, as proposed for the resin of *Larrea dentata* (Rhoades, 1977), it may be that there are multiple functions to be assigned to the polyphenolics of *B. fistulosa*.

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MISIDENTIFICATION BY WILD RABBITS, *Oryctolagus cuniculus*, OF GROUP MEMBERS CARRYING THE ODOR OF FOREIGN INGUINAL GLAND SECRETION

III. Experiments with Mixed Sex Groups and Analysis of Further Data from All-Male and All-Female Groups

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Abstract—The experiments described in this paper are part of a series designed to clarify the behavioral function of the odor of the secretion from the inguinal glands of rabbits, *Oryctolagus cuniculus*. Results are presented of tests carried out on 48 mixed-sex groups consisting of 162 adult wild-type rabbits, 48 males and 114 females. The subordinate females of the groups were smeared with various odorous materials and the reactions of their pen-mates towards them recorded. Eight sources of natural rabbit odors and a commercial perfume were used in 198 tests. A high proportion (60%) of the subordinate females smeared with the inguinal gland secretions from unfamiliar males were attacked, mainly by the males, which were generally the most dominant individuals within the groups. The paper also presents statistical analyses of behavioral data collected during two earlier related experiments with single-sex groups involving 258 rabbits in 300 tests which have not previously been evaluated. The results throughout the whole study indicate clearly that the odor of the inguinal gland secretion of rabbits carries information which affects the acceptance of individuals by their companions in organized groups. Other sources of unfamiliar rabbit odor did not influence the attitude of groupmates toward the individuals smeared with them, or if they did, their effects were only marginal.

Key Words—Wild rabbit, *Oryctolagus cuniculus*, mixed-sex groups, all-male groups, all-female groups, inguinal glands, strange odors, misidentification.

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INTRODUCTION

The experiments reported here are part of a wider study aimed at demonstrating the behavioral function of the secretion from the inguinal glands of wild rabbits, *Oryctolagus cuniculus*. The earlier two papers of this series described the reaction of all-male and all-female groups of rabbits towards members of their own social entities when those members were treated experimentally with various odorous materials including the inguinal gland secretions derived from unfamiliar conspecifics (Hesterman and Mykytowycz, 1982a,b). Both reports dealt only with part of the data collected, presenting analyses of the proportions of tests in which aggression or injuries occurred.

This paper, apart from summarizing, in the same way, the results from experiments with mixed-sex groups, also presents statistical analyses of other behavioral data which were collected throughout the three groups of experiments but were not considered in the first two papers. These additional behavioral measures include incidence and duration of aggression and olfactory investigation of rabbits by their group-mates as well as assessments of the severity of injuries.

METHODS AND MATERIALS

The rationale and general methodology for these experiments have been described in earlier papers dealing with data from all-male and all-female groups of rabbits (Hesterman and Mykytowycz, 1982a,b). Those papers also contained information on the animals and materials used in the experiments with all-male and all-female groups. Therefore the detailed information given in this section relates only to experiments with mixed-sex groups, as these are being reported here for the first time.

Animals

A total of 162 adult, wild-type rabbits, 48 males and 114 females, living in 48 separate groups were used in the mixed-sex group experiments. Of the total of 198 tests performed, 121 were carried out with groups of one male and three females and 77 with groups of one male and two females.

The animals were maintained in pens under conditions identical to those described for all-male and all-female groups.

Odor Sources and Preparation

The following seven sources of odor were used in the mixed-sex group experiments: secretions from the inguinal glands of the lowest ranking female from each group; inguinal gland secretions from unfamiliar males and

females; secretions from the chin glands of unfamiliar males; urine from unfamiliar males and females; and a commercially available perfume.

The preparation of the odor sources is described in detail in the two earlier papers of this series (Hesterman and Mykytowycz, 1982a,b). However, for completeness, a brief summary is given here.

Inguinal Gland Secretions. For tests involving "own" inguinal gland secretion, the sebum contained in the animals' inguinal pouches was collected onto a cottonwool swab and immediately rubbed into the fur on its hindquarters, flanks, and head.

Strange inguinal gland secretions were used in the form of an aqueous suspension prepared from the pooled collections of sebum from at least 20 adult males or females unfamiliar to the test animals. The suspension contained 20 mg sebum/ml and 0.5 ml was used for each test. It was stored in sealed glass ampoules at −18°C for up to three months.

Chin Gland Secretions. Pooled samples of chin gland secretions collected from at least 20 adult male rabbits unfamiliar to the test animals were used. The pooled sample was diluted with water to 8 mg chin secretion/ml and 0.5 ml of this suspension was used for each test. It was stored in sealed glass ampoules at −18°C for up to three months.

Urine. Overnight collections of urine were made from under the cages of male or female rabbits. Precautions were taken to avoid contamination of the urine with fecal pellets. The collections were pooled, thoroughly mixed, and 0.5 ml amounts used for each test. The mixed urine was stored at −18°C in sealed glass ampoules for up to three months.

Perfume. A proprietory brand cologne lotion (Old Spice, Musk for Men, Shulton Inc., Clifton, New Jersey) was employed. Approximately 0.1 ml of the undiluted lotion was used for each test.

Selection of Animals for Application of Odors

The interrelationships between individuals within mixed-sex groups are more complex than within single-sex groups. In mixed-sex groups the females form, among themselves, a clear-cut dominance hierarchy. Their relationships to males in the groups vary according to the behavioral situation but, in general, the high-ranking males play the most active role in the maintenance and protection of the group territories. In the experiments with mixed-sex groups, the male rabbit was therefore considered to be the overall dominant animal.

In line with the procedure adopted in the earlier experiments, the odorous materials were applied in all but one treatment to the lowest-ranking animal of the group, namely the subordinate female. The one exception was when inguinal gland secretion from unfamiliar females was applied to the male of each group.

All odor preparations were applied by means of a cottonwool swab and

were rubbed thoroughly into the fur of the hindquarters, flanks, and head of the selected rabbits.

Experimental Procedures

Tests were conducted in the same way as previously described for single-sex groups, and the same variables were recorded.

The odor under test was applied to the selected individual without removing it from the shelterbox and with minimum possible disturbance to the other animals. The behavior of the group was then observed for 30 min, starting from the time when any member of the group emerged from the shelter box.

The incidences and durations of the following forms of behavior for each rabbit were recorded: agonistic behavior (chasing, biting), following other rabbits, sniffing at other rabbits, mounting, grooming, and exploration.

To assess the long-term effects of the odor applications, all rabbits were examined on each of the three days following the test, and their general conditions and fresh injuries were recorded.

In the present experiments the effect of each odor was tested within 20 groups of rabbits (apart from male chin secretion which was tested in 18 groups), and the testing of any one odor was completed within a period of 10 days.

All tests were carried out during the normal breeding season of the wild rabbit in southeastern Australia (May to December). In the groups used for testing, females were in various stages of pregnancy, but young were never present.

Experiments were conducted during two different breeding seasons separated by a period of three years. Although some individuals were the same, no groups were common to both periods.

Because of the separation in time and the differences in animals and groupings used to test the various odors, one of the key treatments, namely smearing with the inguinal gland secretion from unfamiliar males, was repeated on three occasions.

The order in which odor sources were tested was as follows: July to December 1977—inguinal gland secretion from unfamiliar males, inguinal gland secretion from unfamiliar females, chin gland secretion from unfamiliar males, inguinal gland secretion from unfamiliar males applied to male of group, and inguinal gland secretion from unfamiliar males; April to May 1980—inguinal gland secretions from the subordinate female of each group, a commercial perfume, urine from unfamiliar females, urine from unfamiliar males, inguinal gland secretion from unfamiliar males.

Treatment of Data and Statistical Analysis

To provide comparable information to that already given for single-sex groups (Hesterman and Mykytowycz, 1982a,b), a similar analysis was made

of the results from mixed-sex groups using only the data on the presence or absence of aggression and injuries in the tests.

Additional data from the experiments with all three different sex groupings were analyzed using an analysis of variance. The variables considered were: (1) frequencies and durations of attacks on the smeared and unsmeared animals, (2) severity of injuries to smeared animals, and (3) frequencies and durations of olfactory investigation (i.e., sniffing) of the smeared animals.

The severity of injuries was rated on an arbitrary scale of 0–6 using the following criteria: no injury; 0; patches of fur missing, 1; one or two minor wounds, 2; three to 19 minor wounds, 3; more than 19 minor wounds or one severe wound, 4; more than one severe wound, 5; rabbit in danger of dying, removed from group, 6. Minor wounds were defined as superficial scratches or bites on the skin surface; major wounds as the loss of skin over an area of 1 cm^2 or more.

In many cases the data for particular events were zero for all groups subjected to a treatment. The analysis was therefore carried out in two stages. First the data were analyzed as a series of binary events, in which only the presence or absence of observations of a particular type of activity was considered. The "fitted values" obtained from this analysis represent the probabilities of an activity occurring for any treatment. Activities with a 'small" probability of occurring were eliminated from further analyses. The "small" probability is an estimate of the base level of a particular activity which normally occurs among the groups of rabbits. Hence only those observations which differed from the base level were retained.

Analysis of variance was then carried out on this reduced set of data with treatments as the only factor. The data related to frequencies and durations were analyzed using the transformation $\log_e$ (value + 1). From this analysis any significant effects of treatments could be detected and the mean values for each treatment could be calculated.

RESULTS

Percentage of Tests of Mixed-Sex Groups with Aggression

The percentages of tests with mixed-sex groups in which there was evidence of aggression towards the smeared animals are shown in Figure 1. Evidence of aggression was obtained either from the direct observations made during the 30 min following application of the odor source or from the appearance of injuries on the smeared animal within the three days after the treatment.

It can be seen that in the control treatments, when animals were smeared with their own inguinal secretions or with a perfume, aggression occurred in only 20% of the tests. There was a marked increase, however, in the number of individuals attacked (60%) after they were smeared with the male inguinal

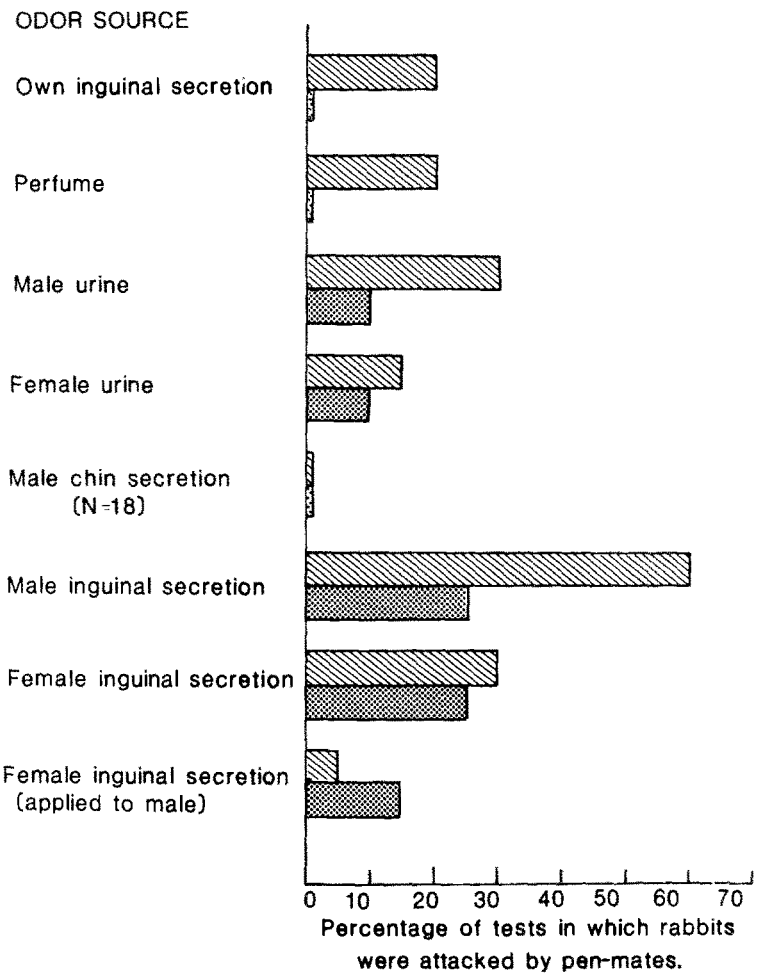

FIG. 1. The percentage of tests in which rabbits, *Oryctolagus cuniculus*, smeared with various odors were attacked by pen-mates in mixed-sex groups. Comparable data for unsmeared members of the groups are also shown. $N = 20$ for each odor treatment except where otherwise indicated. Hatched columns = smeared rabbit. Stippled columns = unsmeared rabbits. Odor sources were applied to the lowest ranking female except where otherwise indicated.

secretion and some increases (30%) after treatment with female inguinal secretion and male urine.

In all treatments in which the odors were applied to the subordinate females, aggression was directed more towards the smeared animals than towards unsmeared ones.

Incidence and Duration of Aggression

In Figures 2, 3, and 4 the mean incidence and duration of attacks on the smeared animals in the different sex groupings and treatments are presented. Attacks carried out by the dominant animal and by animals other than the dominant are indicated separately.

The results of analyses of variance are also summarized in the figures.

All-Male Groups. In all-male groups the highest mean levels of aggression against the subordinate males (approximately two incidences and 4 sec duration per test) was produced by smearing them with male inguinal secretion (Figure 2).

The levels of aggression observed were also higher after smearing with female inguinal secretion than after the control treatments with "own" inguinal secretion and perfume.

The first stage of the analysis eliminated three (own inguinal secretion, male chin secretion, and male urine) of the six treatments from further analysis, thus indicating that the levels of aggressive activities in these three treatments were very low and did not differ from the base level.

Analysis of the data from the remaining three treatments shows that there is a significant difference between treatments for total incidence ($P < 0.01$) and total duration ($P < 0.025$) of aggression. Differences between treatments for the separate aggressive activities of the dominant and nondominant animals are not significant, although there is an indication that the nondominant rabbits were more aggressive towards the subordinate animals smeared with male and female inguinal secretions.

Analysis of the data for aggression against animals other than the smeared rabbit shows a significant difference ($P < 0.05$) between treatments, the levels being highest for treatments with male and female inguinal secretions.

All-Female Groups. It is evident from Figure 3 that treatment with female inguinal secretion greatly increased aggression towards the subordinate females and that this was mainly due to aggression by the dominant females. Thus after treatment with "own" inguinal secretion, the mean levels of aggression were less than one incidence and 1 sec duration per test but rose to a total of over 10 incidences and 16 sec duration per test when the subordinate females were smeared with female inguinal secretion. Of these totals,

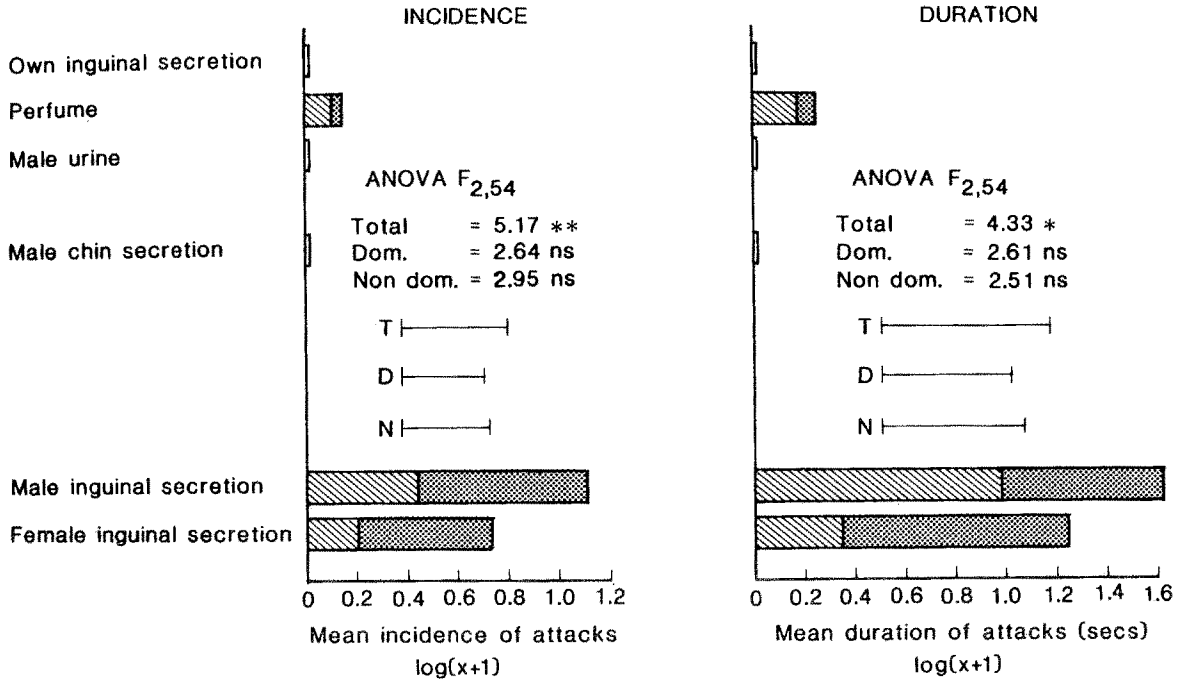

FIG. 2. Mean incidence and duration of attacks by pen-mates on rabbits, *Oryctolagus cuniculus*, smeared with various odors in all-male groups. The results of analysis of variance (ANOVA) are shown. The standard errors of the differences are presented in bar form separately for total (T), dominant rabbits (D), and nondominant rabbits (N). $N = 20$ for each odor treatment. Hatched columns = attacks by dominant rabbit. Stippled columns = attacks by nondominant rabbit. NS = not significant; $*P < 0.05$; $**P < 0.01$.

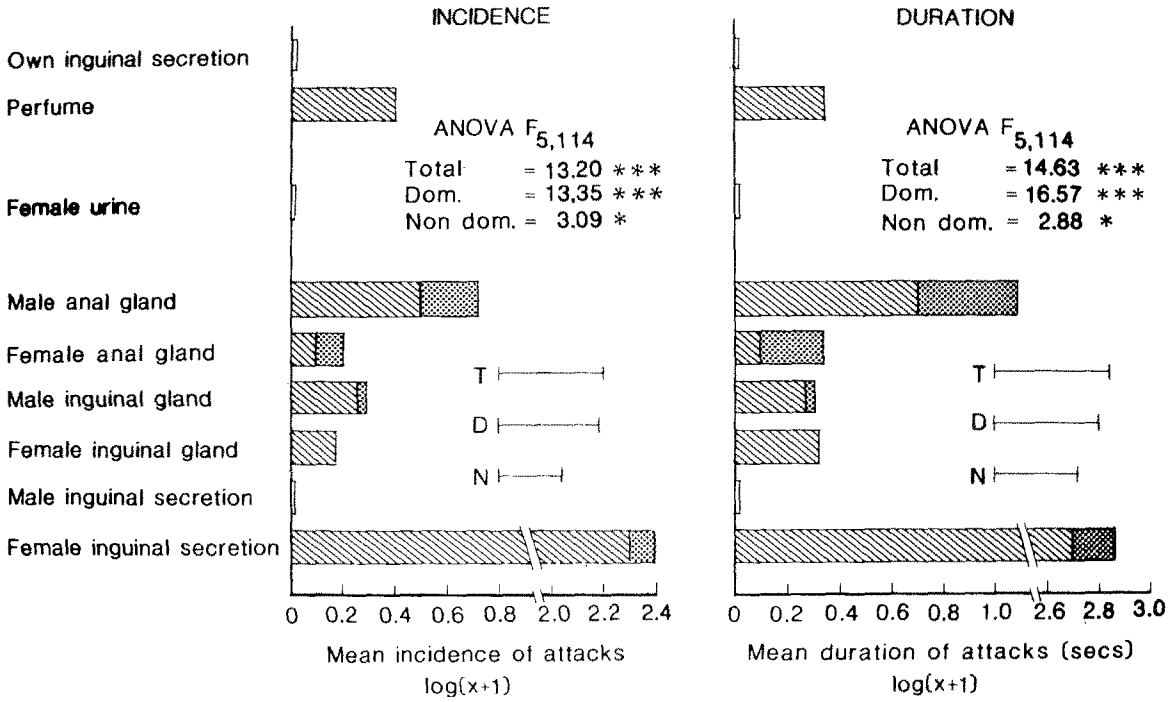

FIG. 3. Mean incidence and duration of attacks by pen-mates on rabbits, *Oryctolagus cuniculus*, smeared with various odors in all-female groups. The results of analyses of variance (ANOVA) are shown. The standard errors of the differences are presented in bar form separately for total (T), dominant rabbits (D), and nondominant rabbits (N). $N = 20$ for each odor treatment. Hatched columns = attacks by dominant rabbit. Stippled columns = attacks by nondominant rabbits. NS = not significant; $*P < 0.05$; $***P < 0.001$.

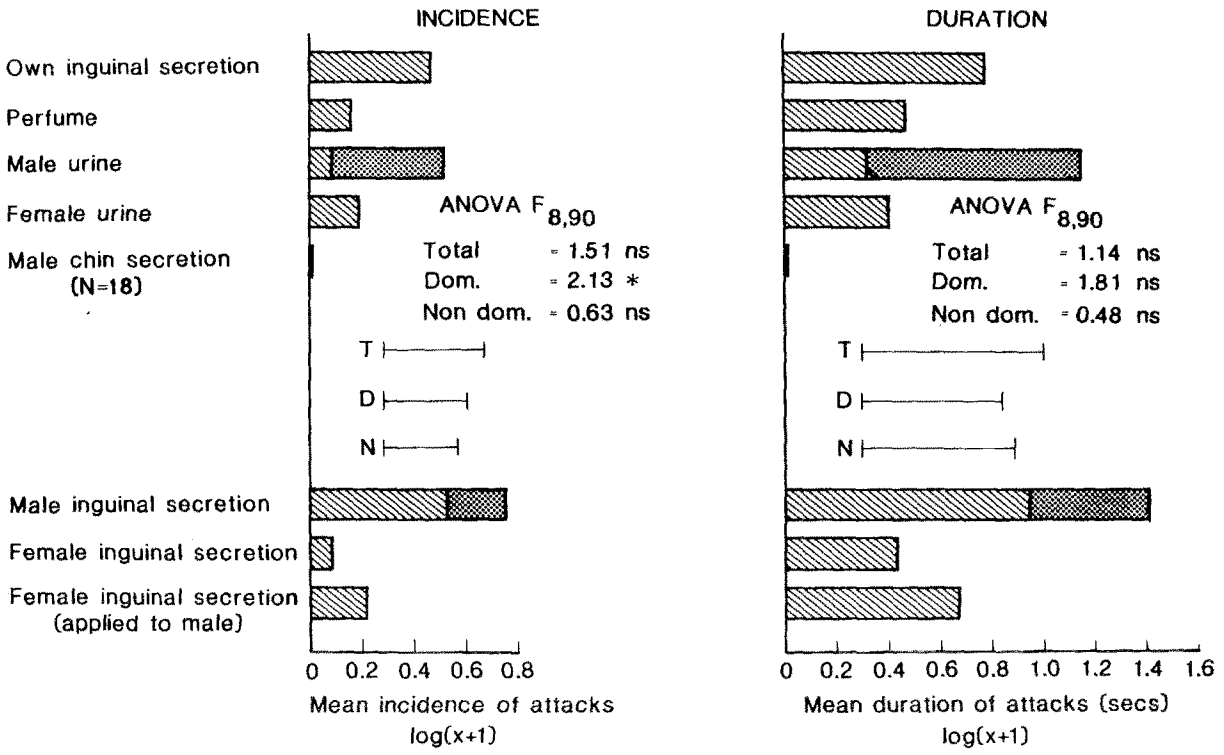

FIG. 4. Mean incidences and durations of attacks by pen-mates on rabbits, *Oryctolagus cuniculus*, smeared with various odors in mixed-sex groups. The results of analysis of variance (ANOVA) are shown. The standard errors of the differences are presented in bar form separately for total (T), dominant rabbits (D), and nondominant rabbits (N). $N = 20$ for each odor treatment except where otherwise indicated. Hatched columns = attacks by dominant rabbit. Stippled columns = attacks by nondominant rabbits. NS = not significant. $*P < 0.05$.

about nine incidences and 13 sec duration were due to aggression displayed by the dominant females.

There are highly significant differences ($P < 0.001$) between treatments for the incidences and durations of aggression towards the smeared rabbits by the dominant females. The levels of aggressive activity displayed by the nondominant females are also just significantly different ($P = 0.05$) between treatments.

Aggression directed towards the unsmeared animals does not vary significantly between treatments.

Mixed-Sex Groups. It can be seen from Figure 4 that the levels of aggression in the different treatments fluctuated less in the mixed-sex groups of rabbits than in the all-male and all-female groups. The only noticeable increase in aggression occurred in tests involving treatment with male inguinal secretion, and this was due mainly to aggression displayed by the males of the groups.

In the analyses of variance of data from the mixed-sex groups, the three replicates of treatment with male inguinal secretion were considered separately, while treatment with male chin secretion was excluded since no aggressive activity was observed. Differences between treatments in the levels of aggression towards the subordinate female are significant only for the incidences of aggression by the males ($P < 0.05$). Durations of aggression by the males also approaches significance ($P = 0.07$).

The results from the three replicates of treatment with male inguinal secretion were compared using standard errors obtained from an analysis including all three treatments. No significant differences were found between the three sets of data for incidence or duration of aggressive activity.

The levels of aggression against individuals other than the smeared rabbit also differ significantly ($P < 0.05$) between treatments due mainly to an increase in the treatment with male inguinal secretion.

Injuries

The mean assessment scores indicating the severity of the injuries sustained by the smeared rabbits are presented in Table 1, which also shows the results of statistical analyses.

All-Male Groups. The severity of injuries was uniformly low for all treatments involving all-male groups. There are no significant differences between treatments in this respect even though there was a clear increase in aggressive activity towards the subordinate male after treatment with male inguinal secretion and to lesser extent with female inguinal secretion and male chin secretion.

All-Female Groups. In all-female groups there is a highly significant difference between treatments ($P < 0.001$). This was due to a large increase

TABLE 1. MEAN SCORES USING ARBITRARY SCALE OF 0–6 FOR SEVERITY OF INJURIES INFLICTED BY PEN-MATES ON RABBITS *Oryctolagus cuniculus*, SMEARED WITH VARIOUS UNFAMILIAR ODORS

Odor source	All-male groups	All-female groups	Mixed-sex groups
Own inguinal secretion	0	0	0.10
Perfume	0.25	0	0
Male urine	0	—	0.70
Female urine	—	0	0
Male chin secretion	0	—	0 ($N = 18$)
Male anal gland	—	0.60	—
Female anal gland	—	0	—
Male inguinal gland	—	0	—
Female inguinal gland	—	0.05	—
Male inguinal secretion	0.15	0	2.23
Female inguinal secretion	0.15	2.15	0
Female inguinal secretion (applied to male)	—	—	0
ANOVA	χ^2_4 = 0.001 NS	χ^2_5 = 132.89***	χ^2_5 = 78.6***

$N = 20$ for each odor treatment except where otherwise indicated, NS = not significant; ***$P <$ 0.001. See description of arbitrary scale in text.

in the severity of injuries sustained by animals smeared with female inguinal secretion.

Mixed-Sex Groups. In the mixed-sex groups there was a marked increase in the severity of injuries to females smeared with male inguinal secretion. The overall difference between treatments is highly significant ($P < 0.001$). This is particularly interesting in view of the lower levels of directly observed aggressive activity in these groups compared to the single-sex groups.

Incidence and Duration of Olfactory Investigation

The treatment means for the incidence and duration of olfactory investigations are presented in Table 2. There are no significant differences between treatments in the incidences or durations of olfactory investigations of the smeared animals within any of the sex groupings.

The females of the mixed-sex groups showed some evidence of an increased olfactory interest in the males when they were smeared with inguinal secretion from unfamiliar females, although this is not significantly higher than in other treatments ($P = 0.07$).

DISCUSSION

Earlier studies confirmed the territorial function of the odors derived from the anal and chin glands, and in the same experimental situation, the

TABLE 2. MEAN INCIDENCES AND DURATIONS OF OLFACTORY INVESTIGATIONS BY PEN-MATES OF RABBITS, *Oryctolagus cuniculus* SMEARED WITH VARIOUS ODORS

	All-male groups		All-female groups		Mixed-sex groups	
Odor source	Incidence	Duration (sec)	Incidence	Duration (sec)	Incidence	Duration (sec)
Own inguinal secretion	0.15	0.37	0.72	1.06	0	0
Perfume	0.38	0.80	0.69	1.19	0	0
Male urine	0.33	0.67	—	—	0	0
Female urine	—	—	0.47	0.99	0	0
Male chin secretion	0	0	—	—	0 ($N = 18$)	0
Male anal gland	—	—	0	0	—	—
Female anal gland	—	—	0.45	0.82	—	—
Male inguinal gland	—	—	0.28	0.50	—	—
Female inguinal gland	—	—	0.84	1.35	—	—
Male inguinal secretion	0.67	1.45	0.48	0.80	0.27	0.53
Female inguinal secretion	0.82	1.53	0.53	0.55	0	0
Female inguinal secretion (applied to male)	—	—	—	—	0.46	1.13
SED	0.29	0.58	0.39	0.61	0.26	0.60
ANOVA	NS	NS	NS	NS	NS	NS

[a]The values are $\log_e (x + 1)$ transformations. Twenty groups were tested in each treatment except where otherwise indicated. NS = not significant.

odor from the inguinal glands was found to have only a slight effect on the territorial confidence of rabbits (Mykytowycz et al., 1976). Those results reinforced speculation that the secretion from the inguinal gland is primarily involved in the identification of individuals. This idea was further supported not only by the observations that there are individual differences in the chemical composition of inguinal sebum (Goodrich and Mykytowycz, 1972), but also by the fact that the application of strange inguinal secretions to rabbit kittens confused their own mothers as to their identity (Mykytowycz and Goodrich, 1974).

The results of the studies reported in the present and previous papers (Hesterman and Mykytowycz 1982a,b) clearly indicate that the odor derived from the inguinal gland carries information that affects the acceptance of individuals by other members of their own social groups. In all three types

of groups used in the experiments (all-male, all-female, and mixed-sex) there was a marked increase in aggression towards the subordinates when they were smeared with inguinal secretion collected from rabbits unfamiliar to the group members. In contrast, the attitude of group members towards the subordinates was unchanged or only marginally affected when they were smeared with other unfamiliar rabbit odors—chin gland secretion and urine and anal gland material.

It was emphasized in an earlier paper of this series (Hesterman and Mykytowycz, 1982a) that it cannot be determined from the results of these studies whether the information carried by the odor of a rabbit's inguinal secretion identifies it as an individual or merely as a group member. However, considering their complex social interrelationships, it would seem essential for rabbits to be able to identify individuals within their own social milieu, and it appears likely that the inguinal odor would be the source of such information.

In comparing the effectiveness of unfamiliar odors from the different sources in stimulating aggression against the animals smeared with them, it is assumed that "equivalent" amounts of the odors were used. The quantities of chin secretion and urine used in the experiments were based on the amounts naturally present on or produced by an individual rabbit. However, in the case of the macerated anal and inguinal gland materials used in the all-female group experiments, the choice of 25-mg amounts was purely arbitrary since we have no knowledge of the amount of secretion present in the glands.

The comparison of "naturally occurring amounts" of odor sources seems to be a useful approach at this stage, when the identity of the behaviorally active components of the odors is unknown. Indeed, it can be argued that this is a more valid basis for comparing the effectiveness of the different odors than a measure of physical concentration which may have no relation to "odor intensity" in terms of the rabbits' olfactory perceptions.

In all-male and mixed-sex groups there was no indication that treatment with male chin secretion increased the level of aggression against the subordinate animals. Females produce very little chin secretion and it was not practicable to collect enough of it for testing. This fact itself suggests that chin secretion is of little importance in forming the individual odor of female rabbits.

Although in mixed-sex groups treatment with male urine resulted in a slight increase in aggression and in the severity of injuries to the smeared animals, it had no effect in all-male groups. Smearing with female urine did not result in any increase in aggression in either all-female or mixed-sex groups.

On balance, the evidence suggests that the odors of a rabbit's chin secretion or of urine are not used by its group mates as a primary means to identify it, although there are reasons to suppose that they can be used as secondary sources of such information (Bell, 1980).

It has been shown (Mykytowycz et al., 1976; Hesterman et al., 1981; Goodrich et al., 1981) that rabbits can recognize their own anal and chin gland secretions and urine, but these odors are used primarily as cues in a different behavioral context, that is, to signify the presence or field of influence of an individual.

Although statistically significant reactions were recorded from the use of the unfamiliar inguinal gland secretions in all sex groupings, there were some differences between them. Since the same standard odors were used throughout all the series of experiments, it is obvious that the same odor stimulus released different responses in different social groupings. It is to be expected that totally different responses will be elicited by the same message, depending on the social context in which it is received and the status of the receiver.

In mixed-sex groups the reaction towards the smeared individuals was shown mainly by the male. The dominant female did not attack. This observation confirms once more that the dominant male is the one mainly concerned with the preservation of the existing order in a social group.

In fact, a dominant male will often intervene to prevent other members of its group from fighting (Mykytowycz and Dudziński, 1972). This is why the levels of aggression against unsmeared animals increased in the mixed-sex groups. The dominant males would not allow the females to attack the smeared animals. In all-male groups there was also a significant increase in aggression against unsmeared animals, and this again was due to the dominant males intervening when the second-rankers attacked the smeared animals.

In all-female groups the dominant females assumed the role of leader in the absence of a male. This they did even more forcefully than the bucks. In these tests the incidence and severity of aggression rose very sharply in response to unfamiliar female inguinal secretion, and it was interesting to see that aggression was more intense in all-female groups than in all-male groups.

The data on olfactory investigations carried out by rabbits in all-female and mixed-sex groups did not suggest the existence of any differences between the various odor sources in their significance for the rabbits. Even where levels of aggression were very high, sniffing rates do not increase in parallel. It seems that if the odor carries important information which can be acted upon immediately, the rabbits will not prolong or repeat the examination.

Olfactory investigation increased when female inguinal secretion was applied to the male in mixed-sex groups. This suggests that such manipulation was perhaps more confusing and required more time for the females to evaluate, possibly with the aid of information from other sources.

Many species of mammals can discriminate between the odors of individual conspecifics (Halpin, 1980). This ability has been demonstrated experimentally in, for example, the house mouse, *Mus musculus* (Bowers and

Alexander, 1967), the gerbil, *Meriones unguiculatus* (Halpin, 1976), the mongoose, *Herpestes auropunctatus* (Gorman, 1976) and the lemur, *Lemur fulvus* (Harrington, 1976) to name but a few. It has to be considered, however, that under natural conditions clues obtained through other sensory modalities could be used to identify individuals. The relative importance of the different sources of information undoubtedly varies from species to species, and from one behavioral situation to another.

In the experiments reported in this series of papers, no attempt was made to prevent the rabbits from receiving visual, acoustic, or tactile signals from their pen-mates. Thus, the ability of the unfamiliar odor to mask, at least temporarily, the smeared animal's true identity emphasizes even more the importance to the rabbit of information obtained through olfactory signals.

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TRIMERIZATION OF *Earias insulana*[1] SEX PHEROMONE, (*E,E*)-10,12-Hexadecadienal, A PHENOMENON AFFECTING TRAPPING EFFICIENCY[2]

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Abstract—The sex pheromone of *Earias insulana*, (*E,E*)-10,12-hexadecadienal, may trimerize extensively to form a crystalline trioxane derivative. The structure of the trimer was deduced from its CI-MS and NMR spectra. Capillary GC analysis resulted in the thermal decomposition of the trimer to the monomer. This process could be studied on a 2-m packed column under specific conditions. A convenient separation between the pheromone and its trimer was achieved by TLC. The trimer was inactive in the field, and it has a harmful effect on the performance of the polyethylene dispenser. Material which contains large amounts of the trimer is unsuitable for field use, even if applied at high dosage. The pheromone should be analyzed by NMR or TLC in addition to GC in order to detect the presence of its trimer. The trimerization process is catalyzed by acid which should therefore be completely eliminated from the storing vessels.

Key Words. *Earias insulana*, spiny bollworm, Lepidoptera, Noctuidae, sex pheromone, (*E,E*)-10,12-hexadecadienal, trimerization, chromatographic analysis, nuclear magnetic resonance, mass spectrometry.

INTRODUCTION

The sex pheromone of the spiny bollworm *Earias insulana* (Boisduval) has been identified as (*E,E*)-10,12-hexadecadienal (*E*10,*E*12-16:Ald) (Hall et al., 1980). The compound has been synthesized in England (Hall et al., 1980)

[1]Boisduval, Lepidoptera: Noctuidae.
[2]Contribution from the Agricultural Research Organization (ARO), No. 675-E, 1983 series.

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and in Israel (Klug et al., 1982). Field tests have shown that funnel traps baited with 2 mg of the synthetic pheromone absorbed into polyethylene vials captured *E. insulana* males effectively (Kehat et al., 1981a). The sensitive pheromone was protected from oxidation by the addition of 10% 2,6-di-*tert*-butyl-4-methylphenol (BHT). Tests in cotton fields in Israel during 1979–1981 gave satisfactory results, and the sex pheromone traps were proposed as a potential means of improving control programs for *E. insulana* (Kehat et al., 1981b). Surprisingly, the pheromone prepared for the 1982 season in Israel and a small sample obtained from England gave erratic and significantly lower male catches than in previous years. Ultimately it was found that these samples trimerized extensively. This paper describes this phenomenon, the analytical procedures for detecting the trimer in *E*10,*E*12–16:Ald before the pheromone is used in the field, and recommendations for preventing this undesirable process.

METHODS AND MATERIALS

Pheromones. Samples A and B were prepared in the Chemistry Department of Ben-Gurion University in Beer Sheva in 1982 (Klug et al., 1982). Samples C and D were received from the Tropical Products Institute, London, England, in 1981 and 1980, respectively (Hall et al., 1980). Sample D was used throughout the 1981 season.

Gas Chromatography (GC). A Varian 3700 chromatograph with flame ionization was used for capillary (splitless mode) and packed column analysis. The conditions for WCOT SP 2100 capillary column (60 m × 0.25 mm ID) were: injector and detector temperature, 250°; injection at column temperature of 100°, purge 45 sec, and programing at 30°/min, 3 min after injection to 200° and then isothermal; helium flow, 1 ml/min. Conditions for the packed 3% OV-1, on Supelcoport 100–120 mesh, glass column (2 m × 2 mm) were: injector temperatures 200°, 250°, and 300°, column at 180° and detector at 250°; He pressure of 30 psi. Injection was directly onto column.

Thin-Layer Chromatography (TLC). Merck (DC-Alufolien, 20 × 20 cm) plates, Kieselgel 60 F_{254}, 0.2 mm thickness, were used with hexane + ethyl acetate 9:1 as eluent, and BHT was used as internal standard. Spots were visualized with iodine vapors.

Nuclear Magnetic Resonance (NMR). Spectra were recorded on a Varian EM-360 spectrometer as $CDCl_3$ solutions with TMS as internal standard. They are reported in δ units.

Mass Spectrometry (MS). The CI-MS analysis was performed on a Finnigan 4021 combination using isobutane as the reagent gas. The sample was introduced into the source via the solid probe at 250°.

Trimerization Experiments. Freshly prepared pheromone samples (20 mg), either neat or as 10% hexane solutions, were exposed to the following

conditions: (1) kept in clean untreated vials; (2) kept in vials which were washed with ethanolic KOH and then with distilled water; (3) BHT was added; (4) *p*-toluenesulfonic acid was added. The samples were kept at room temperature or in the refrigerator for three weeks, and the trimerization process was monitored by TLC.

Field Tests. Field tests were conducted in a cotton field in the Bet Shean Valley. The different pheromone samples were dispensed in polyethylene vials (30 × 16 mm, with 1.5-mm-thick walls), as rubber septa are not suitable for this pheromone. The vials were loaded with 2 mg of pheromone containing 10% BHT in 500 μl of hexane and the tops closed after evaporation of the solvent. The trap used consisted of a plastic container with a plastic funnel (13 cm diam) fixed on its top. The funnel was dusted with talcum powder to make the surface more slippery. A plastic roof was fixed 5 cm above the funnel and the pheromone dispenser was hung below the center of the ceiling. A small piece of plastic impregnated with the insecticide-dichlorovinyl phosphate (DDVP) was placed in each container to kill the trapped males. The traps were fixed at approximately the same height as the cotton plants. Four traps were used for each treatment; adjacent traps were located at least 30 m apart. Captured moths were collected every 2–3 days. Positional bias was reduced by moving the traps one position every 2–3 days. Results were analyzed by Duncan's new multiple-range test for significance at the 0.05 level.

RESULTS AND DISCUSSION

The *E. insulana* pheromone *E*10,*E*12–16:Ald was usually kept in the refrigerator either as neat material or in hexane solution with BHT. Although the material was analyzed at the end of the synthesis by GC and NMR, it was always checked by capillary GC before preparation for field use. Pheromone preparations with a purity greater than 95% gave satisfactory trap catch during the years 1979–1981.

A batch of 15 g, of sample A was prepared in 1982 for monitoring purposes in cotton fields in Israel. This material did not melt at room temperature as did previous samples (1979–1981) after removal from cooling apparatus. A routine capillary GC analysis showed a content of *E*10,*E*12–16:Ald above 98%, and therefore the higher melting point of the material was attributed to the greater purity. When this material was used in the field, the trap catch of *E. insulana* males was drastically lower than in the previous season. Somewhat higher catches were obtained with sample B (batch of 5 g) which was subsequently prepared for this season. Sample C attracted almost no males. The three pheromone samples were compared with pheromone sample D, which was used successfully during the 1981 season.

The results of this comparative experiment are presented in Table 1. It

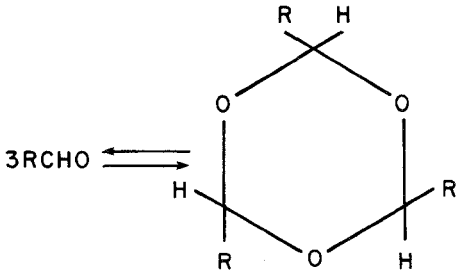
TABLE 1. NUMBER OF *Earias insulana* MALES TRAPPED BY DIFFERENT SAMPLES OF *E*10,*E*12-16:ALD, CONTAINING VARYING AMOUNTS OF TRIMER

Sample	Trimer (%)	*X* males/trap/night[a]
A	80	0.40 bc[b]
B	50	0.96 b
C	>95	0.17 c
D	<5	2.70 a

[a]Four traps were used, each baited with 2 mg of the material tested, during 20 nights (September 9-29, 1982).
[b]Numbers followed by the same letter are not significantly different at $P = 0.05$ (Duncan's new multiple-range test).

is evident that the 1980 pheromone (sample D) was superior to the new samples. The discrepancy between the capillary GC analysis and the field results led to the conclusion that the capillary GC might be erroneous. When the NMR spectrum of the low-catching sample A was analyzed, it was found that the pheromone content of this sample was surprisingly low, only 20% of the total material. The ratio of the aldehydic proton at δ 9.75 to the vinylic protons at δ 6.15-5.15 was 1:20 instead of the expected 1:4 ratio in *E*10,*E*12-16:Ald. In addition there was a new broad triplet at δ 4.75. The NMR spectrum of sample B was similar but the *E*10,*E*12-16:Ald content was about 50%, where sample C contained less than 5% of *E*10,*E*12-16:Ald. In this sample the ratio of the vinylic protons to the new signal at δ 4.75 was close to 4:1. The NMR spectrum of Sample D was consistent with the structure of *E*10,*E*12-16:Ald (1:4 ratio of aldehydic to vinylic protons), as expected from its high field activity. The NMR spectra of samples A, B and, in particular, C, showed that these batches contained a new material which decomposed to *E*10,*E*12-16:Ald in the capillary GC system. The chemical shift of the new triplet at δ 4.75 indicated protons α,α to oxygen. The high field section of the spectrum was very similar to that of *E*10,*E*12-16:Ald. The CI-MS spectrum of sample C (mp 54-55° after recrystallization from hexane) showed that the new compound is a trimer of *E*10,*E*12-16:Ald, with the highest mass at *m/e* 709 (6%) $M^+ + 1$ and a peak at *m/e* 473 (25%) 2/3 $M^+ + 1$ and *m/e* 237 (100%) 1/3 $M^+ + 1$ as base peak.

The structure of the trimer (Figure 1) was deduced by comparison of its NMR spectrum with the published NMR spectra of the trimers of acetaldehyde and priopionaldehyde. Both these aldehydes trimerize to stable trioxanes with a *cis-cis* geometry. The chemical shift of the ring protons is δ 4.92 for trimethyl-trioxane (paracetaldehyde) and δ 4.68 for triethyl-trioxane (parapropionaldehyde) (Jungnickel and Reilly, 1965), which is in excellent agreement with the chemical shift of the ring protons in the trimer of



$R \equiv CH_3CH_2CH_2CH=\underline{CH}CH=\underline{CH}(CH_2)_8$

FIG. 1. Trimerization of the *Earias insulana* sex pheromone (*E*,*E*)-10,12-hexadecadienal.

*E*10,*E*12–16:Ald at δ 4.75, thus showing that the *E*10,*E*12–16:Ald trimer has a trioxane structure.

The formation of trioxane derivatives from aldehydic pheromones has not yet been reported to the best of our knowledge. The main problem with these pheromones has always been their susceptibility to oxidation. The trimerization of simple aliphatic aldehydes was described many years ago (Franke and Wozelka, 1912), and it is known that this process is catalyzed, at room temperature, by acidic reagents such as mineral acids, halogens, and metal halides (Buchanan et al., 1965). The trimers can be decomposed to the corresponding monomers by distillation with a trace of acid or by heating which also causes extensive polymerization (Franke and Wozelka, 1912). This type of conversion occurred with the *E*10,*E*12–16:Ald trimer in the capillary GC system, and therefore analysis of the trimer (Sample C) or mixtures (samples A and B) gave only sharp peaks corresponding to *E*10,*E*12--16:Ald. To overcome this difficulty, the samples were submitted to GC analysis on a 2-m packed column with varying injector temperatures in the hope of detecting both *E*10,*E*12–16:Ald. and its trimer and of studying the detrimerization process in the gas chromatograph (Figure 2). At a constant column temperature of 180°, injection of 0.5 μg of sample A at 250° (injector) gave a sharp peak of *E*10,*E*12–16:Ald. followed by a barely separated broad peak. In some cases, this peak appeared only as a shoulder on the pheromone peak. A plausible explanation for this phemonenon is a slow decomposition of the trimer in the injector block at 250°. The formed monomeric *E*10,*E*12–16:Ald enters the column over a time period producing the broad peak. Injection at 200° of the same amount of material gave only a small peak of *E*10,*E*12–16:Ald due to nonvolatility of the trimer at this

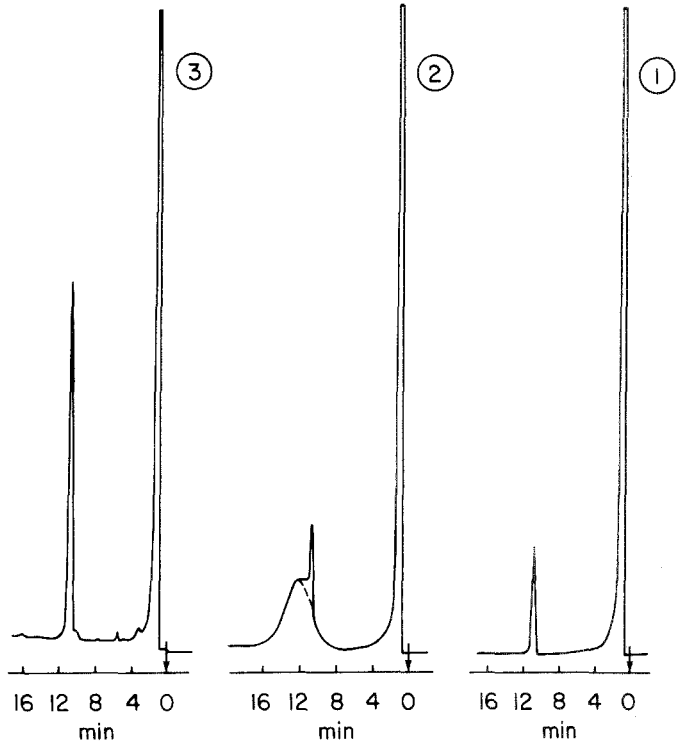


FIG. 2. Chromatograms of a mixture of 20% (*E,E*)-10,12-hexadecadienal and 80% of its trimer on a 2-m, 3% OV-1 column at 180°: (1) injector at 200°; (2) injector at 250°; (3) injector at 300°.

injector temperature. On the other hand, the same injection at 300° resulted in a large peak corresponding to *E*10,*E*12-16:Ald (Figure 2). The last injection shows clearly that the trimer undergoes a thermal detrimerization under the appropriate conditions in the injection block. This type of analysis can now be applied to detect possible trimer formation of *E*10,*E*12-16:Ald. The difference in the peak area obtained from injections at 300° and 200° (injector) gives the approximate amount of trimer in the tested sample.

Another very simple separation of *E*10,*E*12-16:Ald and its trimer was achieved by TLC on commercial Kieselgel plates. Elution with a mixture of hexane and ethyl acetate (9:1) gave excellent separation, with an R_f of 0.66 and 1.23 for *E*10,*E*12-16:Ald and its trimer, respectively, relative to BHT. The trimer has a larger R_f because it is much less polar than the monomer. The advantage of the TLC analysis is its simplicity and mild conditions.

After the simple TLC separation was developed, it was easy to check several old samples from previous years for trimer content. Most samples

contained small amounts of trimer, in the range of 0–10%. These small amounts could not be detected in field tests because an excess of pheromone was always used (Kehat et al., 1981a). However, the variability in trap catches in previous years may be attributed also to varying amounts of the trimer, even if they were low.

The presence of the trimer may also have a damaging effect on the performance of the polyethylene dispenser. Thus, trap catches with samples A and B (Table 1) were similarly low, although A contained 20% pheromone whereas B contained 50% pheromone. When the total amount of sample A per dispenser was enhanced fivefold, the trap catch did not increase as expected but even decreased slightly (Table 2). The harmful effect of the trimer could be due to crystallization in the pores of the polyethylene vial, thus changing the matrix properties of the dispenser.

A preliminary study was made to simulate conditions which could enhance or prevent trimerization of *E*10,*E*12–16:Ald. Thus, addition of *p*-toluenesulfonic acid to the pheromone resulted in trimerization, in some cases even in the refrigerator. The process was faster with the neat pheromone as compared with the hexane solutions. Addition of BHT had no influence on the trimerization process, although it prevented, as expected, the formation of polar oxidation products. Some samples trimerized very slowly, at room temperature, even when kept in untreated new vials without the presence of acid. To eliminate any possible effect of traces of acid, storage vessels should be base-washed. It seems that additional factors, except external acid, might be involved in the trimerization of *E*10,*E*12–16:Ald. One of them could originate from the oxidizing agent pyridinium chlorochromate (Hall et al., 1980; Klug et al., 1982), which is mildly acidic, or from its decomposition products. Accordingly, it will be useful to purify the crude reaction product either by washing with mild base, or by column chromatography prior to distillation.

A check of several additional aldehydic pheromones revealed the pres-

TABLE 2. NUMBER OF *Earias insulana* MALES TRAPPED WITH DIFFERENT AMOUNTS OF PHEROMONE SAMPLE A

Sample	Trimer (%)	Amount (mg)	X males/trap/night[a]
A	80	2	0.60 b[b]
A	80	10	0.37 b
D	>5	2	3.14 a

[a]Four traps were used for each treatment during 14 nights (September 15–29, 1982).
[b]Numbers followed by the same letter are not significantly different at $P = 0.05$ (Duncan's new multiple-range test).

ence of the corresponding trimers in some of them. These findings suggest that this undesirable process should be considered in other aldehydic pheromones before their use in the field.

CONCLUSIONS

In order to avoid difficulties in future field work, the *E. insulana* pheromone (*E*10,*E*12-16:Ald) must be checked for trimer presence by NMR or TLC in addition to GC analysis, before use in the field. Material which contains large quantities of trimer is unsuitable for use, even when applied at high dosage, probably due to the effect of the trimer on the polyethylene dispensers used for the *E. insulana* pheromone.

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CONTACT SEX PHEROMONE IN THE TSETSE FLY *Glossina pallidipes* (Austen) Identification and Synthesis

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Abstract—Adult male *G. pallidipes* attempted to copulate with decoys treated with a branched paraffin obtained from laboratory-reared female flies. The compound causing maximal response was isolated and identified as 13,23-dimethylpentatriacontane. The synthesized compound elicited increasing responses with increasing doses. This sex- and species-specific compound was always present in physiological amounts in females, as it increased from 2 μg at emergence to 10 μg per female at 14 days. It was present in wild-caught females from a wide geographical range.

Key Words—*Glossina, pallidipes*, tsetse fly, Diptera, Muscidae, pheromone, contact stimulant, branched alkane, 13,23- dimethylpentatriacontane.

INTRODUCTION

Glossina pallidipes Austen is sympatric with *G. morsitans morsitans* Westwood across large areas of eastern and central southern Africa. Both may feed on the same host animal, and both are important vectors of trypanosomiasis. Males of *G. morsitans* attempt to copulate with artificial or natural decoys treated with natural (Langley et al., 1975) or synthetic 15,19,23-trimethylheptatriacontane, while much weaker responses were seen to two

dimethyl homologs, 15,19- and 13,17-dimethylheptatriacontane (Carlson et al., 1978). The trimethylheptatriacontane released copulatory attempts from wild male *G. m. morsitans* visually attracted to decoys in the field (Langley et al., 1981). Some interspecific activity was seen, in that male *G. pallidipes* responded to decoys treated with 100 μg of the trimethyl alkane in the laboratory, although this dose is too high to be considered biologically meaningful (Huyton et al., 1980).

The presence of a sex stimulant pheromone in *G. pallidipes* was demonstrated in bioassays of males with live or dead females and female materials, including surface lipid extracts, total hydrocarbons, total paraffins, and the major 35-carbon paraffin isolated from mature females (Langley et al., 1982a; McDowell et al., 1981). In a preliminary report, we identified this material as 13,23-dimethylpentatriacontane and reported on the activity of the synthesized compound (Carlson et al., 1981). Copulatory responses were released in *G. pallidipes* males by newly emerged females, either live or killed by freezing, showing that a physiologically active quantity of sexual stimulant was present in very young females. Behavioral maturation, rather than development of cuticular stimulants, was thus considered responsible for the observation that females were most receptive at 9 days of age (Langley et al., 1982a).

The presence of female-produced sex stimulant pheromones has been demonstrated in a third species, *G. palpalis palpalis* Rob-Des. (Offor et al., 1981), and implied in a fourth, *G. austeni* Newstead (Huyton et al., 1980).

We report the analysis of cuticular paraffins from female *G. pallidipes*, and synthesis and bioassays of several potential pheromones that release sexual behavior in the male on contact.

METHODS AND MATERIALS

Wild flies for analysis were obtained as dried intact specimens shipped in capped vials (Tables 3 and 4). Larger samples were obtained as concentrates of crude ether or hexane extracts of freshly caught and chilled wild flies obtained from CO_2 plus acetone-baited traps operated in the Zambezi River Valley of Zimbabwe (Vale, 1982) (Table 2). Laboratory flies were aged for extraction in rearing cages under conditions described previously (Tables 1 and 4) (Langley et al., 1982a). Lipids from each sample were prepared for analysis by liquid chromatography on silica gel, then argentation liquid and thin-layer chromatography to obtain active paraffins (Carlson et al., 1978) for further analysis by gas chromatography (GC) and gas chromatography–mass spectrometry (GC-MS).

Gas–liquid chromatography (GC) was performed on a Varian model 2100 GC using a glass column (1.8 m $\times$ 2 mm ID) packed with 3% OV-1 on 120–140 mesh Chromosorb W AWDMCS, with flame ionization detector,

and a Hewlett-Packard model 3380A recording integrator. Operating conditions were: injector, 345°; detector, 360°; helium carrier gas, 24 ml/min; and the oven was temperature programed from 150 to 325° at 6°/min. Each peak in samples of individual and pooled flies was quantitated and percentage composition determined. Each sample was reinjected on a 12 m or 25 m × 0.3 mm ID fused silica column (DB-1, J & W) together with *n*-paraffin standards of 22–40 carbons (Analabs, Inc.) and temperature programmed to 300° for determination of Kovats' indices (KI) (Kovats, 1965). Afterward, an internal standard *n*-alkane of 35 carbons was added for quantitations that were not adjusted further. Compounds were separated for bioassay by preparative GC, using a 99:1 splitter in the Varian 2100 and substituting a glass column (1.7 m × 3.2 mm ID) packed with 3% OV-1. Mass spectra were obtained with a Varian/MAT SS-200 data system and a MAT 112S electron impact mass spectrometer coupled via a jet separator to a glass column (3 m × 3.2 mm ID) packed with 3.5% OV-101 on 100–120 mesh Gas-Chrom Q. The carrier gas was helium, and the oven temperature was programed from 180 to 324° at 2°/min. The mass spectra were interpreted as previously described (Nelson, 1978; Nelson et al., 1980, 1981; Pomonis et al., 1980).

For bioassays, *G. pallidipes* or their offspring were obtained from puparia collected in the Zambezi Valley of Zimbabwe that had emerged at Bristol, England (Tables 4 and 5). Nine- to 12-day-old males from Bristol (Tables 4–6), 0- to 19-day-old males from puparia collected originally in Uganda that emerged in Amsterdam (Table 7), or flies from Amsterdam that were maintained in Bristol (Table 8) were used. Decoy objects were either *G. pallidipes* females, *G. morsitans* females, 7- to 8-day-old males freshly killed by freezing then washed with solvent, or rectangular prisms of cork (9 × 3 × 3 mm) mounted on corks. A single test male was brought into physical contact with a decoy and its response scored as 0, 1, 2, or 3 according to the vigor of its response, with 3 being the maximal copulatory response involving curving of the abdomen and engagement of the hypopygium with the decoy. Scores were added because the highest possible score ($3n = 100\%$) is the same as the score for an active female fly (Carlson et al., 1978). Decoys were baited with microliter quantitites of solutions containing extracts of females, fractions of extracts separated by liquid or preparative gas chromatography (Langley et al., 1982a), or solutions of synthetic chemicals in hexane. Bioassay conditions were as described by Huyton et al. (1980).

RESULTS

Analysis

KIs are generally used to designate natural products that are often unseparable mixtures. Names and/or Roman numerals are used for syn-

thesized materials in the text and tables. Analysis of cuticular paraffins of young females showed that the major components at KI 3563 increased by 50% from day 1 to 7.4 μg at day 8. However, the proportions of the four principal peaks at KI 3065, 3563, 3663, and 3763 did not change appreciably with age, as the mean abundances were 21, 39, 6, and 12%, respectively (Table 1). These four components comprised 78% of the paraffin complement, while the other listed minor components comprised 13% of the total, and none changed appreciably with age. Together the materials shown here comprised 91% of the total paraffins. There did not seem to be any other major materials appearing or becoming prevalent in older females, although there was more material in older females.

The average quantity of the major 3563 peak in pooled wild females from Zimbabwe was 13 μg per female, about 31% of the total paraffins (Table 2). There was variation in the quantities of paraffins found in other samples of *G. pallidipes* from various sources. However, the percentage composition of each was fairly consistent, for example, the 3563 peak ranged from 23.9 to 43% but was always the major dimethyl-branched paraffin (Table 3).

GC profiles of paraffins of wild *G. pallidipes* are compared in Figure 1. Previously, paraffins of more than 34 carbons were not found in male cuticle to any extent in virgin Bristol laboratory males (Huyton et al., 1980). In wild males of unknown history collected as adults, there were often similar small amounts of KI 3563; 0.83 μg (6% of paraffins) in Kenya males and 0.63 μg (5% of paraffins) in Zimbabwe males (Figure 1B).

Results of electron-impact mass spectral analysis of paraffins from females showed that the higher-molecular-weight compounds contained two methyl groups. Two components appeared in the KI 3563 peak. Major fragments were found at m/z 196:197 (doublet of peaks), 351, and 505 (M-15) in Kenya flies (Figure 2A). A minor isomeric component, 11,21-dimethylpentatriacontane (I) was indicated by smaller fragments at m/z 168:169, 224:225, 323, and 379. This compound contained 10- and 14-carbon alkyl chains at opposite ends of the molecule, and a 9-carbon methylene bridge between the two methyl groups. The prominent m/z 196:197 doublet, which characteristically contained more of the even-mass ion, and the prominent m/z 351 fragment were derived from the cleavage on either side of the point of methyl-branching at carbon 13 (Nelson, 1978; Nelson et al., 1980, 1981). Thus, the symmetrical structure, 13,23-dimethylpentatriacontane (II), with a 12-carbon alkyl chain at each end of the molecule and 9-carbon methylene bridge between the methyl groups, was indicated. Since some of the fragments at m/z 168:169 and 323 could be contributed by the symmetrical major isomer II, the best estimate of isomeric proportions was made by comparing the intensity of ions at m/z 351 vs. 379, present at a ratio of 13:1. Division of the 351 intensity by two because of molecular symmetry in II gave a 1:6.5 isomeric ratio of I:II in Kenya females (Table 9). Both components I and II

TABLE 1. QUANTIFICATION OF MAJOR PARAFFINS OF *G. pallidipes* FEMALES REARED IN BRISTOL

Kovats' index	Age (days)	Quantities of peaks found, μg/fly (% composition)							
		1	2	3	5	6	7	8	$\bar{X}$
3065		2.73 (22)	3.07 (18)	3.10 (20)	2.87 (25)	3.03 (20)	3.48 (19)	4.07 (22)	3.19 (21)
3165		0.19 (1)	0.22 (1)	0.28 (2)	0.13 (1)	0.17 (1)	0.22 (1)	0.20 (1)	0.20 (1)
3265		0.42 (4)	0.66 (4)	0.70 (4)	0.59 (5)	0.63 (4)	0.75 (4)	0.87 (5)	0.66 (4)
3365		0.56 (4)	0.49 (3)	0.67 (4)	0.37 (3)	0.65 (4)	0.48 (3)	0.41 (2)	0.52 (3)
3465		0.54 (4)	0.52 (3)	0.57 (4)	0.50 (5)	0.74 (5)	0.75 (4)	0.67 (4)	0.61 (5)
3563		4.51 (37)	6.22 (37)	5.99 (38)	4.23 (38)	5.92 (40)	7.45 (41)	7.43 (41)	5.96 (39)
3663		0.58 (5)	1.13 (7)	1.10 (7)	0.66 (6)	0.88 (6)	1.35 (7)	1.28 (7)	1.00 (6)
3763		1.06 (9)	2.66 (16)	2.03 (13)	1.10 (10)	1.39 (9)	2.31 (13)	2.30 (12)	1.83 (12)

TABLE 2. QUANTIFICATION OF PARAFFINS FROM POOLED *G. pallidipes* FEMALES FROM ZIMBABWE

Kovats' index	Quantities of peaks found, μg/fly (% composition)							
No. of flies:	885	715	135	349	500	367	225	$\bar{X}$
2865	0.13 (0.7)	0.25 (0.7)	0.65 (1.1)	0.26 (0.7)	0.25 (0.7)	0.48 (1.0)	0.71 (2.7)	0.39 (0.8)
2965	0.19 (1.1)	0.43 (1.2)	0.78 (1.3)	0.44 (1.2)	0.37 (1.1)	0.51 (1.1)	0.25 (1.0)	0.42 (1.1)
3065	9.67 (21.0)	11.84 (29.0)	13.34 (23.0)	10.68 (29.0)	10.16 (28.0)	11.92 (25.0)	7.33 (28.0)	10.71 (26.1)
3165	1.52 (3.2)	1.77 (3.6)	1.88 (3.2)	0.89 (2.4)	1.37 (3.8)	1.39 (2.9)	0.94 (3.6)	1.39 (3.2)
3265	2.66 (6.0)	3.16 (7.0)	3.62 (6.2)	2.30 (6.0)	2.44 (6.8)	2.82 (6.0)	1.75 (6.0)	2.67 (6.3)
3365	1.44 (3.0)	1.53 (3.0)	1.85 (3.2)	0.91 (2.5)	1.15 (3.0)	1.29 (2.7)	0.67 (2.6)	1.26 (2.9)
3465	1.48 (3.0)	1.38 (2.0)	1.91 (3.3)	0.94 (2.5)	0.89 (2.5)	1.16 (2.4)	0.60 (2.3)	1.19 (2.6)
3563	11.92 (26.0)	13.73 (29.0)	17.08 (30.0)	12.24 (34.0)	12.20 (34.0)	15.03 (32.0)	8.75 (34.0)	12.99 (31.3)
3663	2.60 (6.0)	2.60 (4.0)	3.63 (6.0)	1.59 (5.0)	1.75 (5.0)	2.18 (5.0)	1.29 (5.0)	2.23 (5.1)
3763	5.6 (12.0)	6.11 (11.0)	6.83 (12.0)	4.15 (10.0)	4.40 (12.0)	5.59 (12.0)	3.27 (13.0)	5.13 (11.7)

TABLE 3. QUANTIFICATION OF MAJOR PARAFFINS OF LABORATORY-REARED OR WILD *G. pallidipes* FEMALES FROM DIFFERENT COUNTRIES

	Quantities of peaks found, μg/fly (% composition)									
	Age (days):	1[a]	60[a]	110[a]	1–5[b]	70[b]	77[b]	Wild[c]	28–35[b]	Wild[d]	Wild[e]
Kovats' index	No. of flies:	1	1	1	10	10	10	20	10	10	2
2865		0.55 (2.4)	0.70 (1.1)	-	0.48 (2.8)	0.27 (0.8)	0.17 (0.7)	0.47 (1.9)	0.21 (0.6)	0.64 (1.9)	0.70 (3.3)
2965		0.28 (1.2)	0.48 (0.8)	0.67 (0.8)	0.27 (1.6)	0.29 (0.9)	0.19 (0.8)	0.36 (1.5)	0.42 (1.3)	0.46 (1.4)	0.89 (4.2)
3065		4.03 (18.0)	10.10 (17.0)	14.46 (26.0)	3.29 (19.4)	6.41 (19.4)	4.58 (18.8)	4.43 (18.0)	5.34 (16.5)	10.62 (31.9)	4.08 (19.2)
3165		0.41 (1.8)	1.04 (1.7)	0.75 (1.3)	0.36 (2.1)	0.69 (2.1)	0.55 (2.2)	1.51 (6.1)	0.79 (2.4)	0.53 (1.6)	1.12 (5.3)
3265		0.95 (4.0)	3.44 (5.6)	4.15 (7.5)	0.79 (4.6)	1.99 (6.0)	2.05 (8.4)	1.48 (6.0)	2.65 (8.2)	2.34 (7.0)	1.38 (6.4)
3365		0.89 (3.9)	1.69 (2.8)	1.97 (3.6)	0.79 (4.7)	0.92 (2.8)	0.85 (3.5)	1.13 (4.5)	0.93 (2.9)	0.41 (1.2)	1.02 (4.8)
3465		0.78 (3.4)	2.10 (3.5)	1.80 (3.2)	0.50 (3.0)	1.22 (3.7)	1.24 (5.1)	0.70 (2.9)	1.33 (4.1)	0.57 (1.7)	0.79 (3.7)
3563		9.26 (40.0)	26.17 (43.0)	19.57 (35.3)	5.84 (34.5)	12.90 (39.0)	9.05 (37.1)	7.83 (31.8)	12.12 (37.4)	7.97 (23.9)	5.33 (24.5)
3663		0.90 (4.0)	3.45 (6.0)	1.87 (3.3)	0.72 (4.2)	2.03 (6.1)	1.65 (6.7)	1.10 (4.5)	2.48 (7.6)	1.28 (3.8)	1.29 (6.0)
3763		1.51 (7.0)	7.38 (12.0)	3.47 (6.2)	1.52 (9.0)	3.70 (11.2)	2.86 (11.7)	2.38 (9.7)	4.89 (15.1)	4.51 (13.5)	2.40 (11.3)

[a]Kenya/Austria.
[b]Uganda/Amsterdam.
[c]Kenya (Kiboko).
[d]Zambia (Kakumbi).
[e]Mozambique (Muabsa).

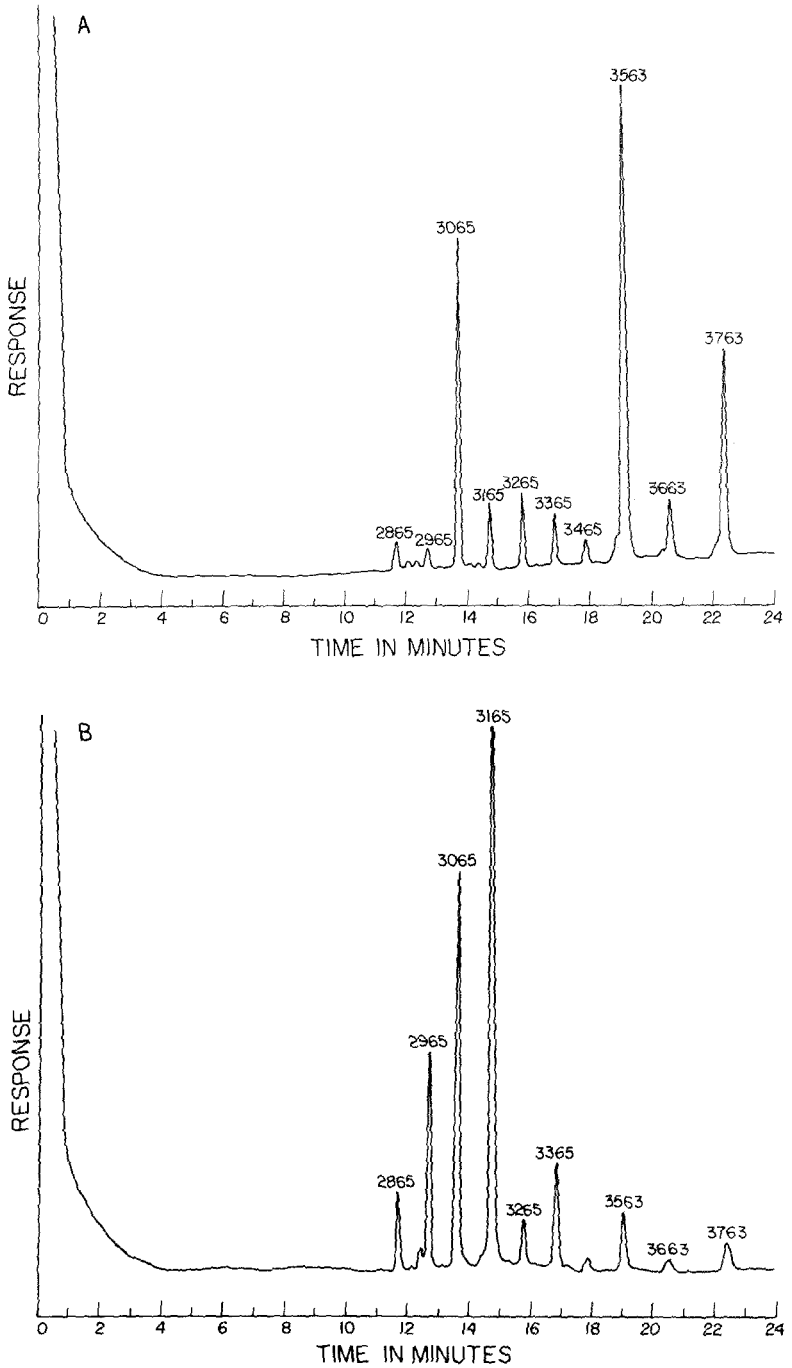

FIG. 1. GC of cuticular paraffins from *G. pallidipes:* (A) Kenya wild females (B) Zimbabwe wild males.

were found in the 3563 peak of all flies analyzed by GC-MS and were present in the following proportions: 1:5 in Bristol flies, trace:1 in Mozambique and Zambia flies, 1:8 in Uganda/Amsterdam flies, and 1:6 and trace:1 in two samples of Zimbabwe flies.

The quantity of II present in the cuticle of females was estimated by combining GC data with isomeric proportions obtained by GC-MS (Table 9). Thus laboratory 8-day-old Bristol females had 5.9 μg (80% of KI 3563 peak of 7.4 μg, Table 1), and Zimbabwe females had 10.8 μg (83% of $\bar{x}$ 13 μg) to 12.3 μg (95% of 13 μg, Table 2). Similarly, levels of II found in wild females from four other countries (Table 3) lay between these values, except for 60- or 110-day-old laboratory Kenya/Austria flies that had 26 μg and 19 μg. For purposes of comparison, an intermediate quantity of 10 μg per female of natural II was assigned. Material that eluted as a shoulder at KI 3579 on the trailing edge of the KI 3563 peak appeared to contain 11,15,21- and 13,17,23-trimethylpentatriacontane and constituted about 4% of the total complement of paraffins from several samples, including Zimbabwe females (Table 9, Figure 1).

Two homologous isomers were present in the KI 3663 peak, 12,22- (III) and 13,23-dimethylhexatriacontane (IV), present in the various ratios (Table 9). The structures were consistent with prominent fragments found at m/z 182:183, 224:225, 337, 379, and 519 (M-15) for III, and 196:197, 210:211, 351, and 365 for IV (Figure 3).

The major isomer present in the 3763 peak of all flies, consistent with the prominent fragments found at m/z 196:197, 224:225, 351, 379, and 533 (M-15) was structure V, 13,23-dimethylheptatriacontane (Figure 4). It was a homolog of the 3563 compounds, having two more methylene units added to the terminal chain.

Other dimethyl compounds with nine methylene units between the branch points identified by GC-MS include 12,22-dimethyltetratriacontane, three compounds present with 38-C backbones, (12,22-, 13,23-, and 14,24-dimethyloctatriacontane), and two compounds present with 39-C backbones (13,23- and 15,25-dimethylnonatriacontane).

Retention indices for synthetic and natural dimethylpentatriacontanes on a nonpolar fused silica column were: natural and synthetic I and II, KI 3563; 13,17-dimethylpentatriacontane, KI 3554; 15,19-dimethylpentatriacontane, KI 3555.

Synthesis of paraffins

1-Bromo-9-[triphenylphosphonio]nonane Bromide (VI). Triphenylphosphine (1.1 equiv, 2.01 g) and 1,9-dibromononane (2.0 g, 7.0 mmol) were brought to reflux in 20 ml of xylene. The mono-Wittig salt began to appear as an oil in 1 hr. GC analysis (3% OV-1, TP 150–325° at 12°/min, T_r dibromononane = 3.2 min) indicated greater than 90% completion in 4.5

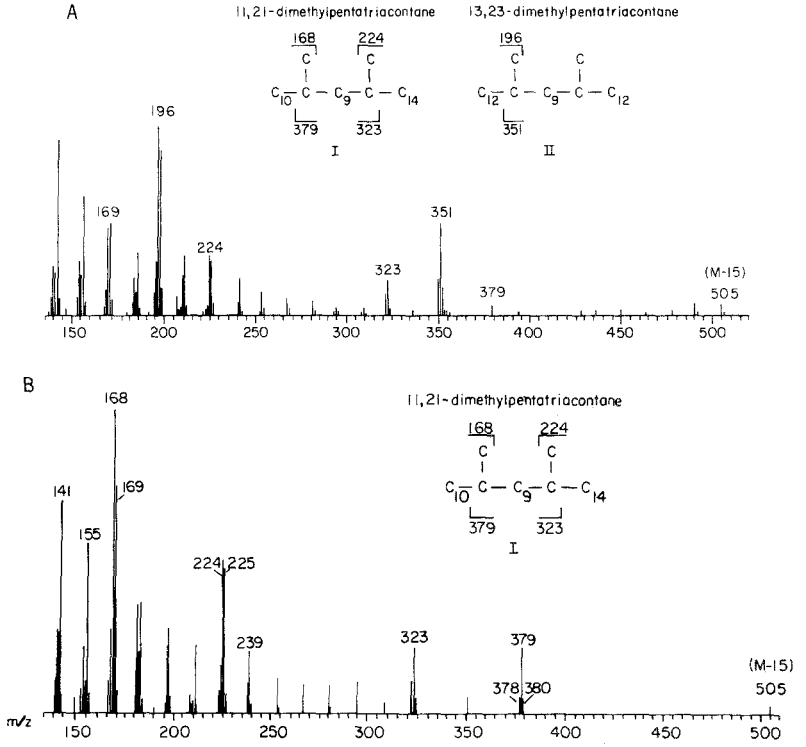

FIG. 2. Mass Spectrum of: (A) KI 3563 peak from *G. pallidipes* females from Kenya, (B) synthetic 11,21-dimethylpentatriacontane, (C) synthetic 13,23-dimethylpentatriacontane.

days. Since the first step was pseudo-second order in either reagent, it was followed by plotting the reciprocal of the concentration of either reagent, as shown by GC. The solvent was removed in vacuo on a rotary evaporator, the product taken up in $CHCl_3$, then precipitated as a viscous oil by addition of diethyl ether. Repeated trituration with ether afforded a colorless, hygroscopic powder which was dried overnight in vacuo to give 3.2 g (83% from the dibromide). The product was further purified by crystallization from methylene chloride/THF, melting with loss of solvent at 129–130°.

The identity of VI was confirmed by the proton NMR spectrum ($CDCl_3$): 7.8 ppm (multiplet = m) 15H, aromatic; 3.7 ppm (m) 4H, $Ph_3P^+CH_2$ and CH_2Br; 2.4 ppm (m) 2H, $Ph_3P^+CH_2CH_2$; 1.2–1.9 ppm (m) 14H, methylene envelope.

1,9-Bis(triphenylphosphonio)nonane Dibromide (VII). Triphenylphosphine (2.4 g, 1.1 equiv) and 4.6 g of VI (8.4 mmol) were combined in a dry 100-ml flask. The flask was purged with argon, equipped with a glass Tru-

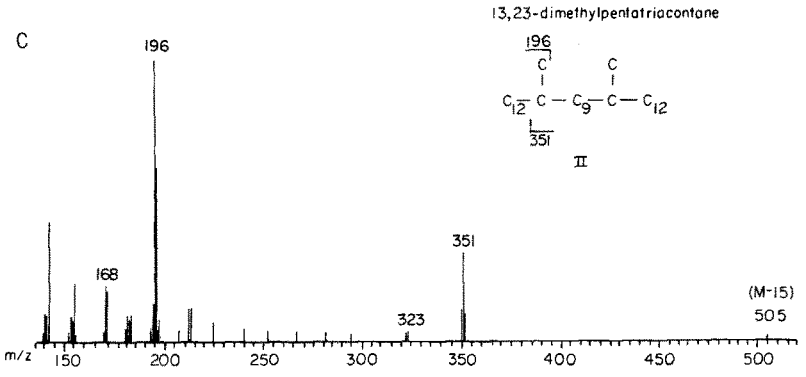

FIG. 2. Continued.

bore stirrer, and heated for 2 hr in a 230–260° sand bath (Mondon, 1957). The melt became viscous, but did not solidify. Upon cooling, the resultant hard, glassy product was dissolved in $CHCl_3$, and the bis-Wittig salt precipitated with ether. Repeated trituration with ether afforded 6.6 g of an off-white powder. NMR analysis indicated the product contained about 20% of the mono salt. Crystallization from methylene chloride–THF gave 6.0 g (74% yield of the pure VII, based upon VI) of the pure bis-Wittig Salt, mp 290–295° (loss of solvent 140–145°).

11,21-Dimethylpentatriaconta-11,20-diene. A 0.1 g (1.2 mmol) portion of bis-Wittig VII in 30 ml of freshly distilled THF was purged with nitrogen and chilled to 5° in an ice bath. Butyllithium was added from a syringe until a faint, persistent yellow color was obtained, then 2 equiv (3.5 ml, Aldrich, 1.6M in hexane) of *n*-BuLi were added dropwise over 10 min below 10°. Immediately after addition, an equal volume of freshly distilled hexamethylphosphoramide (HMPA, bp 90°/2.5 mm, from CaH_2, Aldrich) was added. No change was noted in the brown-orange color of the reaction mixture. To the ylid solution was added 0.868 equiv each of 2-hexadecanone and 2-dodecanone (Chemical Samples, 250 and 192 mg, respectively). The reaction mixture was maintained 1 hr below 10°, 1 hr at 25°, and 2 hr at 65° before being cooled, diluted with water, and the products extracted three times with hexane. The combined hexane phases were washed with brine, dried over sodium sulfate, and concentrated to give 0.45 g of oil. Chromatography on activated silica gel (20 g, V_0 = 55 ml) gave 50 mg of nonpolar material that eluted at 1–1.2 V_0 with hexane. To recover the olefins, chromatography on 20% $AgNO_3$-impregnated silica gel (10 g, Applied Science, Hi-Flosil /Ag, 60–200 mesh, V_0 = 23 ml), eluting with $4V_0$/hexane, then $4V_0$/hexane: 2% ether, gave 2.4 mg (0.4% yield) of a diolefinic fraction at 2–2.7 V_0 of the latter [TLC: R_f = 0.45; 3% ether–hexane, 15% $AgNO_3$-impregnated silica gel (Analabs, 250 μm, 20 cm), where (*E*)-9-tricosene R_f = 0.72 and (*Z*)-9-

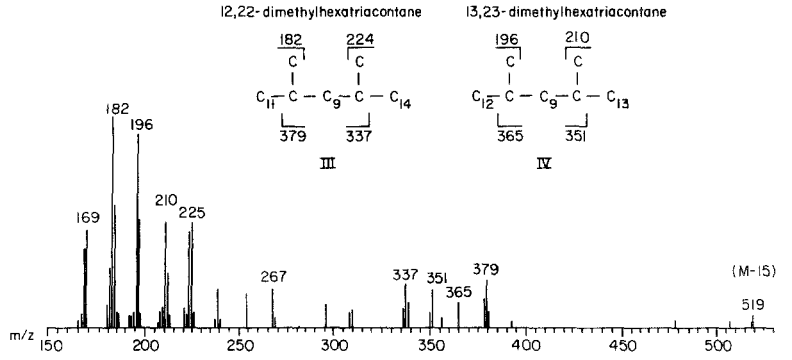

FIG. 3. Mass Spectrum of KI 3663 peak from *G. pallidipes* females from Kenya.

tricosene $R_f = 0.64$.]. The expected mixture of diolefins was produced: 11,21-dimethylhentriaconta-11,20-diene (0.64 mg, three GC peaks at KI 3100, 3148, and 3200), 11,21-dimethylpentatriaconta-11,20-diene (1.10 mg, three GC peaks at KI 3500, 3547, and 3600), and 15,25-dimethylnonatriaconta-15,24-diene (0.64 mg, three GC peaks at 3690, 3737, and 3800).

11,21-Dimethylpentatriacontane (*I*). The diolefin mixture was hydrogenated over 200 mg of prereduced catalyst (Alfa, 5% Pt on carbon) at 40 psi for 48 hr with magnetic stirring in 2 ml hexane, to give ca. 80% reduction. The sample was charged with 100 mg of fresh catalyst and hydrogenation continued overnight. The catalyst was filtered and the concentrated mixture chromatographed on 5 g $AgNO_3$-impregnated silica gel (V_o = 25 ml), eluting with hexane (1–1.75 V_o). The expected mixture of three paraffins was produced: 11,21-dimethylhentriacontane (44%, KI 3267), 11,21-dimethylpentatriacontane (I, 46%, KI 3563), and 15,25-dimethylnonatriacontane (10%, KI 3965).

The desired paraffin (I) (ca. 0.4 mg) was isolated by preparative GC. The

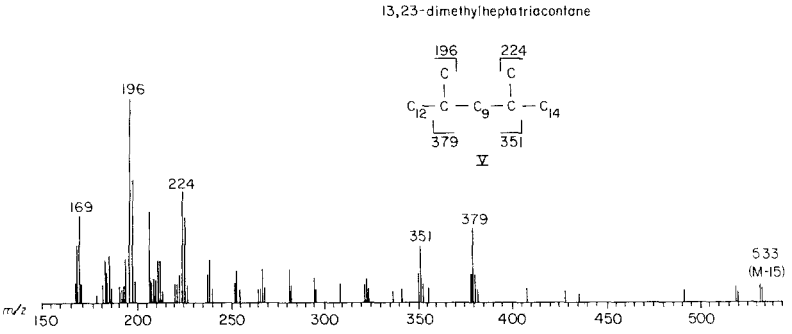

FIG. 4. Mass Spectrum of KI 3763 peak from *G. pallidipes* females from Kenya.

spectrum of synthetic I showed the expected major fragments at m/z 168:169 (100:76%), 224:225 (50:47%), 323 (21%), 379 (21%), and 505 (3%, M-15). Small excesses of ions were seen at m/z 140 and 169 but were not indicative of major homologs (Figure 2B).

13,23-Dimethylpentatriaconta-13,22-diene. Two individual preparations of VII were made using acetonitrile and benzene and combined as a viscous glass that contained some VI. The mixture (31.6 g, 38.9 mmol if all VII) was combined with 175 ml of HMPA in a dry 500-ml 3-neck flask in the dry box. The flask was purged with nitrogen, stirred magnetically, and cooled to 7° during dropwise addition from a syringe of 3 equiv (1.5-fold excess) of 2.4 M *n*-butyllithium (48 ml, 115 mmol) in hexane to give a clear, dark red solution. The ylid solution was held below 10° during addition of 2-tetradecanone (24.5 g, 115 mmol) (Chemical Samples) in 30 ml HMPA. After 3 hr, the flask was allowed to warm to room temperature. The next day the reaction mixture was diluted with 200 ml of water, the organic layer separated with the aid of more hexane, washed with water and brine, dried, and concentrated to give 57 g dark oil. Volatiles were removed from the crude product by vacuum distillation at 186° at 0.5 mm, leaving 18.3 g of brown oil. Chromatography on silica gel of a 1.9-g portion of the crude product gave 0.08 g of nonpolar material eluted at 1.0–1.2 V_o with hexane. It consisted of a mixture containing 13,23-dimethylpentatriaconta-13,22-dienes, which showed three GC peaks at 3500, 3548, and 3600.

13,23-Dimethylpentatriacontane (II). Two crude diolefin preparations (55 g) were added to 50 ml propionic acid in a 100-ml stainless steel bomb together with 1 g of prereduced 5% platinum on charcoal catalyst. The bomb was pressurized to 120 psi and heated for 24 hr at 150–230° with shaking for 96 hr. GC analysis of a cleaned-up hexane-soluble aliquot indicated that the three diolefin peaks had coalesced into one, at KI 3563. The cooled contents of the bomb were then poured into water in a separatory funnel together with bomb washings and partitioned against hexane. The organic layer was washed with water and brine, filtered, dried, and the solvent removed to leave 14 g of viscous brown oil.

To recover II, the crude material was divided into four parts, and each was filtered through a 2 × 45-cm column of silica gel with hexane. The first paraffin-containing fractions (1–2 V_o) were combined and the solvent removed to yield 1.2 g (4.0%) of white solid paraffin, 13,23-dimethylpentatriacontane (II). Argentation TLC using hexane showed that no unsaturated materials were present. GC analysis showed the major peak (KI 3563) comprised 96% of the material present, and several paraffins with lower molecular weight comprised the impurities. The GC retention was the same as that for the natural product. The mass spectrum of synthetic II showed the expected major fragments as m/z 196:197 (100:61%), 351 (31%), and 505 (2%, M-15). Another even–odd doublet was visible at m/z 168:169 (19:17%) from cleav-

age of the terminal alkyl chain of 12 carbons. Fragment ions were also present at m/z 210:211 (9:8%) and 323 (3%) that are real but not explained (Figure 2C).

Bioassay

Wingless, immature (0–1 days old) *G. pallidipes* females killed by freezing were nearly as stimulatory as older (9-day-old) females which released 80–100% copulatory responses in test males (Table 6) Langley et al., 1982a). Live or dead *G. pallidipes* males or *G. morsitans* male decoys never released sexual behavior in test males, allowing use of dead males as convenient decoys for bioassay of test materials for release of sexual behavior.

Using flies which emerged from field-collected puparia in Zimbabwe, a crude extract of females elicited from 37 to 70% responses from test males, and there was little indication of a dose-response relationship at doses of 0.1–10 female equivalents (FE) (Table 4). Similar responses were obtained

TABLE 4. SEXUAL RESPONSES OF TEST MALE *G. pallidipes* TO EXTRACTS OR FRACTIONS OF EXTRACTS OF CUTICLES OF FEMALES

Extract or fraction	Dose (FE)	N	Test male responses[a]				Response (%)
			0	1	2	3	
1. Virgin lab, extract (7 day)[b]	0.1	10	2	2	5	1	50
	0.2	10	3	2	4	1	43
	0.5	10	2	2	5	1	50
	1	10	2	1	3	4	63
	2	10	2	0	3	5	70
	5	10	2	1	6	1	53
2. Virgin lab, extract (7–9 days)[b]	10	5	0	3	2	0	47
	1	10	1	7	2	0	37
3. Hydrocarbons[c]	1	10	2	4	2	2	47
Cholesterol esters	1	10	10	0	0	0	0
Methyl esters	1	10	10	0	0	0	0
Triglycerides, free fatty acids	1	10	10	0	0	0	0
4. Wild caught, extract	5	20	3	4	10	3	55
5. Hydrocarbons[d]	4	6	0	1	3	2	72
Cholesterol esters	4	6	6	0	0	0	0
Triglycerides	4	6	6	0	0	0	0
Free fatty acids	4	6	6	0	0	0	0

[a]0 = no response; 1 = mounting, gripping, and arresting of male on decoy; 2 = characteristic copulatory movement with orientation to the copulatory position, abdomen curving; 3 = flexing of genetalia.
[b]Reared from wild-collected pupae of Zimbabwe origin.
[c]Fractions from virgin lab, extract.
[d]Fractions from wild-caught extract.

with 5 FE of an extract of wild-caught females (55%) and 5 FE of an extract of females emerged in the laboratory (53%). Of fractions obtained by liquid absorption chromatography on silica gel, only the hydrocarbons elicited responses from test males, and these were similar in magnitude to the responses elicited by crude extracts. Further testing showed that only the cuticular paraffins, separated by argentation chromatography from the hydrocarbon fraction, released male responses (Table 4).

Preparative gas chromatography was then employed to separate the cuticular paraffins. Bioassay of these materials showed that optimal activity (41% response) was associated with the KI 3563 peak, while some activity was associated with material collected as KI 3465, 3663, and 3763, perhaps because they are structural homologs of the KI 3563 materials (Table 5). A dose–response relationship could not be established and responses to a range of doses from 1.1 to 11.3 FE were therefore pooled (Table 5).

The two compounds present in the KI 3563 peak were synthesized and, using dead male *G. morsitans* as decoys, their ability to elicit sexual responses from test males of Zimbabwe origin was compared with that of crude cuticular extract, freshly killed *G. pallidipes* females, solvent-washed females, or male *G. morsitans* alone. Only 13,23-dimethylpentatriacontane (II) elicited test male responses including copulatory attempts (level 3). Furthermore, there was a strong indication of a dose–response relationship, although the maximum percentage response at 8 FE was no better than that obtained from 2 FE of crude extract and was lower than that obtained when dead females were used as decoys (Table 6).

TABLE 5. SEXUAL RESPONSES OF 9-DAY-OLD TEST MALE *G. pallidipes* TO ISOLATED PARAFFINS FROM FEMALES DOSED ONTO DEAD MALE DECOYS[a]

Test sample (Kovats' index)	N	Test Male Responses				Total	Response (%)
		0	1	2	3		
2865	5	5	0	0	0	0	0
2965	5	5	0	0	0	0	0
3065	25	25	0	0	0	0	0
3165	16	16	0	0	0	0	0
3265	16	16	0	0	0	0	0
3365	16	16	0	0	0	0	0
3465	16	15	0	1	0	2	4
3563	48	15	9	19	4	59	41
3663	43	37	0	3	0	6	5
3763	42	35	4	3	0	10	8

[a]Responses scored as 0, 1, 2, 3, and summed. Samples collected by preparative GC from female *G. pallidipes* surface hydrocarbons. Dose ranges from 1.1 to 11.3 FE/decoy. R(%) = total response score/3N.

TABLE 6. SEXUAL RESPONSE OF *G. pallidipes* MALES OF ZIMBABWE ORIGIN TO DEAD FEMALES OR DECOYS DOSED WITH SYNTHETIC 13,23-DIMEYHYLPENTATRIACONTANE (II)[a] OR CRUDE EXTRACT OF *G. pallidipes* FEMALES

Test sample[b]	N^c	Test male responses 0	1	2	3	Total	Response (%)	FE[d]	Amount (μg)
G. pallidipes (9 day old)	20	0	2	6	12	50	83.3		
G. pallidipes (washed)	20	16	4	0	0	4	6.7		
G. morsitans (washed)	20	20	0	0	0	0	0	2	
Crude extract	20	3	3	9	5	36	60.0	2	
II	20	5	3	9	3	30	50.0	2	20
II	10	10	0	0	0	0	0	0.1	1
	10	8	2	0	0	2	6.7	0.2	2
	10	8	0	2	0	4	13.3	0.5	5
	10	5	2	3	0	8	26.6	1	10
	10	4	2	2	2	12	40.0	2	20
	10	2	2	3	3	17	56.6	5	50

[a]I showed no responses in any tests tested alone.
[b]All samples treated onto dead 19-day virgin *G. morsitans* males except as noted.
[c]Five male decoys used at each dose.
[d]Based on estimated 10 μg II per female (see text).

TABLE 7. SEXUAL RESPONSE OF *G. pallidipes* MALES OF VARIOUS AGES OF UGANDA ORIGIN TO CORK DECOYS DOSED WITH 13,23-DIMETHYLPENTATRIACONTANE (II) AND DEAD *G. pallidipes* FEMALES (8–10 DAYS OLD), TEST CONDUCTED IN AMSTERDAM

Age of males (days)	Dead females	n	Response of test male (%) Compound II (μg) 25	50	75	100	150	N
0	2	14	0	0	0	0	0	10
1–3	26	14	0	0	0	0	0	6
4–5	60	14	8	11	39	39	33	24
6–7	58	12	13	42	78	69	58	15
8–10	89	12	72	58	84	87	77	23
11–12	83	8	86	83	98	100	100	14
13–14	97	13	97	97	100	100	100	12
15–17	92	8	94	100	100	100	100	6
18–19	79	8	100	88	100	100	100	8

TABLE 8. SEXUAL RESPONSE OF 12–14-DAY-OLD *G. pallidipes* MALES OF UGANDA ORIGIN TO CORK DECOYS DOSED WITH 13,23-DIMETHYLPENTATRIACONTANE (II) AND CRUDE EXTRACT OF *G. pallidipes* FEMALES

		Test male responses					Response		Amount
	N^a	0	1	2	3	Total	(%)	FE	(μg)
Crude extract	6	6	0	0	0	0	0	0.2	3[b]
	6	4	1	0	1	4	22.2	0.6	7
	6	3	0	0	3	9	50.0	1.3	13
	6	2	1	0	3	10	55.5	2.6	27
	6	2	0	1	3	11	61.1	6.7	67
II	6	3	1	0	2	7	38.8	0.2[b]	2
	6	2	1	0	3	10	55.5	0.5	5
	6	1	0	0	5	15	83.3	1.0	10
	6	0	0	0	6	18	100.0	2.0	20
	6	0	0	0	6	18	100.0	5.0	50

[a]Two decoys per dose and three test males per decoy (Amsterdam–Bristol colony).
[b]Based on 10 μg II per fly (see text).

Male *G. pallidipes* of Uganda origin, which have been in laboratory culture in Amsterdam for several years, were used to bioassay synthesized II using cork decoys (see Methods and Materials). Results (Table 7) show clearly that the responsiveness of males increased with age and that during the first week of adult life males of this species do not possess the ability to respond predictably to increasing doses of II. Older males responded maximally to the higher doses, but even the lowest dose tested was in excess of the amount of material which occurs naturally on or in the cuticle of a female fly. These experiments were therefore repeated using 12- to 14-day old Uganda–Amsterdam–Bristol males, and a comparison was made between crude extract of cuticle and compound II at doses of 2–50 μg. The results (Table 8) showed that 83.3% copulatory responses were obtained to 10 μg of II and obligatory (100%) copulatory responses were obtained to 20 and 50 μg, demonstrating its superiority over crude extract.

DISCUSSION

Sexual responses of adult males of *G. pallidipes* were elicited only by natural 35-carbon backbone dimethyl paraffins in the surface cuticular waxes of females. Since formal evidence was obtained for the existence of two major compounds in the KI 3563 fraction, both compounds were synthesized, and in

bioassays only the latter compound elicited sexual responses from males; no synergism was apparent.

Although the amount of 3563 paraffin increases in females as they age, there is sufficient material on or in the cuticle at emergence to elicit almost obligatory copulatory responses from test males towards live or fresh-killed females used as decoys (Langley et al., 1982a). Hence, we conclude that maturation of sexual behavior in females and perhaps also in males of *G. pallidipes* is more important than changing levels of a stimulatory pheromone in regulating the sexual behavior of this species.

Since quantities of natural II ranged from ca. 6 to 12 μg per female in most flies, a level of 10 μg of II was used to compare its activity with that of crude extract (Tables 4, 6, and 8). The inconsistencies observed and the lack of a clear dose–response relationship, except in the case of II in Table 8 where 100% responses of test males were achieved, can probably be explained as follows.

Tsetse emerging from field-collected puparia often behave differently from those which have been in laboratory culture for some time. Differences in reproductive behaviour also are known to exist among separate populations of *G. pallidipes* in the wild (Van Etten 1981). Using *G. pallidipes* emerging from field-collected puparia of Zimbabwe origin, test males never produced obligatory (100%) copulatory responses to extracts of synthetics in bioassay (Tables 4 and 6) and only produced 83.3% response to killed conspecific females (Table 6). Table 7 shows clearly that 100% responses to II were elicited in test males of Uganda origin that had been in laboratory culture for some time, but that the responsiveness to a given dose increased with age, and the doses required to elicit 100% response were in excess of the amounts known to exist on the cuticle of a female fly. Nevertheless, from Table 8, it is clear that synthetic II is superior to crude extract, and using test males of Ugandan origin (12–14 days old) an ED_{50} of around 5μg can be determined.

Ideally an ED_{95} of around 5 μg would put the assignment of II as the sex pheromone of *G. pallidipes* beyond doubt. However, the bioassay system was developed for use initially with *G. morsitans* (Huyton et al., 1980), and stimuli other than chemical may be of relatively greater importance in the initiation of mating behavior in *G. pallidipes* than in *G. morsitans*. The superiority of II over crude extracts may be due to the masking effect of other paraffins present in the extract (Coates and Langley, 1982).

Alternatively, some component synergistic with II may be missing. Synthetic compound I does not appear to be a contributor to activity, but the KI 3579 material, difficult to separate by preparative GC, contains 13,17,23-trimethylpentatriacontane. This candidate structure combines the features of II and of 13,17-dimethylpentatriacontane which is equally active in bioassays (Langley, unpublished data), but which was erroneously identified as the sex pheromone of *G. pallidipes* females (McDowell et al., 1981).

The quantities of II present in females from Bristol (ca. 6 μg), wild females from Zimbabwe (10.8–12.3 μg), and other African countries (5.3–7.9 μg) were in marked contrast to the 80 μg of pheromone per fly quoted by McDowell et al. (1981). The latter observation was probably due to the inclusion of internal lipids in the weighed extract. Inaccurate quantitation of candidate pheromone compounds can lead to spurious conclusions from bioassay data concerning their biological significance.

The element of symmetry in the structure of biologically active sexual stimulants for *Glossina* is therefore again proposed. It is interesting to note that the most active synthetic compounds found for *G. morsitans*, 15,19,23-trimethylheptatriacontane (Carlson et al., 1978) and 15,19-dimethyltritriacontane in *G. austeni* (Huyton et al., 1980), are symmetrical structures, as is II. The nonsymmetrical structures proposed by other workers (McDowell et al., 1981), 13,17-dimethylpentatriacontane (major) and 15,19-dimethylpentatriacontane (minor), are not supported by the mass spectra published in that paper. Characteristic fragment ions that should be present for McDowell's proposed major compound do not appear at m/z 280:281 and 267, and fragment ions that should be present for the minor compound at m/z 252:253 and 295 are again missing from the published spectrum. These compounds with 3-methylenes between the branch points are present in the KI 3550–3555 peak in *G. morsitans* females, but not in any of the females of *G. pallidipes* we have investigated to date. Our magnetic sector mass spectra of paraffins from females from laboratory colonies (Zimbabwe–Bristol and Uganda–Amsterdam) or wild females captured in Kenya, Mozambique, Zambia, and Zimbabwe showed remarkable consistency (Table 9) in that 11,21- and 13,21-dimethyl structures having 35-, 36-, and 37-carbon backbones were the only ones found.

The stereochemical character of structures discussed here may play a role in their function, since a symmetrical molecule with two asymmetric centers such as II may have a *meso* form and a racemic pair of enantiomers: *R,S*-, *R,R*-, and *S,S*-. Because natural II, if optically active, may contain only one of the enantiomers, the unnatural enantiomer or the *meso* form may either be inhibitors of response or passive diluents. Synthesized stereoisomers of *G. morsitans* 17,21-dimethyl pheromone components showed increased biological response in only the *meso* C_{37} dimethyl stereoisomer (Ade et al., 1980), while all the stereoisomers of 15,19,23-trimethylheptatriacontane were found to be equally active (Helmchen and Langley, 1981). Although no stereochemical preference was shown by *G. morsitans*, there is no reason to suppose that this situation obtains for other species. Molecular models indicate that there is sufficient conformational and rotational freedom that in a dimethyl compound having three or nine methylene-bridge carbons, the two methyl groups may be oriented to nearly coplanar positions regardless of whether an *R,R*-, *S,S*-, or *meso* compound has been constructed. It is thus impossible to predict which optical isomer would be most active, or

TABLE 9. POSITION OF METHYL BRANCHING AND PROPORTIONS OF ISOMERS IN PARAFFINS OF FEMALE *G. pallidipes* FROM DIFFERENT COUNTRIES

KI	Position of methyl branching[a]	Proportions of isomers seen[b]						
		Bristol	Kenya	Mozambique	Uganda	Zambia	Zimbabwe	Zimbabwe
3363	11,15-; 13,21-	1.7:1	2.7:1	1.6:1	1:1.6[c]	2.2:1	1.6:1	1:2
3463	11,21-; 12,22-	T:1	1:3	T:1	T:1	T:1[d]	T:1	1:5
3563	11,21-; 13,23-	1:5	1:6.5	1:11	1:8	T:1	1:6	T:1
3579	11,15,21-; 13,17,23-	U[f]	U	U	2.6:1	1:1	2:1	2:1
3663	12,22-; 13,23-	2.1:1	1.4:1	1:1.6	1.7:1	1.4:1	1:1	1.6:1
3763	11,21-; 13,23-[e]	T:1	T:1	T:1	T:1	T:1	T:1	T:1

[a]KI 2865, 2965, and 3065 peaks were universally 2-methyl, as each showed a small M, large M-15, and base peak M-43.
[b]Ratio of fragment intensities used to establish proportions of isomers: (Pk Ht/2) m/z 351 ÷ Pk Ht m/z 379.
[c]Trace of 9,X- in Uganda/Amsterdam flies.
[d]Trace of 2-Me.
[e]T = trace, 5%.
[f]U = trace of trimethyl-branched paraffin present, but branching positions could not be determined.

what advantage one would have over the other, especially since the conformation of receptors is unknown.

Identification and synthesis of the sex pheromone of *G. m. morsitans* led to attempts to exploit its properties for tsetse control in Tanzania (Langley et al., 1981) and Zimbabwe (Langley et al., 1982b). In the latter effort, observations led to the conclusion that the sexual behavior of *G. pallidipes* is different from that of *G. m. morsitans* in that males of the former are not visually stimulated to make contact with decoys on host models as are males of the latter. Also, *G. pallidipes* have not been observed mating in the field in Zimbabwe, in contrast to *G. m. morsitans*, which is frequently observed mating on or around host animals (G. Vale, personal communication). Although *G. pallidipes* has been observed mating in the field in eastern Africa (D. Turner, personal communication), laboratory observation has shown marked differences in behavior between flies from two different regions of Kenya. The two populations differed from each other in copulation time, pupal weight, and age at which the first larva is produced. Both exhibited rearing difficulties. These behaviors are thought to have a genetic basis and to illustrate population diversity in this species (Van Etten, 1981). In spite of such differences in the sexual behavior of *G. pallidipes* from different regions, it appears from the present results that the species uses a single compound as its sex pheromone. Development of control strategies for *G. pallidipes* involving the use of its sex pheromone will undoubtedly require a greater understanding of its reproductive behavior in the field.

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CHEMISTRY OF CEPHALIC SECRETION OF FIRE BEE *Trigona (Oxytrigona) tataira*[1,2]

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Abstract—Analysis of the volatile compounds derived from cephalic glands of the fire bee *Trigona* (*Oxytrigona*) *tataira* by GC-MS was undertaken. The following compounds were readily identified: hydrocarbons: *n*-$C_{11}H_{24}$, *n*-$C_{13}H_{28}$, *n*-$C_{14}H_{30}$, *n*-$C_{15}H_{32}$, *n*-$C_{17}H_{36}$, *n*-$C_{23}H_{48}$, *n*-$C_{15}H_{30}$, *n*-$C_{17}H_{34}$, *n*-$C_{21}H_{42}$, and *n*-$C_{23}H_{46}$; carboxylic acids: palmitic acid, linoleic acid, linolenic acid, stearic acid, and oleic acid; carboxylic esters: dodecyl acetate, tetradecyl acetate, hexadecyl acetate, octadecyl acetate, and dodecyl decanoate; monoketones: 5-hepten-2-one, 3-hepten-2-one, 2-heptanone, and 5-nonen-2-one. Two major components of the mixture were identified as *E*-hepten-2,5-dione and *E*-3-nonen-2,5-dione. Structures of these novel compounds were suggested by their GC-MS behavior and the GC-MS behavior of their dimethoximes and proved by comparison with authentic synthetic samples. Trace amounts of the corresponding *Z* isomers and the saturated analogs, heptan-2,5-dione and nonan-2,5-dione, were also found. The possible functions of these glandular constituents are discussed.

Key words—Fire bee, *Trigona* (*Oxytrigona*) *tataira,* Hymenoptera, Apidae, mandibular gland secretion, enediones, monoketones, carboxylic esters, hydrocarbons, *E*-3-hepten-2,5-dione, *E*-3-nonen-2,5-dione.

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INTRODUCTION

Among stingless bees in the genus *Trigona*, *T.* (*Oxytrigona*) *tataira* and its relatives are particularly noteworthy because of the unusual properties of its mandibular gland secretion. This apid successfully plunders the food reserves from the nests of other species of stingless bees. It also produces a secretion from its extensively developed mandibular gland (Kerr and Costa Cruz, 1961) that produces lesions when administered to the skin of human beings (Wille, 1961). Because of the vesicatory properties of this exudate, it is frequently referred to as the fire bee. Our recent investigations of the robbing *modus vivendi* of *T. tataira* suggest that the secretion from the capacious mandibular glands may constitute powerful allomones that effectively disrupt the defensive behavior of bees whose nests are being plundered.

One of us (T.E.R.) recently observed that workers of *T. tataira* successfully invaded the nests of honeybees (*Apis mellifera*) (Moure, 1946), which were robbed of resources in the absence of any organized resistance on the part of the host bees. During these raids, the air in the vicinity of the hives was redolent with a floral odor which was subsequently shown to be derived from the cephalic secretion of workers of *T. tataira.* In the present study, we report on the chemistry of the cephalic secretion of this bee which is characterized by the presence of several novel insect natural products.

METHODS AND MATERIALS

Bees

Workers of *T. tataira* were collected in Acarigua, Venezuela. The bees were immediately decapitated, and the heads were placed in vials containing methylene chloride. All subsequent analyses were undertaken on these extracts.

Chemical Analyses

Mass spectra and analytical gas chromatographic data were obtained on an LKB-2091 GC-MS system at 70 eV, a source temperature of 270–300°C, separator 270°C, using 50 μA ionizing current. Fused quartz capillary columns [15 m $\times$ 0.33 mm ID coated with SE-30 liquid phase (J and W Scientific Co., Rancho Cordova, California)] were used throughout. Gas chromatographic analyses of synthetic mixtures and preparative gas chromatography were carried out on a GOW-MAC model 169-150 gas chromatograph equipped with 2 m $\times$ 5 mm ID columns packed with 10% SE-30 on Chromosorb W. Infrared spectra were obtained using a Perkin-Elmer model 467 grating infrared spectrophotometer. NMR spectra were obtained at 60

MHz with a Varian A60-A instrument and at 360 MHz with a Nicolet 360 MHz FT instrument. All boiling points and melting points are uncorrected.

Heptan-2,5-dione and Nonan-2,5-dione

These 1,4-diketones were prepared according to the literature (Stetter et al., 1974) and had the following mass spectra: heptan-2,5-dione: see Figure 1a; nonan-2,5-dione MS: m/z (rel. %) 156 (1,M+·), 141(1), 128(2), 115(5), 114(76), 109(2), 99(95), 85(56), 71(70), 57(88), 55(13), 43(100), 42(9), 41(3).

Selenium Dioxide Oxidation of 1,4-Diketones

E-*3-Hepten-2,5-dione.* A mixture containing 5 g of heptan-2,5-dione and 4 g of selenium dioxide in 100 ml of water was heated to reflux for 5 hr (Figure 2). The mixture was steam distilled to give 200 ml of condensate which was extracted with three 70-ml portions of ether. The combined ethereal extracts were dried over anhydrous $MgSO_4$, filtered, and the solvent was removed in vacuo to give 2 g of an oil. Gas chromatographic analysis of the oil showed it to approximate a 1:1 mixture of the starting diketone and a single component with a longer retention time. Preparative gas chromatography provided a pure sample of this component as a waxy solid, mp 35–36° C; NMR (360 MHz): δ = 6.83 (s, 2H, HC=CH), 2.69 (q, 2H, J = 7.2 Hz, $COCH_2$—), 2.63(s, 3H, CH_3CO), 1.13(t, 3H, J = 7.2 Hz, —CH_2CH_3); MS: see Figure 1b. A small amount (ca. 5%) of a component with an identical mass spectrum, presumed to be the Z isomer, was also discovered upon GC-MS analysis of the mixture.

E-*3-Nonen-2,5-dione.* A 5-g sample of nonan-2,5-dione was treated with selenium dioxide as described above. Gas chromatographic analysis of the resulting oil showed approximately a 1:1 mixture of the starting diketone and a single longer retention time component which was obtained by preparative gas chromatography as a waxy solid, mp 36–37° C; NMR (360 MHz): δ = 6.84(s, 2H, HC=CH), 2.66(t, 2H, J = 7.2 Hz,—CH_2CO), 2.47(s, 3H, CH_3CO), 1.64(m, 2H, $COCH_2CH_2$), 1.35(sextet, 2H, J = 7.2 Hz, $CH_3CH_2CH_2$—), 0.92(t; 3H, J = 7.2 Hz, —CH_2CH_3); MS: m/z (rel. %) 154(1,M+·), 139(3), 126(2), 125(1), 112(25), 111(39), 98(12), 97(100), 85(7), 84(2), 83(2), 82(2), 71(6), 70(5), 69(17), 57(17), 55(21), 54(5), 53(3), 43(47), 41(16). As before, a minor component (ca. 5%) with an identical mass spectrum, presumed to be the Z isomer, was also observed upon GC-MS analysis of this reaction mixture.

2-Butyl-5-methylfuran. A mixture containing 3 g of nonan-2,5-dione and a small amount (ca. 0.3 g) of Dowex 50W-4X acid resin was heated to reflux under a nitrogen atmosphere (Figure 2). When the reaction was complete by gas chromatography (ca. 2 hr), the mixture was taken up in ether, dried over anhydrous K_2CO_3, filtered, and distilled to give 1.3 g (50% yield) of a

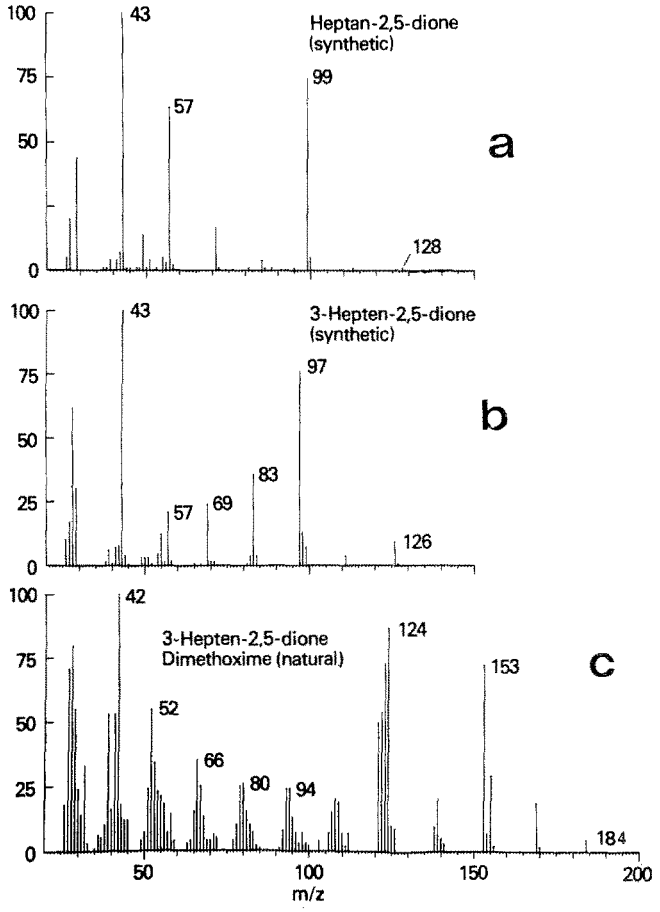


FIG. 1. Mass spectra of (a) heptan-2,5-dione (synthetic), (b) *E*-3-hepten-2,5-dione (synthetic), and (c) earliest eluting isomer (see Figure 4) of dimethoxime of *E*-3-hepten-2,5-dione (natural).

colorless, volatile liquid, bp 165° C; IR: 3052, 1220, 1015, and 770 cm^{-1}; NMR (60 MHz): δ = 5.71(s, 2H, furan 3-H and 4-H), 2.54(t, 2H, J = 7 Hz, furyl-CH_2), 2.20 (s, 3H, furyl-CH_3), 1.45 (m, 4H, —CH_2CH_2—), and 0.95 (t, 3H, J = 7.0 Hz,—CH_3).

2-Ethyl-5-methylfuran. A mixture containing 10 g of heptan-2,5-dione and a small amount of Dowex 50W-4X acid resin was distilled at atmospheric pressure. The fraction distilling between 95 and 100° C was taken up in ether, dried over anhydrous K_2CO_3, filtered, and redistilled to give 6.9 g (80% yield) of a colorless volatile liquid, bp 118°C; NMR (60 MHz): δ = 5.70 (s, 2H,

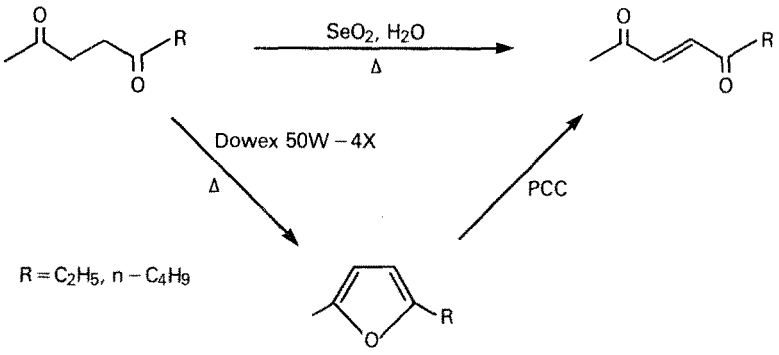


FIG. 2. Synthetic schemes for synthesis of endiones (see text).

furan 3-H and 4-H), 2.59(q, 2H, J = 7.5 Hz,—CH_2—), 2.20(s, 3H, furyl-CH_3), 1.18 (t, 3H, J = 7.5 Hz, —CH_2CH_3).

Pyridinium Chlorochromate Oxidation of 2,5-Dialkylfurans

3-Hepten-2,5-dione and 3-nonen-2,5-dione were prepared from the corresponding 2,5-dialkylfurans in 50% and 60% yield, respectively, by oxidation with pyridinium chlorochromate (Piancatelli et al., 1980). Kugelrohr distillation at 14 mm gave the solid *E*-endiones in greater than 98% purity by gas chromatographic analysis. None of the *Z* isomers were detected. These compounds were identical in all respects to the endiones prepared by selenium dioxide oxidation of the 1,4-diketones.

RESULTS

Upon GC-MS analysis of the methylene chloride extracts of *T. tataira* heads (Figure 3), the following compounds were identified from first principles and confirmed by comparison with mass spectral compilations (Heller and Milne, 1982), spectra of authentic samples, or spectra of simple homologues. Saturated hydrocarbons: *n*-$C_{11}H_{24}$, *n*-$C_{13}H_{28}$, *n*-$C_{14}H_{30}$, *n*-$C_{15}H_{32}$, *n*-$C_{17}H_{36}$, and *n*-$C_{23}H_{48}$; Olefinic hydrocarbons: *n*-$C_{15}H_{30}$, *n*-$C_{17}H_{34}$, *n*-$C_{21}H_{42}$, and *n*-$C_{23}H_{46}$; Carboxylic acids: palmitic acid, linoleic acid, linolenic acid, stearic acid, and oleic acid; Carboxylic esters: dodecyl acetate, tetradecyl acetate, hexadecyl acetate, and octadecyl acetate; Monoketones: 5-hepten-2-one, 3-hepten-2-one, and 5-nonene-2-one. Dodecyl decanoate was identified by noting intense ions at m/z 173 and m/z 168 corresponding to the $(C_9H_{19}COOH+H)^+$ and $(C_{12}H_{24})^+$ fragments expected for this substance, as well as a molecular ion at m/z 340.

Two major components of this mixture eluted at 102°C and 130°C

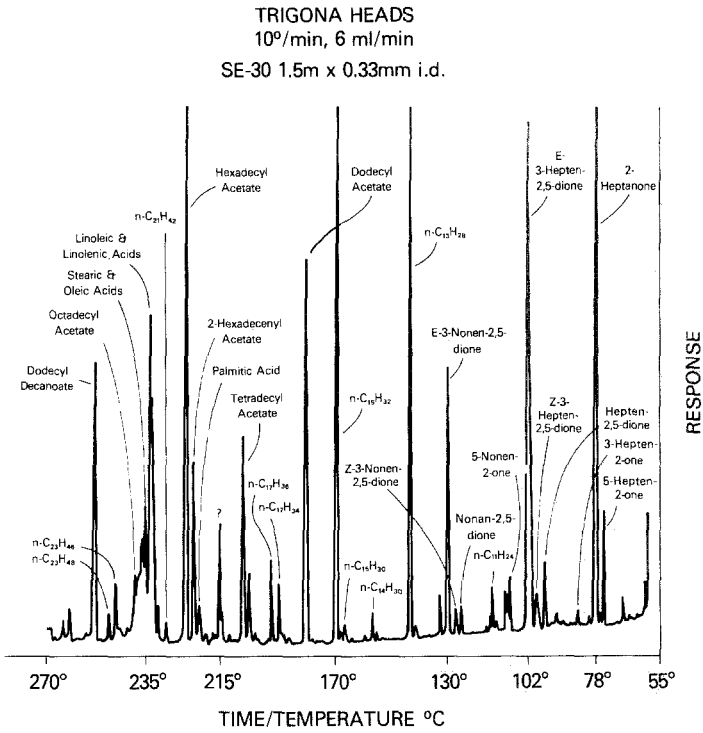
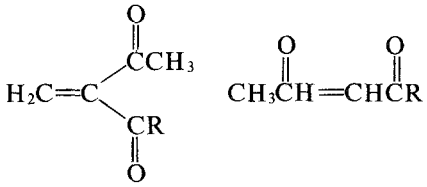


FIG. 3. Gas chromatogram of extracts of *T. tataira* heads. Compounds identified by mass spectra.

(Figure 3). The first had a molecular ion at m/z 126 while the spectrum of the second showed many homologous ions, including a molecular ion at m/z 154, differing by two methylene units.

The structure of the substance responsible for the first peak was deduced as 3-hepten-2,5-dione from its mass spectrum alone, which showed important ions at m/z 43, 83, and 111 (CH_3CO, M-CH_3CO and M-CH_3, respectively) and m/z 57, 69, and 97 (C_2H_5CO, M-C_2H_5CO and M-C_2H_5, respectively), accounting for all of the structure except 26 mass units (C_2H_2). Two structures are possible, Ia and IIa.



I_A R = C_2H_5 II_A R = C_2H_5
I_B R = C_4H_9 II_B R = C_4H_9

The second peak also showed ions at m/z 43, 83, and 111 for the terminal acetyl group, while ions at m/z 85, 69, and 97 represented C_4H_9CO, M-C_4H_9CO, and M-C_4H_9, respectively, indicating structures Ib or IIb. The presence in the same extract of 3-heptene-2-one, 5-heptene-2-one, and 2-heptanone as well as 5-nonene-2-one argued in favor of structures IIa and IIb while formation of dimethoximes (see below) supported the diketone function. Synthesis of the *E* isomers of IIa and IIb (see below) provided samples showing mass spectra and retention times identical with those of the natural products.

Upon closer examination (Figure 3), two minor components were observed which eluted before *E*-3-hepten-2,5-dione at 102°C. The first showed a mass spectrum and retention time identical to that of an authentic sample of heptan-2,5-dione. The second, minor, component showed a mass spectrum identical to that of *E*-3-hepten-2,5-dione, presumably from its *Z* isomer. In analogous fashion, two minor components were observed eluting just before *E*-3-nonen-2,5-dione and were assigned structures of nonan-2,5-dione and *Z*-3-nonen-2,5-dione. The retention times of all these minor components were identical to those of the synthesized samples.

Treatment of the *Trigona* extract with methoxyamine hydrochloride and pyridine followed by GC-MS analysis converted all of the ketones to syn-anti mixtures of their corresponding methoximes (Figure 4). Thus, a pair of peaks eluting at 80°C showed identical mass spectra corresponding to syn- and anti-isomers of 2-heptanone methoxime [MS: m/z (rel. %) 143 (3,$M^{+\cdot}$), 128(1), 114(6), 100(19), 87(68), 57(15), 52(14), 45(7), 43(16), 42(100), 41(31), and 39(17)]. A peak at 78°C represented the methoxime of 5-hepten-2-one (MS: m/z = 141($M^{+\cdot}$) and 126(M-CH_3), and a peak eluting at 115°C corresponded to the methoxime of 5-nonen-2-one [MS: m/z (rel. %) 169(3,$M^{+\cdot}$), 154(2), 138(10), 127(3), 126(7), 112(15), 100(7), 87(66), 57(18), 55(30), 42(100), 41(52), 29(17)].

All four of the theoretically possible syn-anti isomers of the dimethoxime of *E*-3-hepten-2,5-dione were observed (Figure 4, see Figure 1c for the mass spectrum of the isomer eluting earliest), along with three from the dimethoxime of *E*-3-nonen-2,5-dione [MS, m/z (rel. %) 212(38,$M^{+\cdot}$), 197(8), 181(100), 170(14), 166(31), 154(28), 151(4), 140(58), 139(30), 135(22), 125(41), 124(68), 109(4), 108(71), 42(90), 29(71), and 27(65)]. Three of the possible four isomers from the dimethoxime of heptan-2,5-dione appeared between 100°C and 150°C [MS of isomer eluting second: m/z (rel. %) 186(10, $M^{+\cdot}$), 155(45), 140(100), 109(45), 100(43), 54(50), and 42(99)]. The isomeric dimethoximes had very similar mass spectral fragmentation patterns in all cases. The peak eluting before the pyridine peak was due to methoxyamine free base.

A small portion of the *Trigona* extract was hydrogenated in the presence of PtO_2 using methanol as the solvent. All of the olefinic hydrocarbon peaks

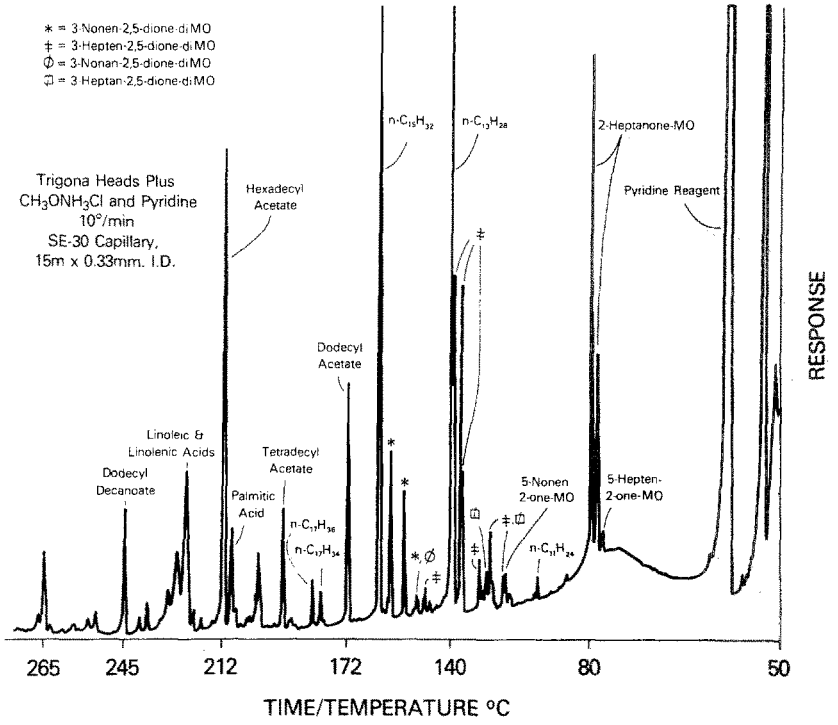


FIG. 4. Gas chromatogram of *T. tataira* heads extract treated with methoxyamine hydrochloride and pyridine. Compounds identified by mass spectra.

and olefinic monoketone peaks disappeared, presumably merging with their saturated counterparts. Likewise, the gas chromatographic peaks for 3-hepten-2,5-dione and 3-nonen-2,5-dione disappeared, and major peaks appeared whose mass spectra and retention times were identical to those of heptan-2,5-dione and nonan-2,5-dione, respectively.

The synthesis of the 3-en-2,5-diones was relatively straightforward and served to prove their structures and stereochemistry. Condensation of the appropriate aldehyde (propanal or pentanal) with methyl vinyl ketone in the presence of a thiazolium salt catalyst produced heptan-2,5-dione and nonan-2,5-dione in good yield (Stetter and Kuhlmann, 1974). Initially, the saturated 1,4-diketones were oxidized directly to the 3-en-2,5-diones with selenium dioxide (Figure 2) (Goldberg and Muller, 1938). This served to provide samples containing the starting diketones as well as both *E* and *Z* isomers of the unsaturated diketones, which were useful for GC-MS comparisons. To produce larger quantities of pure *E*-3-hepten-2,5-dione and *E*-3-nonen-2,5-dione, the two-step sequence shown in Figure 2 was found to be much more

practical. The saturated 1,4-diketones were cyclized by heating in the presence of the acid resin Dowex 50W-4X, (Scott and Naples, 1973), and the resulting 2,5-dialkylfurans were oxidized to *E*-3-en-2,5-diones with pyridinium chlorochromate (this oxidation is known to produce only the *E*-3-en-2,5-dione isomer) (Piancatelli et al., 1980). The products were purified by Kugelrohr distillation. Comparison of the natural 3-en-2,5-diones to the synthetic materials showed that the *Z* isomers are very minor components of the natural mixture.

DISCUSSION

The chemistry of the cephalic secretion of *Trigona* (*Oxytrigona*) *tataira* is radically different from those of *Trigona* species in other subgenera that have been subjected to similar analytical scrutiny. Indeed, only one of the oxygenated compounds detected as a cephalic product of *T. tataira* has been identified as a mandibular product of *Trigona* species in another subgenus. 2-Heptanone, a major secretory product of this bee (Figure 3), is present as one of a large series of methyl ketones which are accompanied by their corresponding carbinols in species in the subgenus *Scaptotrigona* (Luby et al., 1973). On the other hand, species in the subgenus *Geotrigona* synthesize the isomers of citral in their mandibular glands (Blum et al., 1970), whereas a species in the subgenus *Trigona* produces 2-alkanols and long-chain octyl esters in its glandular exudate (Kerr et al., 1981). Therefore, the diversity of products identified in cephalic extracts of *T. tataira* contrasts greatly with the conservatism observed with species in three other subgenera.

In a qualitative sense, workers of *T. tataira* clearly emphasize the production of ketones in their cephalic glands. The presence of 10 ketones, including both saturated and unsaturated mono- and diketones, provides the fire bee with a ketonic profile that is both distinctive and biosynthetically intriguing. The biogenetic relationship between the 2-heptanone, 3-hepten-2-one, 5-hepten-2-one, and *E*- and *Z*-3-hepten-2,5-dione on one hand and 5-nonen-2-one and *E*- and *Z*-3-nonen-2,5-dione on the other hand is particularly interesting. Are the monoketones precursors of the diketones or is the sequence reversed?

Diketones appear to be relatively rare in insects. A 1,3-diketone, 3,6-dimethylheptan-2,4-dione, has recently been discovered as a sex pheromone of the mushroom fly, *Megaselia halterata* (Wood), (Baker and Parton, 1982). Meinwald et al. (1972) and Schildknecht et al. (1972) have identified (*E,E,E*)-3,7-dimethyl-8,11-dioxo-2,6,9-dodecatrienal (gyrinidal, gyrinal) from gyrinid beetles.

The *E*-3-en-2,5-diones might be considered as a form of acyclic *p*-benzoquinone. They undergo similar Michael addition as the first step of their

reactivity toward basic nucleophiles (Piancatelli et al., 1980) and a cyclic *E*-3-en-2,5-dione has been reported to undergo both Lewis acid- and base-catalyzed Michael addition (Danishefsky et al., 1981). Similarly, it has been suggested that the potency of *p*-benzoquinones as defensive compounds may stem from their ability to function as Michael acceptors (Blum, 1981).

The saturated forms of these 1,3-diketones may also provide a clue to the formation of 2,5-dialkylpyrrolidines that are found in the unrelated ant species *Solenopsis fugax* (Blum et al., 1980). Their reductive condensation with ammonia to provide pyrrolidines has, in fact, been used extensively for their synthesis in the laboratory (Jones et al., 1980). However, pyrrolidines with side chains corresponding to those that would result from these particular diketones have not yet been encountered.

The ability of workers of *T. tataira* to plunder the nests of other species of bees with impunity may be related to their possession of a cephalic secretion that functions as a powerful chemical disruptant. Highly reactive compounds such as the endiones may constitute defensive allomones that disrupt the defensive cohesiveness of worker bees whose colonies are under attack, resulting in the pronounced disorientation that characterizes the behavior of bees being raided by the aggressive fire bees. Furthermore, the pronounced vesicatory properties of the cephalic secretion of workers presumably are also identified with specific highly reactive compounds which fortify their glandular exudates. We are now studying the effects of the compounds identified as cephalic products of *T. tataira* in order to ascertain if they are key elements of the chemical weaponry that fire bee workers direct against both honeybees and vertebrates. We also note the close relationship between IIa and 6-(2-formyl-3-methylcyclopentyl)-E-3-hepten-2,5-dione (Miller *et al.*, 1975) and its hemiacetal, gyrinidione (Wheeler *et al.*, 1972).

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NOVEL SEX PHEROMONE COMPONENTS FROM THE FALL CANKERWORM MOTH, *Alsophila pometaria*[1,2]

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Abstract—A sex pheromone extract from fall cankerworm moths, *Alsophila pometaria*, attracted conspecific males in field tests. Four EAG-active components were isolated from the extract and identified by GC-MS, high-field PMR spectroscopy, and microchemical techniques as *n*-nonadecane (I), (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene (II), (*Z*,*Z*,*Z*,*E*)-3,6,9,11-nonadecatetraene (III), and (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-nonadecatetrane (IV). Studies of the behavioral responses of male moths in a flight tunnel to the isolated components showed II, III, and IV were the major components of the sex pheromone. No sex pheromone behavioral responses were observed for I.

Key Words—*Alsophila pometaria* (Harris), fall cankerworm, Lepidoptera, Geometridae, sex pheromone, (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene, (*Z*,*Z*,*Z*,*E*)-3,6,9,11-nonadecatetraene, (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-nonadecatetraene.

INTRODUCTION

In contrast to other major families of Lepidoptera where sex pheromones and sex attractants are known for several species (Inscoe, 1982), the pheromone of only one geometrid species, the winter moth, *Operophtera brumata* (L.), has been identified (Roelofs et al., 1982; Bestmann et al., 1982). The winter moth pheromone, (*Z*,*Z*,*Z*)-1,3,6,9-nonadecatetraene, constitutes another example of a new group of pheromone components, unsaturated hydrocarbons, which have been reported in the last few years from species of Arctiidae (Conner et al., 1980; Hill and Roelofs, 1981; Hill et al., 1982) and

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Noctuidae (Heath et al., 1983; Underhill et al., 1983). Underhill et al. (1982) reported males of 15 geometrid species gave strong EAG responses to one or more unsaturated C_{18-22} hydrocarbons and monoepoxides substituted at positions 3, 6, and 9. These results suggested unsaturated hydrocarbons and/or monoepoxides could be pheromone components for several geometrid species. Included in the group of responding geometrids was the fall cankerworm, *Alsophila pometaria* (Harris).

Larvae of the fall cankerworm are major defoliators of ornamental trees in North America including elm, oak, apple, beech, and linden. In late fall when the temperature nears freezing, adult moths emerge and the vestigial-winged females climb trees or buildings where mating occurs and eggs are deposited. Methods of controlling populations of fall cankerworm have included use of *Bacillus thuringiensis*, insecticides, and banding tree trunks with sticky materials to entrap adult females (Appleby et al., 1975; Johnson and Lyon, 1976.) Population control by mating disruption using the insect's sex pheromone is another alternative. This may be particularly useful for fall cankerworm control since the females cannot fly from non-treated areas into pheromone disrupted areas, a common drawback to this control method (Rothschild, 1982). To this end we set out to characterize the female sex pheromone.

METHODS AND MATERIALS

Insects and Pheromone Extraction. Fall cankerworm adults were collected locally from the field. Males were hand collected after sunrise as they emerged from grass and climbed trees and buildings to expand their wings. Males were kept in an incubator maintained at 10°C for at least 24 hr prior to EAG assays or flight tunnel studies. Females were obtained by entrapment in a corn syrup–corn starch mixture applied around elm tree trunks at a height of 0.5 m. Pheromone extract was prepared from approximately 5000 excised ovipositors by extraction with three portions of dichloromethane. The combined dichloromethane extracts were dried over anhydrous magnesium sulfate, filtered, concentrated to a volume of 4 ml under a stream of nitrogen, and stored at −20°C until required for bioassay or pheromone isolation.

Electroantennography (EAG) and Flight Tunnel. EAG measurements were performed as described previously (Chisholm et al., 1975).

Behavioral responses of males to ovipositor extract and to GC-isolated components were studied using a flight tunnel whose operation and construction have been described elsewhere (Palaniswamy et al., 1983). Field-collected males, tested individually within the first 2 hr of scotophase, were placed in wire release cages located at the downwind end of the tunnel in the path

of the odor plume. After a 3-min baseline period, if the moth remained calm, the odor source was introduced 208 cm upwind. Males were recorded as being nonresponsive if they failed to show wing fanning within 3 min after sample introduction. Moths were considered to have initiated plume-oriented flight if they followed the plume for at least 10 cm after release. Plume-oriented flight was considered terminated when the moth landed on the source or left the plume area and did not reorient in the plume. Odor sources were prepared prior to each day's test by application of 0.35 female equivalents (FE) of ovipositor extract, or an equivalent amount of GC-isolated component, to filter paper disks. Males were exposed only once to a treatment and then discarded. Tests were done on several days and on each day moths were exposed to the nonfractionated ovipositor extract. No specific day-to-day variability in response was observed. Data from all testing days were combined and the percentages were transformed (arcsin $\sqrt{\chi}$). The significance of treatment effects was determined by χ^2 analysis, and the means were compared by Duncan's multiple-range test using a pooled binomial error variance. Means of transformed (ln X) flight time data were compared by Duncan's multiple range test using the error mean square from one way analysis of variance.

Field bioassay. Pherocon ICP® traps (Zoecon Corp., Palo Alto, California) were baited in the afternoon of each day's tests with filter paper disks containing 2 FE of ovipositor extract or with six field-collected females held within a wire screen cage. Traps were set at a height of 1 m on stakes 20 m apart near a line of elms known to have harbored fall cankerworm larvae during the preceding months.

Fractionation of Ovipositor Extract and Pheromone Isolation. The fractionation of ovipositor extract for EAG assays was carried out on a Hewlett-Packard model 5710A gas chromatograph equipped with a 18740B capillary inlet system and an effluent splitter (85:15). A sample which contained 5 FE of ovipositor extract was injected under splitless conditions onto an OV-17 column (30 m × 0.32 mm ID, Quadrex Corp., New Haven, Connecticut) programed from 90° to 230°C at 4°C/min. Fifteen-second fractions were collected in glass capillary tubes (126 × 0.65 mm ID), cooled with Dry Ice, and assayed by EAG shortly after collection. Fractions which elicited an EAG response were then rechromatographed on a DB-5 column (30 m × 0.32 mm ID, J&W Scientific Inc., Rancho Cordova, California), 90° to 230° at 4°C/min, and the fractionated material (15-sec fractions) assayed by EAG. Submicrogram quantities of EAG-active components were similarly obtained for flight tunnel studies and microchemical reactions by repetitive fractionations.

Compounds III and IV were isolated for proton magnetic resonance (PMR) spectroscopy by a combination of HPLC and GC. HPLC was performed on a Spectra-Physics SP8700 liquid chromatograph equipped with a

Spectroflow 773 spectrophotometer. A sample of concentrated ovipositor extract (60 μl, 1500 FE) was loaded onto a Partisis PXS 5/25 column (25 cm $\times$ 4.6 mm ID) and eluted with isopropyl alcohol in hexanes (0.1% from 0 to 10 min, then gradient to 2.0% at 20 min) at a flow rate of 1 ml/min. The effluent was monitored at 215 nm (0.005 AUFS), and 1-ml fractions were collected. This procedure was repeated on a second sample of ovipositor extract (1500 FE) and material which eluted between 4 and 5 ml was collected from both fractionations and concentrated for purification on the DB-5 column under the conditions described previously. Compounds III and IV were washed directly from the collection tubes into 5-mm PMR tubes, which were constricted at the bottom end (50 $\times$ 1.77 mm OD, Wilmad Glass Co. Inc., Buena, New Jersey), with 99.9% deuterated benzene (Sigma Chemical Co., St. Louis, Missouri).

GC Quantitation of Pheromone Components. GC quantitation of pheromone components was carried out by the internal standards method with *n*-octadecane as the internal standard. No corrections were made for differences in response factors.

Spectroscopic Methods. Chemical ionization (CIMS, isobutane reagent gas) and electron impact (EIMS) mass spectral analyses were obtained using Finnigan model 3300 and 4000 mass spectrometers, respectively, equipped with DB-5 columns (60 m $\times$ 0.32 mm ID). PMR spectra of pheromone components were obtained using a Bruker WH-400 spectrometer. Chemical shifts were calculated from benzene [δ(TMS) = δ(benzene) + 7.20 ppm].

Microscale Epoxidation and Hydrogenation of Pheromone Components. The position and geometry of double bonds in polyunsaturated hydrocarbon pheromone components was determined by an epoxidation-hydrogenation procedure (Underhill, in preparation) carried out as follows. To 0.5 μg of isolated pheromone component in 50 μl of dichloromethane was added 2 μl of a 0.065% solution of *m*-chloroperbenzoic acid in dichloromethane. The reaction mixture, which was contained in a conical-bottom glass vial, was thoroughly mixed and then kept at 22° C for 18 hr. The product, consisting primarily of unsaturated monoepoxidized hydrocarbons was hydrogenated over a trace of 5% platinum on neutral alumina with stirring for 30 min at 22° C. The hydrogenated material was then filtered through a small plug of glass wool to remove catalyst and concentrated for GC-MS analysis to determine the location of the epoxide moieties in the saturated monoepoxides (Bierl-Leonhardt et al., 1980). The geometries of the epoxides, and hence of the double bonds from which they were derived, were assigned by comparing their GC retention times (DB-5, 60 m $\times$ 0.32 mm ID) with those of reference standards. Catalytic hydrogenation without prior epoxidation was carried out under conditions identical to those described above.

RESULTS AND DISCUSSION

Field tests established the extract of female fall cankerworm ovipositors possessed pheromone activity. Conspecific males were observed entering extract-baited traps from the time they were placed in the field at 1500 hr (MST) until approximately 2030 hr, which was 2 hr into scotophase. Traps baited with 2 FE of extract on filter paper captured fall cankerworm males specifically, the mean daily capture per trap was 11.1 ± 7.9 ($N = 7$), while female-baited traps caught 7.7 ± 4.2 ($N = 3$). Blank traps caught no males. Although the numbers of males caught in extract- and female-baited traps were similar, the values are not comparable because the number of unmated females used to prepare the extract and to bait traps was not determined. Pheromone release from extract-impregnated filter papers was inadequate for male attraction after one day in the field. Freshly prepared baits consisting of rubber septa treated with 2 FE of ovipositor extract were ineffective for capturing males, probably because the release rate from rubber septa was lower than from filter paper.

EAG assays of 15-sec fractions obtained from the GC fractionation (OV-17 column) of ovipositor extract revealed the presence of four well-resolved peaks which elicited strong EAG responses. These peaks for components I, II, III, and IV eluted at 24.55, 25.21, 27.19, and 27.88 min, respectively. Standards of *n*-octadecane and *n*-heneicosane eluted at 21.78 and 29.80 min. The approximate quantities of the EAG-active components in the ovipositor extract was determined by GC to be 13.7, 12.0, 27.2, and 7.0 ng per FE for I, II, III, and IV, respectively.

The behavioral responses of field-collected males to the EAG-active components were studied in a flight tunnel. The four components, which were collected from both the OV-17 and the less polar DB-5 columns, yielded single symmetrical peaks on the DB-5 column with retention times of 25.03, 25.73, 26.30, and 26.83 min for components II, I, III, and IV, respectively (*n*-octadecane 23.10 min, *n*-heneicosane 30.67 min). Flight tunnel behavioral tests (Table 1) showed lures containing I and II alone or together did not induce a wing-fanning response in males, while those containing III or IV alone or III and IV together elicited wing fanning and plume-oriented upwind flight. The onset of wing fanning was rapid for all treatments and occurred within 1–5 sec of the plume reaching test males. More than 55% of moths which initiated plume-oriented flight in response to III or IV alone and to III + IV landed on the source as did all males exposed to the whole extract or components I + II + III + IV. Although additions of component II to lures containing III + IV did not result in a significant difference in the percentage of males which landed on the source, a marked difference in their behavior to these sources was noted, particularly their flight behavior as

TABLE 1. FLIGHT TUNNEL BEHAVIORAL RESPONSES OF FALL CANKERWORM MALES TO OVIPOSITOR EXTRACT OR ISOLATED EAG-ACTIVE COMPONENTS

Treatment[a]	Wing fanning		Plume-oriented flight initiation (%)[c,d]	Landing on source (%)[c,e]	Flight time within 35 cm of the source	
	N[b]	Response (%)			N[b]	$\bar{X}$ (sec)[c]
Whole extract	7	100 a	86	100 a	5	4.0 c
I + II + III + IV	5	100 a	100	100 a	5	5.2 bc
II + III + IV	19	100 a	100	90 ab	8	4.1 c
III + IV	16	94 a	80	58 b	7	15.3 a
III	17	100 a	88	60 b	7	18.1 a
IV	14	79 a	82	56 b	6	10.2 ab
I + II	5	0 b				
I	5	0 b				
II	5	0 b				

[a]0.35 FE of ovipositor extract or equivalent of isolated components; I = *n*-nonadecane; II = (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene; III = (*Z*,*Z*,*Z*,*E*)-3,6,9,11-nondecatetraene; IV = (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-nonadecatetraene.
[b]Number of males tested.
[c]Values followed by the same letter in a column are not different ($P = 0.05$).
[d]Percentage based on the number of moths which fanned; values were not different ($P = 0.05$).
[e]Percentage based on the number of moths which initiated plume-oriented flight. Plume-oriented upwind flying moths that reached the source but did not stay on the source for at least 2 sec were not considered to have landed.

they neared the source. In response to lures containing III or IV or III + IV, males exhibited hesitant forward progress as they came within 35 cm of the source. This was characterized by reduction in ground speed or even complete arrest of forward progress followed by looping flights in and around the plume area. No such hesitation was observed for males responding to lures in which II had been added, thus accounting for their shorter flight time within 35 cm of the source. Furthermore, in choice tests between sources containing II + III + IV and III + IV located 8 cm apart, eight of 10 males tested landed on the three-component lure. This indicates that II may be involved in maintaining upwind flight at close range and in landing. No recognizable behavioral effect could be attributed to I. The results of our behavioral studies suggest that the fall cankerworm utilizes a three-component pheromone blend for sex attraction.

The CIMS of the EAG active components indicated the molecular weights of 268, 262, 260, and 260 for I, II, III, and IV, respectively. The EIMS of I was characteristic of an unbranched, saturated hydrocarbon. Comparison of the GC retention times (two capillary columns) and EIMS of I with those of an authentic standard confirmed that I is *n*-nonadecane.

Catalytic hydrogenation of II, III, and IV all yielded *n*-nonadecane, thus demonstrating that these components are unbranched, unsaturated C_{19} hydrocarbons. Component II was identified as (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene based on comparisons of GC retention times and EIMS of the natural material and synthetic material (Underhill et al., 1983). The spectrum of II (Figure 1) contains prominent ions at *m*/*z* 55 $(CH_3CH_2CH{=}CH)^+$, 108 $[CH_3CH_2(CH{=}CH)_3H]^+$, and 206 $[CH_3(CH_2)_8(CH{=}CH)_3H]^+$ which indicate the 3 and 9 positions of the two outer double bonds in a methylene-interrupted triene (Karunen, 1974, and references therein). Partial epoxidation followed by catalytic hydrogenation of II gave a mixture of three monoepoxynonadecanes whose relative retention times (RRT, relative to *n*-nonadecane, 60 m DB-5 column) and EIMS alpha-cleavage ions (Bierl-Leonhardt et al., 1980) coincided with those of *cis*-3,4-epoxynonadecane (RRT = 1.178, alpha-cleavage ions at *m*/*z* 71 and 253), *cis*-6,7-epoxynonadecane (RRT = 1.162, *m*/*z* 113 and 211), and *cis*-9,10-epoxynonadecane (RRT = 1.158, *m*/*z* 155 and 169). The *trans*-epoxynonadecanes elute prior to, and clearly separated from, the *cis* isomers.

The EI spectra of III (Figure 1) and IV were similar and contained prominent ions at *m*/*z* 55 and 108 which indicated a double bond at C_3 as part of a methylene-interrupted series of double bonds. The structures of components III (7.4 μg) and IV (1.6 μg), collected from the DB-5 column, were clearly revealed by 400-MHz PMR spectroscopy (Figure 2). The presence of eight proton signals in the olefinic region of the spectrum showed that the four unsaturations were accounted for by four double bonds. Since there are only four allylic protons and four bis-allylic protons, three of the double bonds must be methylene interrupted and one must be conjugated. A comparison of the spectrum of synthetic II, in which all the vinyl protons have signals in the 5.25–5.45 ppm region, with that of III confirms the presence of a conjugated diene since three of the vinyl proton signals appear at the lower field (Cárdenas, 1969). The positions of the double bonds were determined by decoupling experiments. Irradiation at the frequency of the upfield allylic methylene (δ 2.06) caused the simultaneous simplification of the downfield methyl triplet and the multiplet for the two vinyl protons at δ 5.47, thus confirming the presence of the 3,4 double bond. The collapse of two vinyl proton multiplets (δ 5.47 and δ 5.52) as a result of irradiation at the frequency of the upfield bis-allylic methylene (δ 2.89) showed the presence of the 6,7 double bond. Irradiation at the frequency of the downfield bis-allylic methylene resulted in simplification of the signals at δ 5.52 and δ 5.42, thus demonstrating the presence of the 9,10 unsaturation. These experiments established the 3,6,9 methylene-interrupted triene system. The assignments of the three low field signals to H-12, H-10, and H-11 of a 9*Z*,11*E* conjugated diene system were based on chemical shifts, coupling constants, and decoupling experiments. Thus irradiation at the frequency of H-10 resulted in

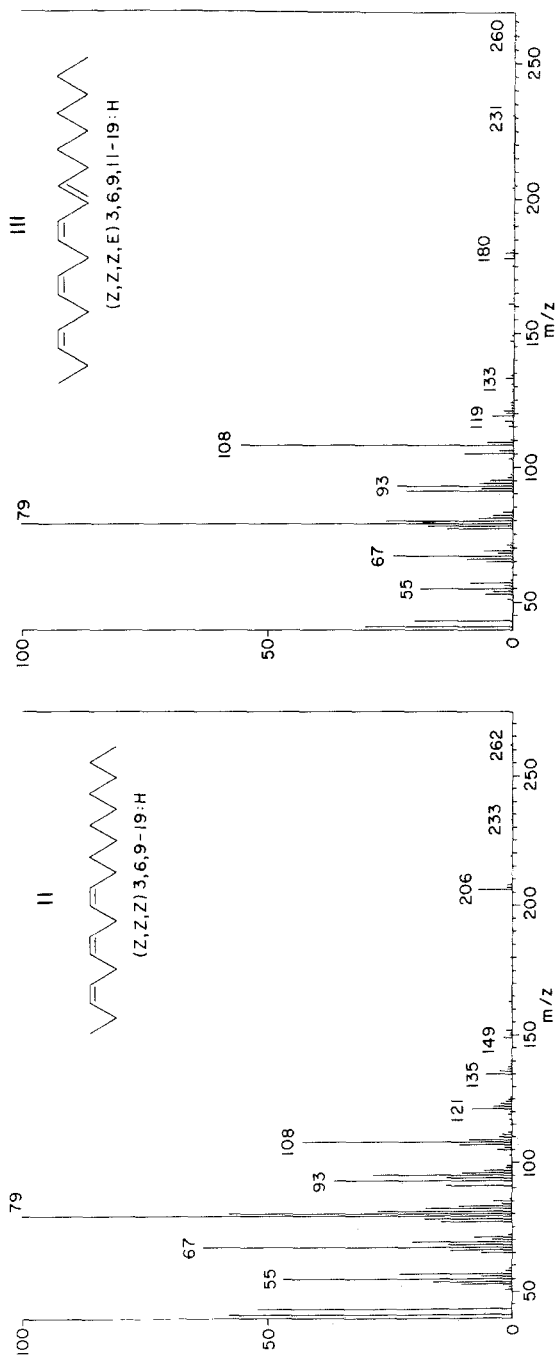

FIG. 1. EIMS of (*Z*,*Z*,*Z*)-3,6,9-nonadecatriene (II) and (*Z*,*Z*,*Z*,*E*)-3,6,9,11-nonadecatetraene (III).

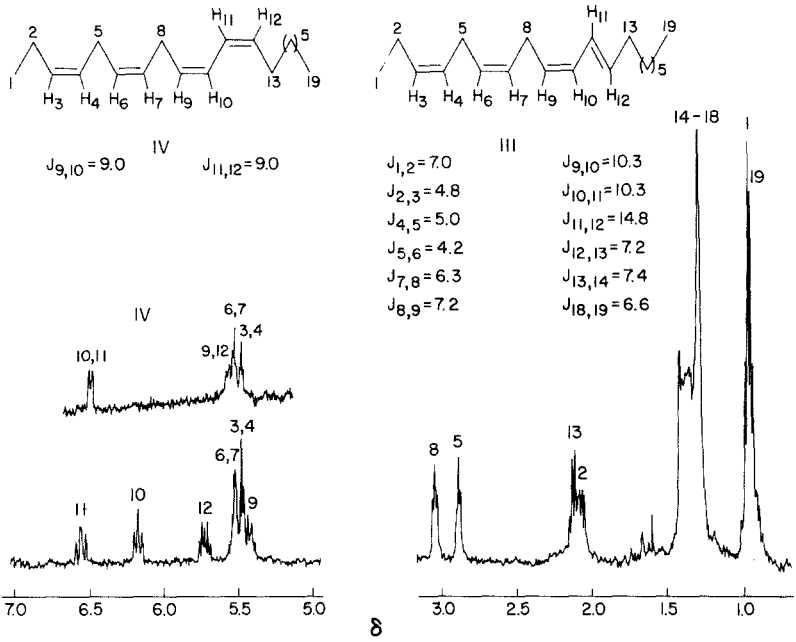

FIG. 2. 400-MHz PMR spectrum (C_6D_6) of (*Z,Z,Z,E*)-3,6,9,11-nonadecatetraene (III) and (inset) partial spectrum of (*Z,Z,Z,Z*)-3,6,9,11-nonadecatetraene (IV) [*J* in Hz].

simplification of the signals for H-9 and H-11, while irradiation at the frequency of H-11 caused simplification of the signals for H-10 and H-12. These results indicated that the double bonds in III were located at the 3,6,9, and 11 positions. Partial epoxidation of isolated III, followed by catalytic hydrogenation, gave four monoepoxynonadecanes whose structures were consistent with the assignment of double bonds by PMR. The retention times and alpha-cleavage ions derived from three of the monoepoxynonadecanes were the same as those obtained from component II confirming a *Z*3, *Z*6, *Z*9 configuration. The retention time of the fourth monoepoxynonadecane (RRT = 1.146), with alpha-cleavage ions at *m/z* 141 and 183, corresponded with authentic *trans*-8,9-epoxynonadecane (equivalent to *trans*-11, 12-epoxynonadecane). Thus III was identified as (*Z,Z,Z,E*)-3,6,9,11-nonadecatetraene.

The PMR spectrum of IV is similar to that of III, δ (C_6D_6) 0.96 (3H, t, $J_{18,19}$ = 6.0 Hz, CH_3, 19), 0.97 (3H, t, $J_{1,2}$ = 7.2 Hz, CH_3, 1), 1.25–1.47 (10H, m, CH_2, 14–18), 2.06 (2H, m, CH_2, 2), 2.20 (2H, m, CH_2, 13), 2.87 (2H, m, CH_2, 5), 3.02 (2H, m, CH_2, 8), 5.46 (2H, m, CH, 3,4), 5.50 (2H, m, CH, 6,7), 5.55 (2H, m, CH, 9,12), 6.47 (2H, m, CH, 10,11). The presence of the two

methyl triplets, methylene envelope, two allylic methylene multiplets, two bis-allylic methylene signals, and the signals for eight vinyl protons suggested that IV is also a 3,6,9,11-nonadecatetraene. Only the olefinic region of the spectrum (Figure 2 inset) differed greatly from the corresponding region in the spectrum of III. The deshielding of H-10, relative to its chemical shift in the spectrum of IV, is presumably due to the effect of the C-13 methylene brought closer to H-10 by a *Z* configuration of the 11,12 double bond (Cárdenas, 1969). GC-EIMS analyses of the monoepoxynonadecanes derived from partial epoxidation and catalytic hydrogenation provided further evidence which indicated the structure of IV as (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-nonadecatetraene. The notable difference in the monoepoxides derived from components III and IV was the presence of *cis*-8,9-epoxynonadecane with a retention time of 1.158 (relative to *n*-nonadecane) in IV.

Confirmation of the structures of III and IV was achieved by synthesis of the components. The reaction of *n*-octyltriphenylphosphorane with (*Z*,*Z*,*Z*)-2,5,8-undecatrienal gave a mixture of III and IV which was identical to isolated III and IV on the basis of GCMS analyses. Details of the syntheses will be reported elsewhere.

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DEFENSIVE BEHAVIOR AND TOXICITY OF ASCOGLOSSAN OPISTHOBRANCH *Mourgona germaineae* MARCUS

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Abstract—The ascoglossan (= sacoglossan) opisthobranch *Mourgona germaineae* Marcus secretes a viscid mucus and autotomizes cerata when mechanically disturbed. Other small invertebrates, i.e., sea anemones, amphipods, and other ascoglossans, will die when placed with these autotomized cerata or in the water in which they have been autotomized. The toxin is methanol-soluble and water-soluble and thus is probably a small molecule. Simultaneous TLC of chloroform and methanol–water extracts of *M. germaineae* and of its food alga, *Cymopolia barbata* indicates that the toxin is most likely of dietary origin.

Key words—Ascoglossa, chemical defense, toxicity, diet-derived toxin, *Mourgona germaineae, Cymopolia barbata.*

INTRODUCTION

Shell-less mollusks are known to secrete toxic or noxious defensive secretions (Thompson, 1960; Edmunds, 1966, 1968; Lewin, 1970; Ros, 1976). The increased interest in marine natural products in the fields of toxicology and pharmacology has made it important to elucidate the nature of these defensive secretions (for reviews of marine natural products see Faulkner and Fenical, 1977; Scheuer, 1978). One group of opisthobranch mollusks, the dorid nudibranchs, derive their toxic and pharmacologically active substances mainly from the sponges on which they feed (Burreson et al, 1975; Fuhrman et al., 1979, 1980; Kitting, 1981; Cimino et al., 1982; Hellou et al., 1982; Hochlowski et al., 1982; Schulte and Scheuer, 1982; Thompson et al., 1982; Walker and Faulkner, 1981). Sea hares, *Aplysia* spp., contain toxic sub-

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stances derived from their food algae (Stallard and Faulkner, 1974; Stallard et al., 1978; Andersen and Sum, 1980; Hollenbeak et al., 1979; Schmitz et al., 1979, 1980).

Ascoglossans (= sacoglossans), another group of opisthobranch mollusks, are generally stenophagous herbivores, feeding on the cell sap of siphonaceous green algae (Jensen, 1980, 1981). Many of these algae contain toxic or noxious substances (Doty and Santos, 1970; Hellebust, 1974; Högberg et al., 1976; Sun and Fenical, 1979; Paul et al., 1982). These substances are thought to act as herbivore feeding deterrents (Stallard and Faulkner, 1974; Sun and Fenical, 1979; Paul et al., 1982), and, indeed, ascoglossans seem to be the only herbivores feeding on some of these algae (personal observations). Several ascoglossans excrete noxious defensive secretions (Lewin, 1970; Ros, 1976), but the noxious substances have not been identified and neither have their sources of origin. Several unique metabolites have been identified in ascoglossans (Ireland et al, 1978, 1979; Ireland and Scheuer, 1979; Ireland and Faulkner, 1981), but the toxicity of these substances has not been tested.

Mourgona germaineae Marcus was originally described from one specimen found in an aquarium in Puerto Rico (Marcus and Marcus, 1970), and it has only been found in one locality since then, namely at Geiger Key, Florida (Jensen, 1981; Jensen and Clark, 1983). It feeds exclusively on cell sap of the green alga *Cymopolia barbata* (Jensen, 1981). This alga contains a number of bromohydroquinones with toxic or antibiotic effects (Högberg et al., 1976). Like most other ascoglossans, *M. germaineae* will excrete copious mucus when irritated mechanically (personal observation), and on previous occasions it has been noted that other animals, including other ascoglossan species, placed in jars containing *M. germaineae* will become moribund or die within 8–36 hr.

This study investigates the toxicity of *M. germaineae* by determining lethal doses to other marine invertebrates. Some sublethal effects are described also, and an attempt is made to identify the toxic substance(s).

METHODS AND MATERIALS

Mourgona germaineae were collected in June and October 1982, and in January 1983, at Geiger Key Florida along with their food alga *Cymopolia barbata.* Animals and algae were kept for up to 8 weeks at approx. 20° C in 30-liter aquaria with subgravel filters and illuminated by 40-W fluorescent bulbs when not used for experiments. *Aiptasia* sp., *Gammarus mucronatus,* and *Ercolania fuscata* were collected weekly in the Indian River Lagoon, Florida, and kept in 30-cm fingerbowls with excess food.

Cerata were teased off *Mourgona germaineae* by touching a small steel spatula to the dorsal surface of the animal. The autotomized cerata would

generally stick to the spatula, which had to be vigorously shaken into seawater or extraction fluid to dislodge the cerata. For toxicity experiments, the autotomized cerata were placed in Millipore-filtered seawater (MFSW) (approximately 1 ml/animal for *Aiptasia* sp. and 1.7 ml/animal for *G. mucronatus* and *E. fuscata*) and an experimental animal would be added (volume determined accurately at end of each experiment). Experiments lasted for 1/2 to 24 hr, after which any surviving animals were placed in fresh MFSW and observed for another 24 hr. Dead animals, *M. germaineae* cerata, and surviving animals after 24 hr in clean seawater were dried at 80° C for 48–96 hr, and their dry weights determined. Control animals were placed in MFSW concurrently with the experiments. Doses were calculated by dividing dry weight of *M. germaineae* cerata by volume MFSW in vial and multiplying this by the duration of the experiment in hours.

Some cerata and one whole *M. germaineae* were fixed in 4% neutral formaldehyde, dehydrated in a series of EtOH , and embedded in paraffin. Sections were cut at 5 μm and stained with Mallory's trichrome stain. A few formalin-fixed cerata were placed directly in Sudan black or Alcian blue for 30 min, rinsed briefly in water and embedded in glycerin jelly.

To characterize the toxin, *M. germaineae* cerata were placed in a boiling water bath and bioassayed as above. Also, *M. germaineae* cerata were extracted overnight in distilled H_2O, MeOH, or a mixture of $CHCl_3$ and MeOH (2:1). The solvents were decanted off, and the cerata resuspended in MFSW and bioassayed as above. $CHCl_3$ and H_2O were added to MeOH extracts, and solvents were evaporated. The residues were then resuspended and/or redissolved in MFSW and bioassayed as above.

For identification of toxin, cerata were teased off a number of *Mourgona germaineae* and extracted twice in MeOH for 24–36 hr at −5° C. The combined MeOH extracts were centrifuged at 12,000 rpm for 30 min. $CHCl_3$ was added to the supernatants in the ratio of 2:1 (v/v), and after 1–4 hr at −5° C the phases were separated by the addition of H_2O. $CHCl_3$ was evaporated at 60° C. The organic residue was redissolved in 100 μl $CHCl_3$ and applied to a silica gel TLC plate, which was subjected to ascending chromatography with $CHCl_3$ as solvent. Spots were detected by spraying with H_2SO_4, and R_f values calculated. MeOH–H_2O extracts were likewise evaporated at 60° C, redissolved in MeOH, and separated by TLC, using *n*-butanol–glacial acetic acid–H_2O (12:3:5) as solvent. Protein was determined in the MeOH-insoluble residues.

Samples of *Cymopolia barbata* were extracted twice in MeOH. Insoluble material was removed by centrifugation as above, and the supernatant separated between $CHCl_3$ and MeOH by the addition of H_2O. After evaporation of solvents at 60° C, the residues were cochromatographed with the corresponding extracts of *M. germaineae* cerata.

RESULTS

Defensive Behavior. Mourgona germaineae is cryptically colored but can be fairly easily dislodged from its algal substrate unless it is in the process of feeding. When dislodged or mechanically disturbed, it rolls up in a ball, covering the entire body surface with its cerata and secreting a viscid mucus. If the disturbance is violent, multiple cerata are autotomized. Sometimes this ball of autotomized cerata is larger than the remaining animal, and will easily confuse a potential predator. The autotomized cerata secrete a sticky, milky-white, viscid mucus from their entire surface. This mucus diffuses slowly into the surrounding seawater; the white color disappears in less than one minute,

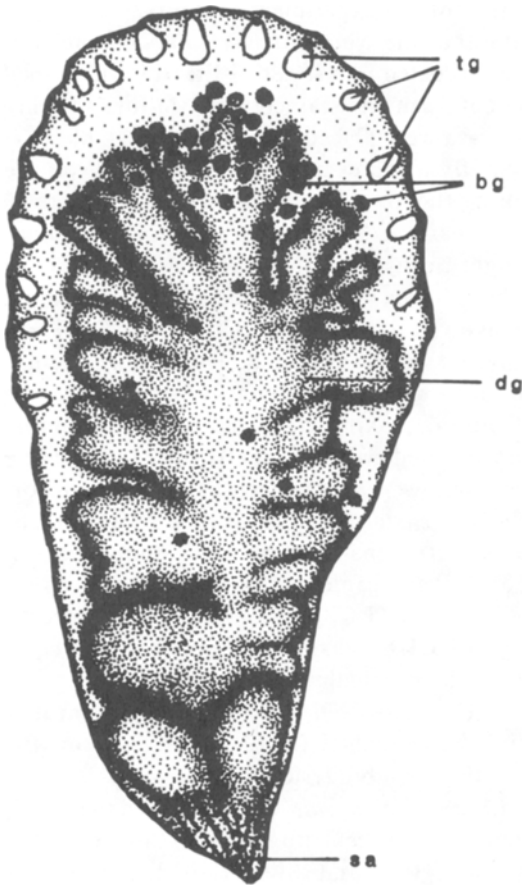

FIG. 1. Whole ceras of *Mourgona germaineae.* Total length ca. 4 mm; bg, light brown glands; dg, digestive gland; sa, site of autotomy; tg, transparent glands.

but the viscosity does not change noticeably for at least an hour. Older cerata, which are located mediodorsally, are most readily autotomized. The digestive gland occupies relatively less space in these cerata and is often paler green than in the younger, more ventral cerata.

Histology of Cerata. The cerata of *Mourgona germaineae* are flat, leaflike structures containing a branch of the digestive gland (Figure 1). A number of transparent, subepithelial glands are located along the border of the cerata, and a number of light brown, granular glands are located around the branched digestive diverticulum. A section through a ceras shows the branches of digestive gland (Figure 2) containing peculiar tetrahedral "crystals," which stained brightly orange with Mallory's trichrome stain. These crystals could also be seen in squash preparations of freshly autotomized cerata, but were never seen in sections of the part of the digestive gland located in the body of the slug. Occasionally a few crystals were seen in the rectum, indicating that they are indigestible and periodically excreted with fecal material. The crystals stained weakly with Alcian blue, and not at all with Sudan black, indicating that they do not contain lipid material, but possibly a small amount of acid mucopolysaccharides. The location of these crystals

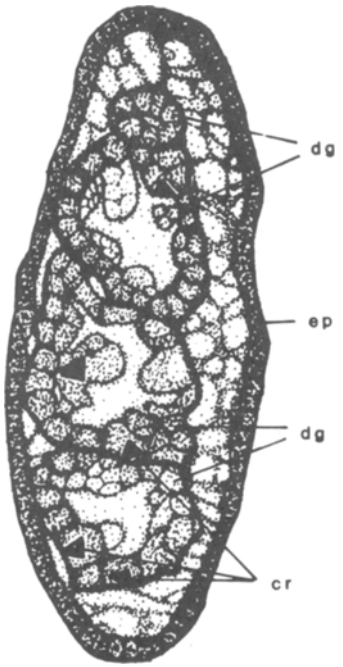

FIG. 2. Longitudinal section through ceras of *Mourgona germaineae.* Total length ca. 0.2 nn; cr, tetrahedral "crystals"; dg, digestive gland; ep, epithelium.

TABLE 1. MORTALITY IN *Aiptasia* SP., *Gammarus mucronatus* AND *Ercolania fuscata* SUBJECTED TO VARYING DOSES OF *Mourgona germaineae* CERATA.[a]

Time (hr)	Dry wt. cerata (mg)	Conc. cerata (mg/ml)	Dry wt. exp. animal (mg)	Dose (conc. × t)	M% (N)
Aiptasia sp.					
17	1.18	0.06	n.d.	1.02	25 (20)
24	1.70	0.09	n.d.	2.16	20 (20)
24	0.68	0.07	n.d.	1.68	67 (3)
				Dose (dwc × t)/dwa[e]	M
Gammarus mucronatus[b]					
17	1.48	n.d.[d]	0.7	35.94	+
17	1.05	n.d.	0.52	34.33	+
17	0.93	n.d.	0.76	20.8	+
12	0.63	n.d.	0.7	10.8	+
5	1.81	n.d.	1.05	8.62	+
5	1.48	n.d.	1.00	7.4	+
5	1.05	n.d.	1.13	4.65	+
5	0.93	n.d.	1.14	4.08	+
4.5	0.65	0.43	n.d.		+
4.5	0.47	0.28	n.d.		+
4.5	0.25	0.16	n.d.		+
4.5	0.25	0.18	n.d.		−
4	0.93	0.58	1.11	3.35	−
4	0.88	0.52	0.78	4.51	+
4	0.72	0.42	1.09	2.64	+
4	0.18	0.11	0.95	0.76	+
3	0.79	0.61	1.00	2.37	+
3	0.53	0.28	1.03	1.54	+
3	0.49	0.29	1.36	1.08	−
3	0.33	0.22	1.31	0.76	−
2	0.91	0.7	0.61	2.98	−
2	0.71	0.55	0.96	1.48	+
2	0.51	0.32	0.94	1.09	−
2	0.34	0.23	1.12	0.61	+
Ercolania fuscata[c]					
18	0.55	0.37	0.22	45.0	+
18	0.48	0.4	0.23	37.56	+
18	0.31	0.22	0.09	62.0	+
12	0.71	0.51	0.26	32.77	+
12	0.45	0.3	0.2	27.0	+
12	0.3	0.21	0.17	21.18	−
10	1.07	0.63	0.18	59.44	−
10	0.97	0.61	0.27	35.93	+
10	0.56	0.33	0.17	32.94	+
6	0.56	0.33	0.14	24.0	−
6	0.53	0.31	0.04	79.5	+

TABLE 1. CONTINUED

Time (hr)	Dry wt. cerata (mg)	Conc. cerata (mg/ml)	Dry wt. exp. animal (mg)	Dose (dwc × t)/dwa[e]	M
6	0.45	0.26	0.19	14.21	−
3	1.65	1.03	0.08	61.88	+
3	1.26	0.7	0.1	37.8	+
3	0.96	0.6	0.08	36.0	+
3	0.27	0.16	0.17	4.76	−
3	0.26	0.16	0.15	5.2	−
3	0.25	0.13	0.08	9.38	+
2	0.41	0.26	0.12	6.83	−
2	0.36	0.26	0.07	10.29	+
2	0.25	0.16	0.09	5.56	−
2	0.21	0.14	0.07	6.0	−
1	0.67	0.39	0.06	11.17	−
1	0.39	0.24	0.17	2.29	+
1	0.31	0.18	0.14	2.21	+
1	0.18	0.1	0.16	1.13	−
1	0.16	0.09	0.13	1.23	−
1	0.14	0.07	0.05	2.8	+
0.5	0.42	0.25	0.19	1.11	−
0.5	0.3	0.17	0.34	0.44	−
0.5	0.3	0.17	0.21	0.71	−
0.5	0.22	0.12	0.18	0.61	−

[a] Mortality (M) is indicated in % for *Aiptasia* sp. and by +(dead or −(alive 24 hr after transfer to clean seawater) for *G. mucronatus* and *E. fuscata.*
[b] Mann-Whitney U = 17.5; $n_1 = 15$, $n_2 = 5$; $P < 0.05$
[c] Mann-Whitney U = 51; $n_1 = 16$, $n_2 = 18$; $P < 0.01$.
[d] n.d.—not determined
[e] dwc, dry weight of cerata; dwa, dry weight of experimental animal.

suggests that they may be part of the chemical defense, or at least that they are toxic to the animal itself. The transparent glands appeared empty on sections of the cerata, indicating that their contents were dissolved during dehydration and embedding.

Toxicity. Mortality for small marine invertebrates exposed to autotomized *Mourgona germaineae* cerata is shown in Table 1. The experimental animals generally became entangled in mucus secreted by the cerata fairly quickly. Sea anemones (*Aiptasia* sp.) would immediately contract tentacles touched by the autotomized cerata, and if this was not enough to disentangle them, they would actually "jump" away from the cerata (sometimes even this was unsuccessful). Animals affected by the toxin would remain contracted and often expel clumps of zooxanthellae and nematocysts, even after transfer to clean seawater. Amphipods (*Gammarus mucronatus*) would fight vigor-

ously to free themselves, thus possibly exhausting themselves until no locomotion was seen. Heart-rate became irregular, and finally death resulted. When amphipods were removed to clean seawater at a relatively early stage of "mucus-entanglement," they would usually recover within 24 hr. The ascoglossan *Ercolania fuscata* would try to crawl out of the experimental vial before getting entangled in mucus, but when they became entangled, movement stopped rather rapidly. Heart-rate slowed down and became irregular, and the sense of equilibrium was lost, so that the animals were not able to right themselves if accidentally turned on their backs; later epithelial damage occurred, melanistic pigment was lost, and eventually the animals died. If *E. fuscata* was removed to clean seawater before any epithelial damage had occurred, it sometimes recovered within 24 hr, but epithelial damage sometimes occurred even after the animals had been removed from the experimental vials.

A Mann-Whitney U test showed that lethal doses were significantly larger than nonlethal doses in *G. mucronatus* and *E. fuscata* ($P < 0.01$).

Nature of Toxin. The toxicity of autotomized *Mourgona germaineae* cerata was not affected by boiling the cerata for up to ½ hr. Toxicity was greatly diminished or lost by extraction in distilled water, methanol, or a methanol–chloroform–water mixture (2:4:1). The toxicity was retained in MeOH and H_2O extracts after solvent evaporation and resuspension in MFSW (Table 2). Thus the released toxin is a small molecule and apparently is not protein-bound. Testing the mucus with pH paper wetted in distilled H_2O showed that its reaction was neutral (pH,6–7).

TABLE 2. MORTALITY OF *Ercolania fuscata* SUBJECTED TO VARIOUS EXTRACTS OF *Mourgona germaineae* CERATA (DURATION OF EXPERIMENTS WAS 24 HR)

	Mortality, % (N)
Cerata after extraction	
$CHCl_3$	20(5)
MeOH	0(10)
H_2O	0(4)
Cerata after boiling	55(11)
Controls	8(12)
Extract	
$CHCl_3$	0(4)
MeOH	67(9)
H_2O	55(9)
Boiled seawater from cerata	100(3)
Seawater in which cerata had been autotomized	100(12)

TABLE 3. R_f VALUES FOR EXTRACTS OF *Cymopolia barbata* AND *Mourgona germaineae*

Chloroform extracts			Methanol–water extracts	
	C. barbata	*M. germaineae*	*C. barbata*	*M. germaineae*
I	0.99	0.99	A 0.85	0.85
II	0.96	0.96	B 0.58	absent
III	trace	0.82	C absent	0.26
IV	0.69	0.68		
V	0.61	0.60[a]		
VI	0.45	0.45		
VII	0.30	0.29		
VIII	0.07	0.06		
IX	0.05	0.04		

[a] This fraction was only present in two of seven animals.

R_f values for TLC of $CHCl_3$ extracts are shown in Table 3. One substance (III) was seen to be greatly concentrated in *Mourgona germaineae* cerata compared to *Cymopolia barbata,* but this substance was not detected in the mucus produced by *M. germaineae*. Dry weight of $CHCl_3$-soluble material averaged 5.33 ±3.25 mg/mg protein ($N = 6$).

DISCUSSION

The green siphonalean alga *Cymopolia barbata,* which is the sole food of the ascoglossan opisthobranch *Mourgona germaineae* (Jensen, 1981), contains a number of secondary metabolites, i.e., cymopol, cymopol monomethyl ether, cyclocymopol, cyclocymopol monomethyl ether, cymopolone, and cymopochromenol (Högberg et al., 1976). Some of these substances have antibiotic effects (Högberg et al., 1976), and at least one, cymopol, acts as a feeding deterrent (Targett and McConnell, 1982; McConnell et al., 1982a). Field observations confirm that indeed very few herbivores are found on *C. barbata* (personal observation). This suggests that *M. germaineae* has developed either a tolerance for these toxic substances or a mechanism to detoxify them. TLC of chloroform and methanol extracts of *C. barbata* and *M. germaineae* showed almost identical bands, indicating that the algal metabolites are not chemically altered in the mollusk. The presence of "crystals" in the digestive diverticula of the cerata, but not in other parts of the digestive system, indicates that certain dietary metabolites are displaced from the body of the animal into the readily autotomized cerata.

Autotomy of cerata is almost universal among eolidiform opistho-

branchs (Ascoglossa as well as Nudibranchia) (Edmunds, 1966; Ros, 1976). It has been sugested that leaving part of the body behind will confuse a potential predator, allowing the prey to escape. However, it must be assumed that a predator is able to learn to recognize a particularly palatable prey, even if it autotomizes part of its body. If, on the other hand, the autotomized body parts are distasteful or toxic, predators may become conditioned to avoid this particular prey organism. Autotomy also would serve to increase contact time, especially if the cerata are adherent. Most shell-less mollusks have surprisingly few predators, and many are known to store or secrete toxic or noxious substances, often of dietary origin (Thompson, 1960; Edmunds, 1966, 1968; Stallard and Faulkner, 1974; Ros, 1976; Fuhrman et al., 1979, 1980; Cimino et al, 1982; Hochlowski et al., 1982; Thompson et al., 1982).

These preliminary studies showed that the ascoglossan *Mourgona germaineae* secretes a substance, when disturbed, which is toxic to other small invertebrates. The toxin is methanol-soluble, water-soluble, heat stable, and of a neutral pH. Thus it is probably a small molecule. It is apparently secreted by the cerata even after these have been autotomized, because there was a small decrease in mortality after boiling the autotomized cerata and then transferring them to clean MFSW. The water in which they had been boiled retained the toxicity (Table 2).

Unfortunately R_f-values for TLC are not reliable for identification of organic molecules, and therefore none have been given in the literature concerning the toxic substances of *Cymopolia barbata* (Högberg et al, 1976; Targett and McConnell, 1982; McConnell et al., 1982a,b). However, in the present study almost all of the secondary metabolites found in *C. barbata* were also found in *Mourgona germaineae* (Table 3). This indicates that the toxic substance(s) secreted by autotomized *M. germaineae* cerata is very likely of dietary origin, although no experiments were made to test the toxicity of the various substances after chromatographic separation.

Targett and McConnell (1982) and McConnell et al. (1982a) have shown that extracts of *C. barbata* as well as pure cymopol have feeding deterrent effects on the snail *Littorina irrorata* and the sea urchin *Lytechinus variegatus.* Cymopol is the largest component of the ether-soluble fraction of *C. barbata* (Targett and McConnell, 1982; McConnell et al., 1982a). In the present study the broadest and darkest staining band on the TLC plates was that of fraction V ($R_f = 0.61$). However, this fraction was not present in all *M. germaineae* tested (Table 3). Fraction III ($R_f = 0.82$) was found in much higher concentration (darker staining; broader band) in *M. germaineae* than in *C. barbata,* thus indicating that this substance is concentrated by the animal. If this substance was just a nondigestible and nontoxic part of the assimilated food, it should not occur in the animal in consistently higher concentrations than in the food plant. Thus it is likely that *M. germaineae* actively concentrates this substance for defensive purposes. Fractions VI and

VII were found in about the same concentration in the ascoglossan as in its food plant, although occasionally only one or the other was present. It is not possible from the present experiments to make any conclusions about which fraction(s) is responsible for the toxicity detected in the autotomized *M. germaineae* cerata.

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INSECT PREDATOR-PREY COEVOLUTION VIA ENANTIOMERIC SPECIFICITY IN A KAIROMONE-PHEROMONE SYSTEM[1]

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Abstract—Insect predators can be guided to their prey by a kairomonal response to the prey pheromone. We found this phenomenon to be highly specific in the bark beetle predator *Thanasimus dubius*. Olfactory responses and behavioral tests revealed that the predator is guided to its major prey *Dendroctonus frontalis* by the primary enantiomer of the pheromone of the prey, (1*S*, 5*R*)-(−)-frontalin. These and other findings suggest the coevolution of a kairomone system of the predator and the pheromone system of its prey.

Key Words—*Dendroctonus frontalis*, Coleoptera, Scolytidae, *Thanasimus dubius*, Cleridae, southern pine beetle, kairomone, behavior, olfaction, coevolution, predator, enantiomer.

INTRODUCTION

Coevolution has been defined as reciprocal evolutionary responses in each of two or more species, each response having been activated by an evolutionary change in the other species (Futuyma, 1979). We found enantiomeric specificity at the olfactory receptor and behavioral levels in an insect predator for the aggregation pheromone of its prey as evidence for coevolution of the species.

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The clerid beetle, *Thanasimus dubius* (F.) (Coleoptera: Cleridae) is a common predator of the southern pine beetle, *Dendroctonus frontalis* Zimmerman (Scolytidae) (Hopkins, 1899). The behavior of *T. dubius* is so closely aligned to that of its prey that as adult *D. frontalis* attack host trees, adult predators arrive simultaneously to feed upon their prey (Thatcher and Pickard, 1966; Vité and Crozier, 1968; Dixon and Payne, 1979). This predator–prey synchrony is the result of the response of *T. dubius* to the aggregation pheromone released by female *D. frontalis* attacking host trees (Vité and Williamson, 1970). Reeve et al. (1980) found populations of the two species highly coaggregated, which they proposed was based upon the kairomonal response of *T. dubius* to its prey.

The fact that behavioral chemicals of some bark beetle species act as kairomones for their predators has been recognized. Wood et al. (1968) reported the first discovery of this phenomenon in *Enoclerus lecontei* (Walcott), a predator of *Ips paraconfusus* (Lanier). Subsequently, many other reports of kairomonal attraction have been made for predators of other scolytid species (see Bakke and Kvamme, 1981). The pheromones of most bark beetles are enantiomeric in composition (Silverstein, 1977). In fact, a given species may produce and be attracted to only one enantiomeric form, while its antipode is inhibitory or inactive (Wood et al., 1976; Vité et al., 1978; Borden et al., 1980). The aggregation pheromone in *D. frontalis*, frontalin (F), is produced as a mixture of 85% (1,*S*, 5*R*)-(−)- and 15% (1R, 5S)-(+)-frontalin (Stewart et al., 1977). The beetles respond to both enantiomers; however, maximal response can be elicited by (−)F alone but not the antipode (+)F (Payne et al., 1982). Pheromonal chirality can be significant in bark beetle behavior, and recently antennal olfactory responsiveness was obtained from the predator *T. formicarius* L. in response to pheromonal enantiomers of the scolytid beetle *Ips typographus* (L.) (Hansen, 1983). However, the significance of the chirality of kairomones to predator response at the antennal olfactory and behavioral levels remained unknown. The following investigation was carried out to provide that information on *T. dubius* and to determine if its response to *D. frontalis* pheromone is evidence of coevolution.

METHODS AND MATERIALS

Electrophysiology. T. dubius were obtained in the field from racemic frontalin-baited funnel-barrier traps (Rose et al., 1981). Antennal olfactory responses were measured using electroantennograms (EAG) (Schneider, 1957). EAGs were recorded from three male and four female *T. dubius* to the following treatments: (1) 2 μl pentane control, (2) 100 μg (±)F, (3) 100 μg (−)F and (4) 100 μg (+)F. (±)F was 99% chemically pure via GLC. Optical purity of the enantiomers was $\geqslant$98% via chiral shift NMR and optical rota-

tion. Compounds were prepared in nanograde pentane, placed on a filter paper and delivered in a 1 liter/min airflow via a solenoid operated valve (Payne, 1975).

Behavioral Tests. Behavioral tests were conducted in a mixed pine-hardwood forest in the Sam Houston National Forest, Texas. Racemic (±)F and the enantiomers (−)F and (+)F were released undiluted from glass planchets (3 × 21 mm) at 0.5 mg/hr (Payne et al., 1978). (+)-α-Pinene, a synergist of frontalin in the attraction of *D. frontalis*, was used to elicit response by *D. frontalis* to the enantiomers (Payne et al., 1982). It was eluted from a separate glass container (1 dram vial 33 mm high) at 2 mg/hr and was 98% chemically pure (Aldrich, $[\alpha]^{22}$ 42° neat) via chiral shift NMR and optical rotation. The elution containers were placed individually on the center shafts of four-vaned, sticky wing traps. Traps were placed ca. 10 m apart within an active *D. frontalis* infestation. Beetles were removed from traps and trap positions were rotated every 30 min per replicate.

RESULTS AND DISCUSSION

The olfactory systems of both male and female *T. dubius* were highly specific for (−)F compared to (+)F (Table 1). In fact, response to (+)F, which was significantly less than to (−)F, was elicited from only one female and may have been due to the small percentage of (−)F present in the (+)F sample. The significant response to (±)F indicates that (+)F did not block or inhibit response to (−)F. Field results substantiate the electrophysiological data (Table 2). Significant response was obtained to (−)F but not to (+)F. The significant response to (±)F, which was nearly equal to (−)F, further indicates the absence of an antagonistic effect by (+)F.

Specificity for the primary olfactory signal of its prey provides *T. dubius* with an efficient mechanism for locating its food resources which are mostly patchy in location and scarce in abundance. Adult *D. frontalis* occur in scattered infestations and are only available to *T. dubius* during the few days while mass attack takes place on the host tree by *D. frontalis* (Coster et al., 1977). Subsequently, adult *D. frontalis*, which successfully attacked the host tree, have entered the bark and thus avoid further predation. Through their strong attraction to (−)F, *T. dubius* adults are able to arrive on trees almost simultaneously with *D. frontalis* females as they attack the tree and release their pheromone. Adult *T. dubius* then consume adult *D. frontalis* as they arrive on the host tree. The opportunity for locating food resources by the predator is thus maximized.

In addition to prey location, *T. dubius* may use the kairomone in mate location, since there is no evidence that it produces and uses aggregation or sex pheromones (J.L. Frazier and T.E. Nebeker, personal communication)

TABLE 1. PERCENT EAGS FROM *T. dubius* TO FRONTALIN (F) ENANTIOMERS RELATIVE TO STANDARD[a]

Replicate	EAG (%)		
	(±)F	(+)F	(−)F
Male			
1	100	0	179
2	100	0	100
3	100	0	100
$\bar{X}\% \pm$ SE	100	0	126 ± 26
Female			
1	100	0	111
2	100	0	67
3	100	18	82
4	100	0	100
$\bar{X}\% \pm$ SE	100	5 ± 5	90 ± 10

[a]Standard = 100 μg (±)F, 100 μg/stimulus.

or aggregates elsewhere than on beetle-attacked trees for the location of food resources and mates. Once on the host tree, males and females mate, and the females deposit their eggs beneath bark scales. The resulting larvae enter the gallery systems of *D. frontalis* as well, and feed on the immature bark beetles under the bark (Fronk, 1947). This high degree of olfactory and behavioral specificity for the primary enantiomer of its prey suggests coevolution of the prey location behavior of *T. dubius* and the aggregation behavior of *D. frontalis*.

T. dubius perceives and responds to the pheromones of *Ips* bark beetle species and feeds upon the beetles as alternate prey (J.C. Dickens and T.L. Payne, unpublished observations; Mizzell and Nebeker, 1982; Turnbow,

TABLE 2. FIELD RESPONSE OF *T. dubius* TO TRAPS BAITED WITH ENANTIOMERS OF FRONTALIN (F) PLUS (+)-ALPHA-PINENE (α-P), SAM HOUSTON NATIONAL FOREST, TEXAS

Treatment	$\bar{X}$ No. *T. dubius* caught[a]
(±)F +α-P	2.5 a
(+)F +α-P	1.0 b
(−)F +α-P	2.3 a
Blank wing trap	0.7 b

[a]Means followed by the same letter are not significantly different. $P = 0.5$, Mann-Whitney test. 27 replicates.

1979). Significant EAGs were recorded from male and female *T. dubius* to the bark beetle pheromones *endo*-brevicomin, verbenone, *trans*-verbenol, *cis*-verbenol, ipsenol, and ipsdienol, as well as the host tree terpenes (+)-α-pinene and myrcene. However, behavioral response to *Ips* pheromones was generally low, and in competition with synthetic frontalin and/or *D. frontalis*, response to other pheromones or scolytids was insignificant. As pointed out by Turnbow (1979), the fact that *T. dubius* perceives and responds to pheromones of different bark beetle species as kairomones makes it a facultative, yet preferential predator for *D. frontalis*. We found that (−)F mediates this preference.

Our findings support the hypothesis that *D. frontalis* and *T. dubius* are coevolving so that *T. dubius* is able to use (−)F to locate its prey. As a result of predation pressure *D. frontalis* may be evolving so that (+)F becomes an aggregation pheromone to which the predator does not yet respond at the behavioral level (Table 2).

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COMPARISON OF TANNIN LEVELS IN DEVELOPING FRUIT BUDS OF TWO ORCHARD PEAR VARIETIES USING TWO TECHNIQUES, FOLIN-DENIS AND PROTEIN PRECIPITATION ASSAYS

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Abstract—As part of a study into dietary selection by avian pest species, the levels of phenolic material in pear buds have been determined using two approaches, the Folin-Denis assay and an assay based on the precipitation of bovine serum albumin by astringent phenols. The results of these two assays do not correlate over the sampling period and their overall significance to ecological studies is discussed.

Key Words—Pear buds, bullfinches, *Pyrrhula pyrrhula*, tannin, polyphenol, Folin-Denis, protein precipitation.

INTRODUCTION

As part of a continuing investigation into the reasons behind dietary selection by avian pest species such as the bullfinch (*Pyrrhula pyrrhula* L.), the levels of phenolic and polyphenolic substances in two varieties of orchard pear fruit buds, Conference and Doyenne du Comice, have been determined. The former variety can provide a significant part of the winter–early spring diet of the bullfinch, while Comice buds are a less favored variety (Newton, 1964). The economic severity of bullfinch damage to commercial orchards is well documented (Wright and Summers, 1960; Wright et al., 1980) and, although many methods of reducing the damage have been tried, none has proved entirely successful.

Previous papers (Grieg-Smith et al., 1983; Wilson et al., 1983) have studied the nutritional composition of pear fruit buds and its significance to bullfinch diet selection, but the intervarietal differences found do not appear sufficient to account for the observed feeding pattern.

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Tannin-like substances have been implicated in dietary selection in a number of species (Green, 1974; McKey et al., 1978; Gartlan et al., 1980), and it has been suggested that their mode of action may be due to their bitter and/or astringent properties. Bitterness is a subjective sensation and difficult to determine with a nonhuman species, but astringency is reflected by the ability of a polyphenol to precipitate protein in an in vitro situation. Polyphenols of a particular molecular size, e.g., C_{45}–C_{120}, anthocyanidin oligomers are able to bind to protein and cause its precipitation, whereas small anthocyanidins and simple phenolics cannot (Bullard, 1981; Goldstein and Swain, 1963). Many methods of determining this protein binding or precipitating property such as the β-glucosidase precipitation assay (Goldstein and Swain, 1965) or hemanalysis assay (Bate-Smith, 1973) are too cumbersome to use on multiple samples, but the bovine serum albumin (BSA) precipitation assay developed by Martin and Martin (1982) is both repeatable and rapid. These authors also suggest that it may present a truer picture of the biological activity of extractable phenols as it reflects the astringence of the material.

This paper describes the levels of phenolic substances in fruit buds as determined by two different approaches, the Folin-Denis assay of total phenol hydroxyl and the precipitation of BSA by astringent polyphenols, and discusses the significance of the data obtained.

METHODS AND MATERIALS

Materials. Pear fruit buds were collected from a commercial orchard site in southeast England between mid-November 1981 and mid-March 1982, and stored (maximum time, 2 hr) in ice until they could be dried. A randomized collection program was used to ensure that buds were collected from all areas of the orchard on each sampling date. On each occasion, the samples from one variety were bulked prior to drying, and all subsequent analyses were carried out on subsamples of the dried powder produced. Drying was carried out in a current of air at $<50°C$ for 3 days, after which the whole buds were powdered in a hammer mill and stored desiccated at $-20°C$. Chemicals were obtained from a variety of sources and were of Analytical Regent quality.

Sample Preparation. Dry bud powder (100 mg) was extracted in 0.5 M HCl in 50% (aqueous) methanol (10 ml) overnight. Acid was present in the extraction medium to ensure a high level of solubilization of phenolic material. The extract was filtered and a 200-μl aliquot removed for the Folin-Denis assay. The remaining filtrate was evaporated to dryness under reduced pressure at less than 40° C and the residue resuspended in 0.5 M HCl in 50% (aqueous) methanol (500 μl).

Folin-Denis Assay. The reagent was prepared using the Association of Official Analytical Chemists method (Horwitz, 1975) and diluted 1:5.6 with water before use. Crude sample extract (50 μl) was added to 2.5 ml of the

reagent in a cuvette and mixed. After 90 sec, a saturated sodium carbonate solution (100 μl) was added and the mixture incubated at room temperature for a further 18.5 min. At the end of this period, the absorbance at 725 nm was determined against a reagent blank and quantified using a standard curve of 0–500 μg tannic acid/cuvette. All assays were run in triplicate, and the results of the determinations are expressed at mg/g dry wt tannic acid equivalents (TAE).

BSA Precipitation Assay. Concentrated bud extract (200 μl) was added to 1.5 mg/ml BSA (crystallized, lyophilized) solution (1.0 ml) in sodium acetate buffer (0.2 M, pH 5.0) containing 0.17 M sodium chloride. The mixture was gently mixed to avoid foaming and incubated at room temperature for 15 min. The mixture was centrifuged at 5000 *g* for 60 min, and an aliquot of the supernatant (~200 μl) carefully removed for subsequent analysis. Supernatant (25 μl) was added to a 1:4 aqueous dilution of Bio-Rad protein determination reagent (2.5 ml) (Coomassie brilliant blue) in a cuvette and the mixture incubated at room temperature for 9 min. At the end of this period, the absorbance at 595 nm (A_{595}) was determined against a reagent blank.

This value was subtracted from the A_{595} for a BSA solution to which 200 ml of 0.5 M HCl in 50% (aqueous) methanol was added in place of the sample to give the δA_{595} for that sample.

A standard curve was prepared to which 0–500 μg tannic acid in 0.5 M HCl in 50% (aqueous) methanol (200 μl) was added to 1 ml of 1.5 mg/ml BSA and treated in the same manner as the samples. The δA_{595} was obtained for eagh value of tannic acid added by subtracting the A_{595} for that concentration from the A^{595} obtained for the BSA control. All assays were performed in triplicate.

Martin and Martin (1982) suggest that a clean-up stage using Sephadex PD-10 (G-25) columns should be employed between the centrifugation of the BSA–sample mixture and the protein estimation to remove any extraneous A_{595} absorbing material. This method was tried with pear bud extracts but was found to make no difference to the final result. Thus in this case it was omitted to make the assay more suitable for application to multiple, routine analysis.

RESULTS AND DISCUSSION

Figure 1 shows the levels of extractable phenolic material (expressed as mg TAE/g dry wt; mean of triplicate determination) as measured by the Folin-Denis assay. This reagent reacts in a nonstoichiometric way with phenolic hydroxyl groups, so these data reflect the total levels of both the simple phenolic acids and the more complex polyphenols. It would appear that Conference buds, the variety preferentially selected by the bullfinch, contain higher levels of phenolic material than do Doyenne du Comice buds.

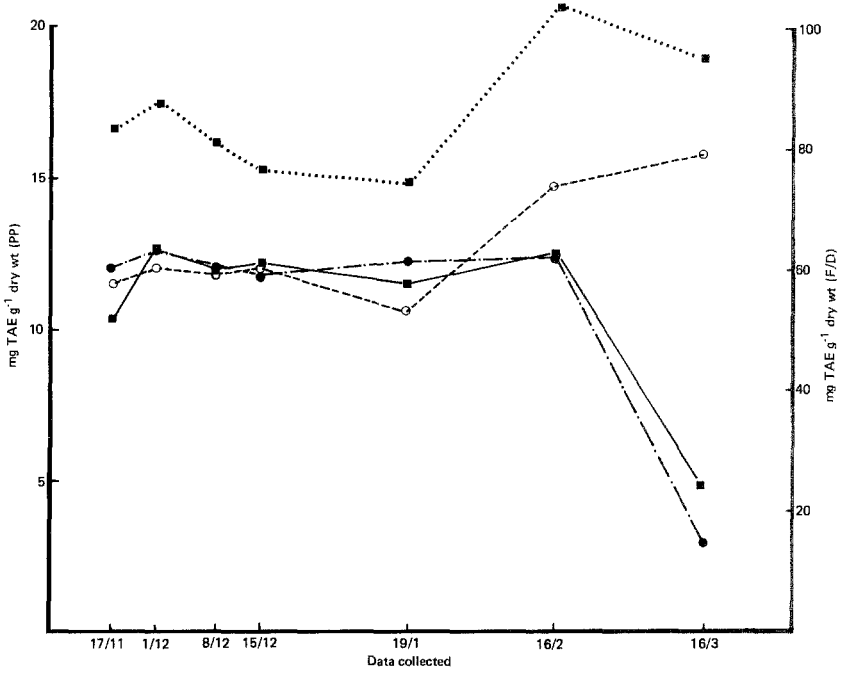

FIG. 1. The changes in the levels of phenolic material in developing pear fruit buds, collected between November 1981 and March 1982, as determined by the Folin-Denis assay(■····■ Conference, ○---○ Comice) and by the precipitation of the bovine serum albumin (PP)(■——■, Conference, ●-·-● Comice). All results expressed as mg/g dry wt tannic acid equivalents.

The levels in both varieties decrease slightly in January and then rise to about 100 mg/g dry wt and 80 mg/g dry wt, respectively, by the end of the sampling period in mid-March. Figure 1 also shows the levels of extractable phenolic material as measured by BSA precipitation for the same samples. Clearly, only a proportion of the total phenolic material is able to precipitate BSA and, unlike intervarietal differences shown by the Folin-Denis assay, extracts of both varieties seem to be equal in their ability to precipitate the protein. While the level of the phenolic material rises toward the end of the sampling period as determined by the Folin-Denis assay, the same samples show a marked decrease in protein precipitating ability. The latter is more likely to influence the astringency and therefore the palatability of the material to a pest species. The degree of agreement between the replicate analyses (C.V. 2% by Folin-Denis and C.V. 1% by protein precipitation) is good, thus error bars have not been included in Figure 1.

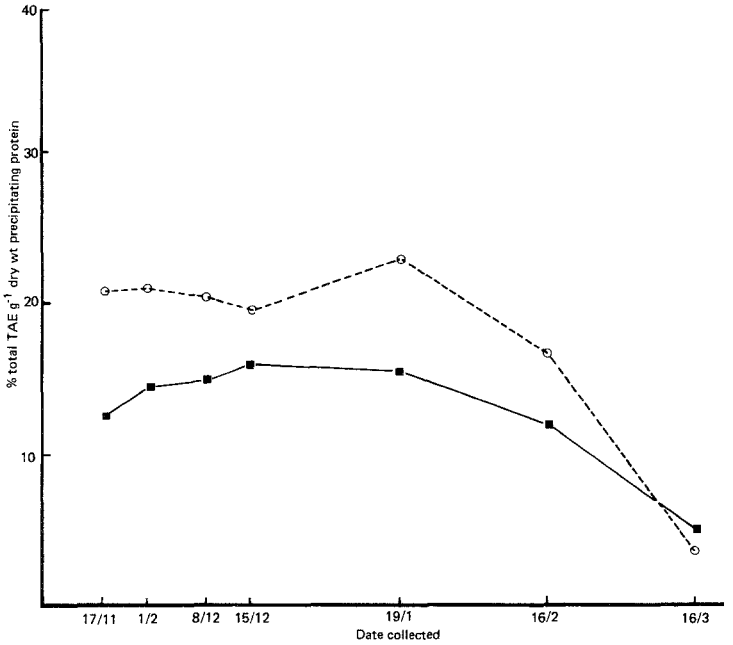

FIG. 2. The changes in the proportion of the total extractable phenolic material able to precipitate bovine serum albumin during the development of the fruit buds of two orchard pear varieties, Conference (■——■) and Comice (○---○).

Figure 2 summarizes the difference between the two assay techniques. It would appear that the bud variety least favored by the bullfinches, Doyenne du Comice, contains a lower total level of extractable phenolic material throughout the sampling period, but of this a higher proportion is capable of precipitating protein. In their paper on oak leaf tannin content and moth larval predation, Martin and Martin (1982) argue that chemical assays alone do not present an accurate picture of the tannin levels in plant material in relation to its pest predation. They found that the leaves with the highest total extractable phenol do not give the greatest precipitation of protein. The data in this paper confirm their findings with respect to the different plant species. Although the exact chemical composition of the phenolics and its significance to dietary self-selection has yet to be investigated, the data presented in this paper demonstrate that the method used for the determination of phenolic material is crucial to any investigation of chemoecological relationships. The purely chemical assay, while helpful, should be used together with a measure of biological activity such as protein precipitation or more specific chromatographic determinations of the individual com-

ponents of the phenolic material. It should also be pointed out that the choice of BSA as a protein is an arbitrary one based on availability and ease of use. A recent paper suggests that the leaf protein, ribulose-1,5-bisphosphate carboxylase can be used in such studies (Martin and Martin, 1983) to reflect the effect of tannins on ingested plant material. Such experiments could also be carried out using protein from the taste receptors of the pest species under study but, as in the majority of cases, this is impractical; a convenient substitute such as BSA must be used.

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DISTRIBUTION OF BIRCH (*Betula* SPP.), WILLOW (*Salix* SPP.), AND POPLAR (*Populus* SPP.) SECONDARY METABOLITES AND THEIR POTENTIAL ROLE AS CHEMICAL DEFENSE AGAINST HERBIVORES

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Abstract—Isoprenoids and phenolics, major metabolites of important browse species, are reviewed in regard to concentrations, distribution within tissues, and between species. Seasonal variation of specific substances and changes with age of the plant are also considered. The distribution of substances may affect food selection and feeding behavior of animals. Wild mammalian herbivores tend to avoid plant parts rich in these substances, in spite of high nutritional content of the plant tissue. Possible mechanisms for defense by plants against depredation by mammalian herbivores are discussed within the framework of the plants' biochemistry.

Key words—*Betula*, *Salix*, *Populus*, isoprenoids, phenols, mammals, herbivores, ecology, defense.

INTRODUCTION

Diet selection by herbivores may be limited by the biochemical characteristics of both plants and animals. Plants may vary in mode of defense according to succession, growth form, browsing pressure, etc., while animals may vary in tolerance of secondary chemicals of a specific food plant (Freeland and Janzen, 1974; Cates and Orians, 1976; Haukioja and Hakkala, 1975; Maiorana, 1978). It is believed that herbivores in general select their food plants according to protein, mineral, and energy content (Lindlöf et al., 1974; Westoby, 1974; Marten, 1978; White, 1978; Ullrey et al., 1978; Mattsson, 1980; Belovsky, 1982). Recent studies show that food selection is more or less influenced by plant secondary chemistry (Markham, 1971; Radwan, 1972; Swain, 1977; McKey et al., 1978; Bryant and Kuropat, 1980; Bryant, 1981; Picman, 1982; Radwan et al., 1982).

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Woody plants such as birch (*Betula* spp.), willow (*Salix* spp.), and poplars (*Populus* spp.) are important food species for wild herbivores in boreal forests (Cederlund et al., 1980). Herbivores which make their living on these plants are exposed to a great number of different plant substances. Observations on the feeding behavior of hare (*Lepus* spp.) and moose (*Alces alces*) show that their selection within and between these plants are not solely explained by the nutritional content (Barikmo, 1976; Pehrson, 1981;Bryant, 1981; Hjeljord et al., 1982).

This paper discusses the distribution and biological activity of isoprenoids and phenolics in the birch and willow families, because these chemical classes are by weight the main secondary substances occurring in these species. Substances belonging to these chemical classes can potentially be involved in the plants' self-defense against herbivores.

Many terpenoids have an ecological function in acting as attractants for insect pollinators, while others are involved in metabolic processes in plants. Some terpenoids serve as a chemical defense against various plant antagonists (Banthorpe and Charlwood, 1980). Mono- and sesquiterpenes in particular have been shown to have feeding deterrent and toxic properties to insects (Scholl et al., 1977; Rice et al., 1978; Langenheim etal., 1980). These chemical groups also act as browsing deterrents to several mammalian herbivores such as deer and squirrels (Radwan, 1972; Radwan and Ellis, 1975; Schwartz et al., 1980a, b; Farentinos et al., 1981; Radwan et al., 1982; Picman et al., 1982).

Phenolic compounds are widely distributed, and all plants have a characteristic phenolic pattern in their tissues (Wagner, 1979). In spite of this, phenolics as a chemical group have not been extensively studied as to their defensive properties (Levin, 1976). Most work so far has regarded the function of tannins, proposed as a "quantitative defense" by Feeny (1976), because of their ability to make complexes with protein and cellulose (Harborne, 1979; Haslam, 1979). However, their function has recently been questioned by Zucker (1983), who discusses tannin structure in relation to biological activity.

Much less is known about the action of free phenolics and phenolic glycosides. Many simpler phenolics exhibit considerable biological activity. Some have antifungal and antibacterial properties (Swain, 1977), others act as estrogens when ingested by mammals (Shutt, 1976; Berger et al., 1977; Labov, 1977), and many make plants unpalatable (Markham, 1971; Oates et al., 1977; McKey et al., 1978).

The genus Betula

In Scandinavia this genus has four species: dwarf birch (*Betula nana* L.), common birch (*B. pubescens* Ehrh.), mountain birch (*B. tourtuosa* Led), and silver birch (*B. pendula* Roth.). Their size ranges from small shrubs to

medium-sized trees which prefer open habitats. They are associated with plant communities characteristic of early successional stages (Olson, 1957; Lid, 1974; LeResche et al., 1974; Wolff, 1978). *B. pendula* and *B. pubescens* grow fairly rapidly and their foliage can, within a few years, be out of reach of most mammalian herbivores. The former species grows in dry habitats, while the latter prefers a more moist soil. *B. pendula* differs morphologically from its relatives in the occurrence of resin glands on twigs of young individuals. *B. pubescens* has hairy twigs (Lid, 1974). These morphological characteristics correspond to *B. papyrifera* and *B. allegheniensis*, respectively, in North America (Mitchell, 1974).

Mono- and Sesquiterpenes (C_{10}–C_{15} *Compounds*). Only small amounts of these compounds are reported. Inki and Väisäinen (1980) measured the isoprenoid content of birch and found in leaves of *B. pendula* about 0.1% dry matter, whereas *B. pubescens* has about twice that level. The isoprenoids of the former species are mainly monoterpenes, and sesquiterpenes occur less often. In *B. pubescens* the chief compounds are sesquiterpenes; monoterpenes are rare. No seasonal variation was detected in these isoprenoids (Inki and Vaisainen, 1980).

Interestingly, the mountain birch shows high levels before leaf burst (7.9%) and a rapid decline during leaf development to about 0.3% in late July. The only identified monoterpene of *B. alba* (= *pendula*) leaves so far isolated is hydroxylinalool glucopyranoside (Tschesche et al., 1974) (Figure 1). The biological activity and function in the plant of this and related mono- and sesquiterpenes are not known. However, studies on other plant genera show that these isoprenoids have high biological activity to browsing animals (Radwan and Ellis, 1975; Schwartz et al., 1980a, b).

Di- and Triterpenes (C_{20}–C_{30} *Compounds*). No reports on the occurrence of diterpenes in birch are available. Most of the work on birch isoprenoid compounds is conducted on the distribution of triterpenoids. In bark the most commonly occurring triterpenes are betulin and lupeol (Figure 1) (Hejno et al., 1965; Rimpler et al., 1966). These substances occur as glycosides, e.g., saponins in plant tissues (Grunwald, 1980). Only small amounts of these chemicals have been detected, and a concentration of about 0.6% is found in birch bark (Rimpler et al., 1966). Related compounds that occur in small amounts are allobetulin and β-sitosterol (Figure 1) (Hejno et al., 1965; Rimpler et al., 1966). The latter compound is a precursor of saponin synthesis (Grunwald, 1980). Mentioned triterpenes have structural similarities with mammalian and insect sex hormones, suggesting potential estrogenic activity (Harborne, 1982). They have not yet been tested on mammalian herbivores with regard to this effect. Reichardt (1981) isolated a triterpene idenitfied as papyriferic acid (Figure 1) from current-year shoots of *B. papyrifera* ssp. *humilis*. He proposes this compound to be an active component of the herbivore deterrent extract of this species.

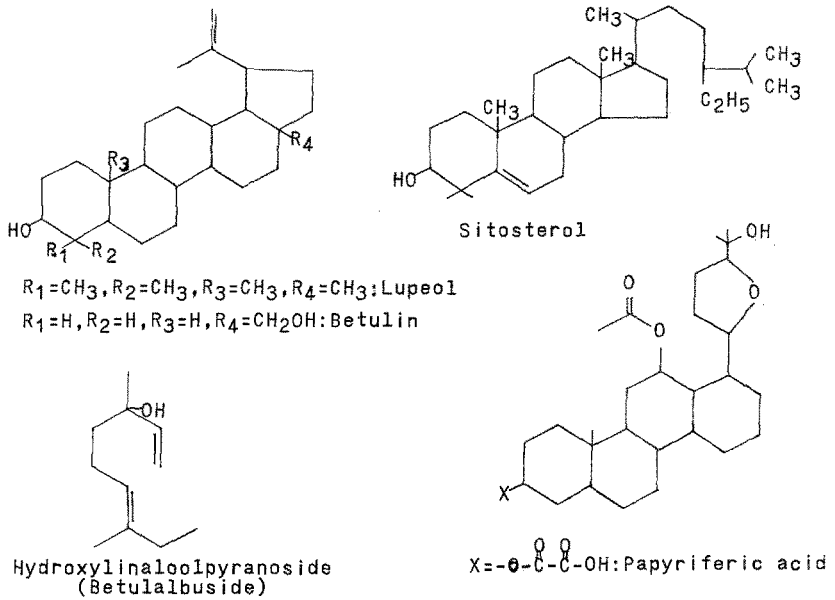
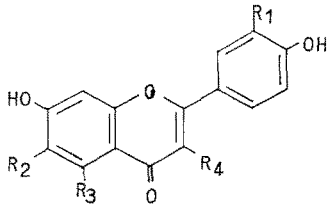


FIG. 1. Examples of some isoprenoid structures identified in birch (from Hejno et al., 1965; Tschesche et al., 1977; Reichard, 1981).

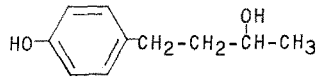
Monocyclic Phenols (C_6 *Compounds*). Few investigations on simpler phenolics are reported. Pawlowska (1980a) found caffeic, gallic, and chlorogenic acids in leaves of *B. pendula*. The ecological function of these acids remains to be tested. Caffeic acid is reported to have antivitamin activity (Reese, 1979). High concentrations of chlorogenic acid are positively correlated with the food preferrence of deer (Tucker et al., 1976). Santamore and Vettel (1978) investigated the distribution of the phenol rhododendrin (Figure 2) in birch and its significance to prevent attack by the bronze birch borer (*Agrilus anxius*). They found no correlation between the attack and the rhododendrin content. The concentration of the compound in bark ranges from 0.1% to 0.3% dry weight (Klischies and Zenk, 1978).

Flavonoids and Tannins ($C_6C_3C_6$)*n*. Buds of birch secrete a lipophilic coat consisting of terpenoids and phenolics. Characteristic phenolic compounds are the methyl derivatives of kaempferol, quercitin, apigenin, and naringenin (Pawlowska, 1980a, b; Wollenweber, 1975, 1977). Methylethers and some 20 unidentified flavonoids also occur in trace amounts (Tissot and Egger, 1972; Gibbs, 1974; Wollenweber, 1975, 1977). The flavonoids of different birch species are listed in Table 1.

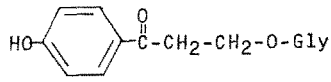
Morino (1979) studied the influence of environmental factors on the flavonoid production of *B. alba*. She found that these compounds reach their



R_1=H, R_2=H, R_3=OH, R_4=OH: Kaempferol
R_1=OH, R_2=H, R_3=OH, R_4=OH: Quercetin
R_1 = H, R_2 = H, R_3 = OH, R_4 = H: Apigenin



Rhododendrin



Hydroxypropiophenone

FIG. 2. Examples of major phenolic structures found in tissues of birch species (From Tissut and Egger, 1972; Tschesche et al., 1972; Wollenweber, 1977).

highest concentrations in young leaves, followed by a decline which parallels leaf maturation. She also showed that stress on the plant by UV light at high altitudes increases flavonoid production. Pawlowska (1976) noticed diurnal variations in flavonal glycoside concentration of *B. humilis*. Highest concentrations occurred in midday. Gibbs (1974) reported the occurrence of protocatechuic acid and syringic acids. Nowak (1966) mentioned that bark of *B. lenta*, a birch species native to southern Canada, is very rich in methylasalicylaldehyde and aromatic alcohols. This gives the bark very high antiseptic properties.

Birch bark is reported to contain only small amounts of tannins (Rowe and Conner, 1979). For example, *B. papyrifera* and *B. allegheniensis* contain about 1.5% on average, according to these authors. Others have detected seasonal variations in the tannin content. The maximum tannin content of *B. pendula* bark was observed in September (5.77–6.17%) and the lowest during winter (4.11–4.75%). Apart from seasonal dynamics, individual variability in tannin content under the same ecological conditions was also observed (Chernyaeva et al., 1982).

Quantitative Measurements. Few investigations have been published regarding amounts and quantitative variations of specific compounds in birch. However, some data exist on measurements of total phenolics.

Leaves of *B. tourtuosa* just emerging showed a concentration of total phenolics of 7% dry weight. Autumn leaves (September) have a maximum of approximately 15% (Haukioja and Niemälä, 1976; Haukioja et al., 1978). Palo et al. (1983b) measured total phenolics of water–acetone extracts of twigs from *B. pendula*. The highest amounts were found in winter twigs (3%). As the leaf starts to develop in the middle of May, the phenolic content of the twigs with leaves declines rapidly to a concentration about 0.9% in late June. No qualitative changes between free phenolic compounds seem to occur during

TABLE 1. FLAVONOID SPECTRUM IN BUDS OF SOME *Betula* SPECIES.[a]

Flavonoid Compound	*Betula pubescens*	*B. pendula*	*B. nana*	*B. nigra*	*B. ermanii*
Kaempferol	+[b]	+	+	(+)	+
3-Me	(+)	(+)	−	−	(+)
7-Me	+	+	+	+	+
4-Me	+	+	+	−	+
3,4-Me	+	+	(+)	+	+
3,7-Me	−	−	−	+	−
7,4-Me	+	(+)	+	+	+
Kaempferol-6-OH	−	−	−	−	−
6,4-Me	+	+	+	−	+
3,6,4-Me	+	+	+	−	+
6,7,4-Me	−	−	−	+	−
Quercetin	−	+	−	−	+
7-Me	(+)	−	−	−	+
3-Me	+	+	+	+	+
7,3-Me	+	−	−	−	−
Apigenin	+	+	−	(−)	+
7-Me	+	+	−	−	−
4-Me	+	+	+	−	+
7,4-Me	+	+	−	−	+
Scoutellarein-6,4-Me	+	+	+	+	+
Rhamnocitrin	−	−	−	(−)	−
Ermanin	−	−	−	(−)	−
Isorhamnetin	−	−	−	(−)	−
Dachypodol	−	−	−	(−)	−
Retusin	−	−	−	(−)	−
Combretol	−	−	−	(−)	−
Luteolin	−	−	−	−	−
Myricetin	−	−	−	(−)	−
7-Me	−	−	−	−	+
4-Me	−	−	−	−	+
7,4-Me	−	−	−	−	+
Chrysin	−	−	−	−	−
Galagnin	−	−	−	−	−
Pinocembrin	−	−		−	−

[a]from Wollenweber (1975, 1976, 1977).
[b]+ main component, (+) minor part, (−) trace amounts, − not detected.

that time (K. Sunnerheim and T. Palo, unpublished observations). Winter twigs of mature growth forms of *B. pubescens* in the middle of Sweden showed a concentration of about 2% (Palo et al., 1983a).

The production of phenolics is shown to be induced by herbivore attack. Haukioja (1980, 1982) and Niemälä et al (1979) demonstrated that attack of the autumn moth (*Oporinia autumnata*) on mountain birch leaves increased

the phenolic production of leaves up to three years after the attack. Bergström and Danell (personal communication) report, on the other hand, that *B. pendula* responds to simulated browsing by lowering its phenolic content in shoots produced the year after browsing. No large differences were observed for *B. pubescens*. However, looking at the water–acetone-soluble fraction, the extractable dry matter is reduced by browsing, but the phenolic content of that fraction is increased at a level of 50% of the annual shoot browsed. The two birches respond quite differently to browsing. *B. pubescens* increases its production of phenolics while *B. pendula* lowers its phenolic concentration.

The Family Salicaceae

The family has only two genera in Scandinavia, *Salix* spp. and *Populus* spp. The genus *Salix* consists of 44 specimens and many hybrids. They range from low shrubs to small trees. A characteristic of most species is their rapid growth rate. However, large variations exist in the life forms of this genus. The sexes are separated on different plants. They are the only tree species in Scandinavian boreal forests which are insect pollinated.

During the 1960s, intense studies were carried out on the distribution and identification of phenolic glycosides of the family. Phenolics are so far the only chemical class of secondary substances reported to occur within the family. Quantitative amounts and types of phenolics in European species were investigated by Thieme (1964a–c, 1965a–d, 1967, 1971) and Thieme and Benecke (1967, 1969a,b, 1970, 1971) and in North American species by Pearl and Darling (1967, 1968a–c, 1970, 1971). Analytical methods are described by Steele and Bolan (1969, 1972).

Distribution of Phenolic Glycosides in Different Tissues of Salix *spp.* Table 2 gives a summary of phenolic glycosides in bark and leaves of different species as reported by Thieme and coworkers. The characteristic chemical marker of the whole family is salicin (Figure 3). This component is found in the bark of all species in the family, but often in small amounts and never as the main glycoside (Thieme, 1965a–d; Jaggi and Haslam, 1969). Some of the glycosides have herbivore-deterrent properties. Markham (1971) observed that some species of *Salix* spp. exhibited a strong natural resistance to attack by opossum (*Didelphis* sp.). It was established that high levels of salicin derivatives are correlated with both high relative bitterness and unpalatability to opossum.

Another characteristic compound is salicortin in bark (Figure 3) (Dytkowska, 1967). This substance is present in all species but *S. cinerea*, *S. fragilis* and *S. triandra* (Thieme, 1965a–d).

In bark of *S. caprea*, *S. cinerea*, and *S. viminalis* the main substances are picein and triandrin. They occur in quantity in several other *Salix* species but are not the main substances. Bark generally shows higher phenolic concentra-

TABLE 2. PHENOLIC GLYCOSIDES IN BARK (FEBRUARY) AND LEAVES (MAY) OF SOME *Salix* SPECIES.[a]

		Glycoside[b]																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total (%)
Salix alba	Bark	+	2+	–	–	–	–	(+)	+	–	–	–	–	–	–	–	–	2.7
	Leaf	No phenolic glycosides																
S. aurita	Bark	+	+	–	–	–	–	–	+	–	–	–	(+)	–	–	–	–	0.7
	Leaf	No phenolic glycosides																
S. caprea	Bark	+	(+)	–	–	–	–	–	4+	–	2+	–	–	–	–	–	–	4.2
	Leaf	No phenolic glycosides																
S. cinerea	Bark	+	–	–	–	–	2+	–	2+	–	–	–	+	–	–	–	–	3.3
	Leaf	No phenolic glycosides																
S. fragilis	Bark	+	–	–	–	–	–	+	+	–	+	–	–	–	–	–	–	4.9
	Leaf	+	2+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.9
S. myrsinifolia	Bark	+	+	–	–	–	4+	–	2+	–	–	–	–	–	–	–	–	6.9
	Leaf	3+	3+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	5.8
S. pentandra	Bark	+	+	–	–	–	–	+	+	–	–	–	–	–	–	–	–	2.5
	Leaf	2+	2+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	2.8
S. purpurea	Bark	2+	5+	2+	–	–	–	(+)	–	–	–	–	–	–	–	–	–	11.1
	Leaf	3+	3+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	7.5
S. repens	Bark	+	3+	2+	–	–	–	(+)	–	–	–	–	–	–	–	–	–	4.8
	Leaf	3+	3+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	6.4
S. triandra	Bark	(+)	–	(+)	2+	–	–	(+)	+	–	–	–	–	–	–	–	–	1.6
	Leaf	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	0.2
S. viminalis	Bark	(+)	(+)	–	–	–	–	–	5+	–	–	–	(+)	–	–	–	–	6.8
	Leaf	No phenolic glycosides																

[a] From Thieme (1965a–c, 1971).

[b] 1. salicin, 2. salicortin, 3. salireposide, 4. tremulacin, 6. picein, 7. grandidentanin, 8. triandrin, 9. tremuloidin, 10. fragilin, 11. trichocarpin, 12. vimalin, 13. nigracin, 14. isolariciresinoglycoside, 15. kaffeic acid glycoside, 16. l-*p*-coumarylglycoside.

[c] (+) < 0.1%, + = 0.1–1%, 2+ = 1–2%, 3+ = 2–4%, 4+ = 4–6%, 5+ = 6%.

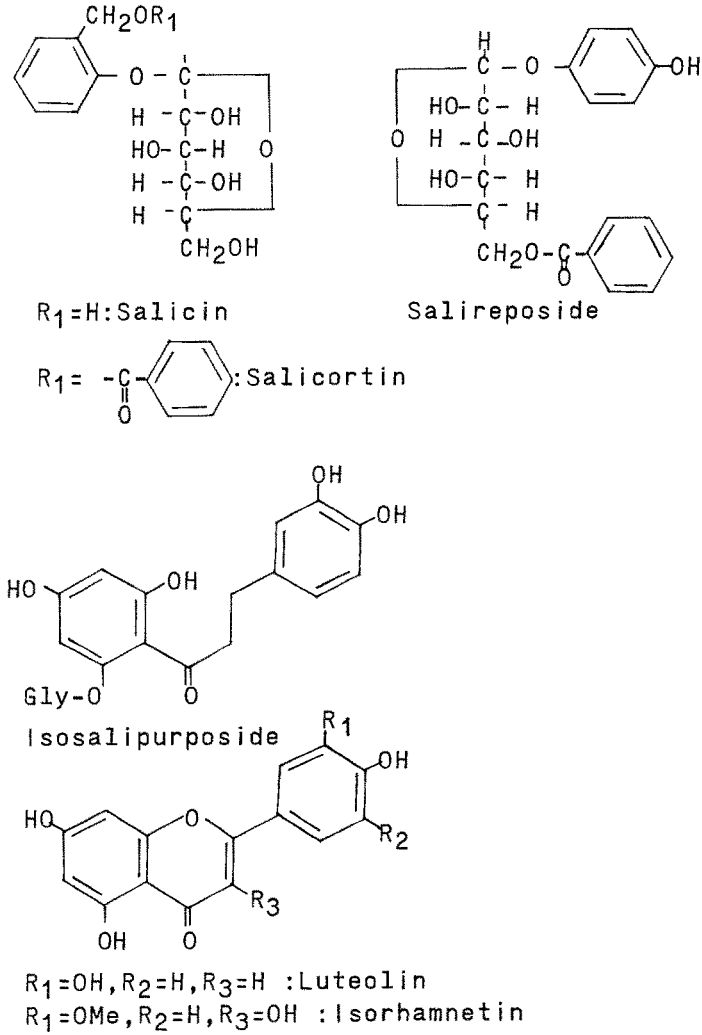


FIG. 3. Structural formulas for some phenolic constituents in *Salix* and *Populus* species (From Thieme, 1967; Thieme and Benecke, 1971; Harborne, 1979).

tion and higher glycoside diversity than leaves (Table 2). Many species do not have any phenolic glycosides in the leaves. In leaves where glycosides occur, salicin and salicortin are most common. In *S. triandra* these two are replaced by the uncommon glycoside salidroside (Thieme, 1965).

Differences between Sexes in Phenolic Production. The chemistry of *Salix* is made more complex by the fact that female and male plants are different individuals. This is shown in the different chemistry of the two sexes

(Thieme, 1965c). Table 3 gives the characteristic glycosides in bark of different sexes.

Generally female plants show higher concentrations of phenolic glycosides in bark than males, and only *S. alba* shows the reverse. The largest difference in concentration is observed in *S. caprea*, whose females have almost twice the male level. In *S. purpurea* and *S. triandra* about equal amounts occur in the two sexes.

The leaves of female plants also generally have higher amounts of glycosides than males. The quantities range from 0.15 and 0.23% (male and female) in *S. triandra* to 6.39 and 7.48% in *S. repens*. The only tissue in which the female plant has lower amounts of phenolics is in the reproductive organs (Thieme, 1965c).

Flavonoids and tannins of Salix. All species show the presence of flavonol glycosides, e.g., quercetin derivatives (Jaggi and Haslam, 1969; Thieme, 1969; Zucker, 1982).

Jarrett and Williams (1967) described differing flavonoid patterns in bark compared to leaves in *S. purpurea*. In young bark the flavonoid isosalipurposide predominates, while in older bark naringenin is predominant (Figure 3). Leaves show a different flavonoid pattern and luteolin dominates together with small amounts of eriodyctiol-7-gly. Kompantsev and Gaidash (1980) report that the flavonoid fraction of *S. alba* x *babylonica* leaves amounts to 3.5% of dry weight. The dominating compounds were luteolin and isorhamnetin (Figure 3).

Vinkurov (1979) isolated pyrocatechol, naringenin, and chalconaringenin from bark of *S. rubra* and *S. acutifolia*.

Salix species contain high amounts of leucoanthocyanidins (cyanidin and delphinidin) as well as catechin and gallocatechin (Jaggi and Haslam, 1969; Binns et al., 1968). There are conflicting reports on the occurrence of anthocyanidins in *S. triandra*. Jaggi and Haslam (1969) say that no such compounds occur in this species, while Bridle et al. (1970) report their presence. In view of the large differences in chemistry with age and sex of the genus, perhaps their plant material is not comparable.

Distribution of Phenolic Glycosides within Populus spp. Most *Populus* species are fairly large, rapidly growing trees. About 30 species are common in the northern temperate zone. *Populus tremula* is the only representative of this genus native to boreal forests in Scandinavia. Several related species in North America have a fairly large distribution (Mitchell, 1974). A common characteristic of the genus is the rapid dispersal by root suckers and a high regrowth capacity after browsing (Wolff, 1978).

The genus is chemically characterized by the occurrence of the phenolic glycosides salireposide and tremulacin (Figure 3). These two substances are only lacking in *P. nigra* bark (Kinsley and Pearl, 1967; Pearl and Darling,

TABLE 3. SEXUAL DIFFERENCES IN GLYCOSIDE PRODUCTION OF *Salix* BARK.[a]

		GLYCOSIDE[b]																
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total (%)
S. alba	♂	+	2+	–	–	–	–	(+)	+	–	–	–	–	–	–	–	–	2.71
	♀	+	+	–	–	–	–	(+)	+	–	–	–	–	–	–	–	–	1.66
S. caprea	♂	+	(+)	–	–	–	–	–	3+	–	–	–	–	–	–	–	–	2.24
	♀	+	(+)	–	–	–	–	–	4+	–	–	–	–	–	–	–	–	4.28
S. cinerea	♂	+	(+)	–	–	–	2+	–	+	–	–	–	+	–	–	–	–	2.74
	♀	+	(+)	–	–	–	2+	–	2+	–	–	–	+	–	–	–	–	3.33
S. purpureao	♂	2+	5+	2+	–	–	–	+	–	–	–	–	–	–	–	–	–	11.17
	♀	2+	5+	2+	–	–	–	+	–	–	–	–	–	–	–	–	–	11.13
s. repens	♂	+	3+	2+	–	–	–	(+)	–	–	–	–	–	–	–	–	–	3.91
	♀	+	3+	2+	–	–	–	(+)	–	–	–	–	–	–	–	–	–	4.80
S. triandra	♂	(+)	–	(+)	2+	–	–	(+)	+	–	–	–	–	–	–	–	–	1.68
	♀	(+)	–	(+)	+			(+)	2+	–	–	–	–	–	–	–	–	1.53

[a] From Thieme (1965c).
[b] For concentrations and compounds see Table 2.

1968a–c, 1971; Thieme and Benecke, 1970). However, salireposide is also found in *Salix* species, but so far only in *S. purpurea* and *S. repens* (Thieme, 1967, 1969). As opposed to *Salix* spp. there are no glycosides, triandrins, vimalins, or salidrosides in the bark of *Populus* (Thieme, 1967) (Table 4).

Differences in phenolic production among tissues and even within the same tissue are reported. Pearl and Darling (1968b) inform us that the nature of individual chemical components varies far more in twig bark than in trunk bark. In general, the twig bark has a much richer concentration of components but a narrower spectrum of substances than trunk bark. As an example, the concentration of trichocarpin in twig bark of *P. balsamifera* was twice as high as in trunk bark, while salicin content was seven times as great in the twig bark (Pearl and Darling, 1968b).

Zucker (1982) reports that individual leaves of *P. angustifolia* do not have the same concentration of phenolics. He studied the infestation of a leaf-galling insect and showed it colonized the largest leaves with the lowest phenol concentration. He reported that the characteristic glycosides of the genus also occur in this species.

Klimzak et al. (1972) investigated the free phenolic acids in *P. tremula.* They found chlorogenic acid in bark and chlorogenic, caffeic, ferulic, and *p*-coumaric acids were present in buds.

Flavonoids and Tannins of Populus *spp.* Buds of *Populus* secrete an oil containing the flavonols chrysin, galagnin, pinocembrin, and quercetin derivatives (Figure 3 and Table 5). Most flavanones and flavonols are methylated (Thieme, 1969; Wollenweber, 1975b; Thamas et al., 1979). Methylation makes the molecule more lipophilic (Harborne, 1979, 1980). Thamas et al. (1979) found 19 spots of unidentified flavonoids on thin-layer chromatography of *P. tremuloides* buds. He also reported that the concentration of flavonoids in buds amounted to 8.1% of dry matter. No studies seem to have been made on the occurrence of tannins in *Populus* species.

Changes in Chemistry with Season and Age of Salix *and* Populus. Seasonal changes in phenolic production are common in both *Salix* and *Populus* species. The concentration of phenolic glycosides in bark generally increases towards the winter, with the highest values during winter dormancy. The lowest values occur in September–October (Thieme, 1965d). In buds, on the other hand, the highest concentrations are found in late May just prior to leaf burst. As the leaves mature, the phenolic concentration declines continuously until September (Thieme, 1965d; Pearl and Darling, 1968a,b; Thieme and Benecke, 1971).

The substances also show diurnal variation, with higher values in early morning, decreasing during the day. In *S. fragilis* and *S. purpurea* the glycoside concentration decreases 20% and 40%, respectively, during the day (Thieme, 1965d).

TABLE 4. SPECTRUM OF PHENOLIC GLYCOSIDES AND AMOUNTS IN BARK AND LEAVES OF *Populus* SPP.[a]

Species		Glycoside[b] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	Total (%)
Populus tremula	bark	+	2+	+	–	2+	–	(+)	–	–	–	–	–	–	–	–	(+)	3.4
	leaf	+	+	–	–	–	–	–	–	(+)	–	–	–	–	–	–	–	2.2
P. tremuloides	bark	+	3+	+	–	4+	–	(+)	–	–	–	–	–	–	–	–	(+)	10.0
	leaf	2+	2+	–	–	3+	–	–	–	(+)	–	–	–	–	–	–	–	7.0
P. alba	bark	+	2+	(+)	–	(+)	–	(+)	–	–	–	–	–	–	(+)	–	–	2.3
	leaf	2+	2+	–	–	(+)	–	(+)	–	(+)	–	–	–	–	(+)	–	–	2.5
P. nigra	bark	+	+	–	–	–	–	–	–	–	–	–	–	+	(+)	–	–	1.3
	leaf	(+)	(+)	–	–	–	–	–	–	–	–	–	–	(+)	(+)	(+)	–	0.02
P. trichocarpa	bark	3+	5+	+	–	5+	–	–	–	–	(+)	–	2+	–	–	–	–	23.0
	leaf	3+	5+	+	–	(+)	–	–	–	–	–	–	3+	–	–	–	–	16.0
P. candicans	bark	3+	5+	2+	–	–	–	–	–	–	–	–	3+	–	–	–	–	19.5
	leaf	2+	4+	(+)	–	–	–	–	–	–	–	–	2+	–	–	–	–	8.0

[a] From Thieme (1967, 1969, 1971).
[b] For concentrations and compound see Table 2.

TABLE 5. FLAVONOIDS IN BUDS AND LEAVES OF SOME *Populus* SPECIES.[a]

Compound	*P. tremula*	*P. tremuloides*	*P. candicans*
Kaempferol	–[b]	–	–
3-Me	–	–	–
7-Me	–	–	–
4-Me	+	–	–
3,4-Me	–	–	
3,7-Me	–	–	–
7,4-Me	–	–	–
Quercetin	–	+	–
7-Me	–	–	–
3-Me	–	–	+
7,3-Me	–	–	–
Apigenin	–	–	–
4-Me	+	–	–
Luteolin	–	–	+
Myricetin	–	–	+
7-Me	–	–	+
Chrysin	+	+	–
Galagnin	+	–	–
Pinocembrin	+	+	–

[a] From Wollenweber (1975), Thamas et al. (1979), and Thieme (1969).
[b] For explanation of signs see Table 1.

The production of phenolics by the plant changes as it ages (Thieme, 1965c,d). Thus in *S. caprea* the levels of salicin and salicortin decrease with tree age, whereas the production of triandrin increases. In this species the overall phenol concentration increases with higher age, but the chemical diversity narrows (Thieme, 1965c,d; Thieme and Benecke, 1971). A similar pattern is also shown for *S. viminalis* and *S. cinerea*. When the latter species is young, triandrin is the main glycoside; picein dominates in older trees. In years 1 and 2 triandrin makes up 80% of the phenolics, decreasing to 9% in year 3 and 7% in year 4. Picein shows the reverse pattern (Thieme, 1965c,d).

Some species show a decline in total concentration of phenolic glycosides with age. Examples are *S. purpurea* and *Populus candicans*.

DISCUSSION

We can conclude that the woody boreal plants discussed in this paper appear to distribute the largest amount of substances into tissues which make a large contribution to plant fitness (photosynthesis and growth). High concentrations of specific substances occur, for example, in current-year

shoots and buds. The predominant chemical class seems to be phenolics of varying structure, perhaps because they are readily metabolized into polyphenols and tannins or catabolized into simpler constituents by the plant (McKey, 1979; Vickery and Vickery, 1981).

The plant accumulates large quantities of compounds during winter dormancy, with a rapid decline during time of growth. This pattern leads to the question of why, if secondary chemicals in these woody plants serve as defense substances, do they accumulate during times when most herbivores and pathogens are absent? One explanation would be that the substances act as growth inhibitors during winter or serve as a nutritional storage. However, these explanations are not satisfactory because of the high biological activity and toxicity observed in both plants and animals by the compounds (e.g., phenols) (Harborne, 1982). The compounds are mostly coupled to a sugar to prevent autotoxicity. Without excluding multiple functions of these plant substances, one major function appears to be to protect the plant against depredation (Janzen, 1981; Harborne, 1982). The external tissues of plants are the first to come into contact with herbivores and pathogens. High concentrations in peripheral tissues are therefore likely (McKey, 1979).

The very short vegetation period in northern latitudes probably makes the insect and pathogen pressure less important than browsing by vertebrates (Bryant and Kuropat, 1980). Vertebrate browsing has a great impact on the plant during winter (Danell, 1983). This could be the reason why the plant allocates large amounts of resources into substances in apical parts during winter. Furthermore, the plant may not be able to defend itself during time of rapid growth, which results in seasonal changes in quantity and biological activity of substances (Palo et al., 1983b). Moreover, mere growing perhaps helps the plant to escape in time and space, diminishing its apparancy to herbivores (Feeny, 1976). During summer, the diversity of different plants potentially available to herbivores is much larger than during winter, decreasing the overall browsing pressure. In such circumstances the need for a chemical defense would be lessened.

The preference of mammalian herbivores for mature growth forms over juvenile plants may be related to plant chemistry (Bryant, 1981; Pehrson, 1981). In young *Salix* and *Populus* species salicin derivatives make the plant taste bitter and hence repellent to herbivores (Markham, 1971). The shift of phenolic production in mature willow perhaps shows that damage on a full grown plant is of less importance than on a juvenile, making the need for an effective defense less acute.

Leaves of young birch have long been used as a drug in folk medicine because of its powerful diuretic effects (Nowak, 1966; Rowe and Conner, 1979). Mountain hares (*Lepus timidus*) showed high sodium losses via urine

when fed on 1.5-mm winter twigs of birch (Pehrson, 1981, 1983). The animals also lost body weight rapidly and did not survive many days on this diet. Bryant (1981) indicated that resin from *B. papyrifera* is repellent to browsing by snowshoe hare (*L. americanus*). He also showed the existence of species-dependent resins which differ in biological activity. Furthermore, Bryant (1981) and Pehrson (1981) independently noted that mature growth forms of birch are more preferred than juveniles or root suckers, also indicating an age-related change in quality in birch. Hjeljord et al. (1982) recorded differences in digestibility of organic matter by moose between *B. pendula* and *B. pubescens*, the latter being more digestible (21.1% vs. 24.2%). The chemical basis for these observations is not well known. The snowshoe hare-repellent resin consists, according to Reichardt (1981), principally of isoprenoids. However, in Scandinavian species phenolics may be of importance as antinutritional agents in mountain hares and ruminants (Palo et al., 1983a,b).

The substances of birch may interfere with the metabolism in animals. In the hare, for instance, sodium metabolism is disturbed (Pehrson, 1981).

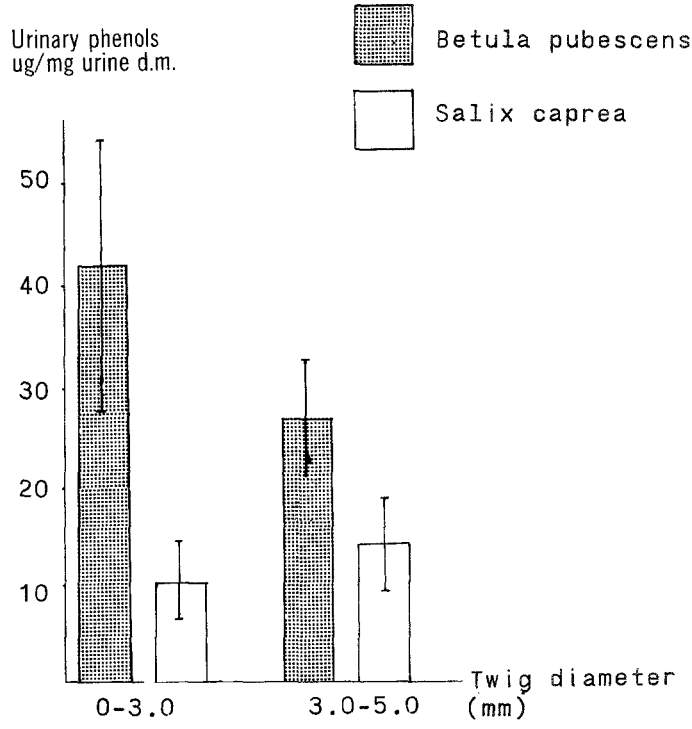


FIG. 4. Amounts (±SD) of urinary phenols excreted by hares fed on winter twigs of different diameters. (Palo, unpublished results.)

Sodium is regarded as a limiting mineral for boreal herbivores (Botkin et al., 1973; Belovky, 1978; Seastedt and Crossley, 1981). Plant substances that interfere with mineral metabolism are probably an effective defense against browsing by mammalian herbivores. The general deterrent properties that have been shown by Bryant (1981) in the snowshoe hare by Alaska birch, may be explained by the resin content of the latter.

Differences in production of phenols between sexes of willow species may well display different investments into defense. Danell et al. (1983) noted that voles, feeding on bark, prefer male plants over female plants. This behavior perhaps is a result of higher defensive investment in female plants.

Most willow species are preferred to birch species by herbivores (Southwood, 1961; Lindlöf et al., 1974; Wolff, 1978; Pease et al., 1979). This behavior by the vertebrate herbivore may be related to differences in defensive tactics by these plants or to a higher capability by the herbivores to detoxify and excrete willow secondary metabolites. Hares fed on different twig diameters of birch and willow exhibited higher phenolic excretion on birch diet than on willow diet, independent of the diameter of the twigs (Palo et al., 1983a) (Figure 4). This observation may indicate a higher physiological cost associated with consumption of birch than willow. Hypothetically, this could be a reason for selective feeding by the herbivores on these plants.

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BEHAVIORAL AND BIOLOGICAL RESPONSES OF *Cotesia marginiventris* TO KAIROMONES OF THE FALL ARMYWORM, *Spodoptera frugiperda*

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Abstract—Potential kairomone sources of the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), were bioassayed with females of *Cotesia marginiventris* (Cresson) in Petri dishes (10 cm diam). Mated *C. marginiventris* females, ranging in age from 1 to 3 days exhibited the most intense bioassay responses to potential sources of kairomone. Contacting a FAW-damaged corn leaf modified the pattern of movement in *C. marginiventris* from random to one exhibiting a significant increase in klinokinesis. No significant differences were present in kairomone responses of nonconditioned and conditioned parasitoids and parasitoid response to kairomones did not change throughout the photophase (0800–1800 hr). Removal of one, both, or the first eight antennal segments reduced or eliminated the response of the parasitoid to kairomones. Female parasitoids did not exhibit a preference for corn leaves damaged by a particular fall armyworm instar and parasitization rates were highest in larvae 48 hr old.

Key Words—*Cotesia marginiventris*, Hymenoptera, Braconidae, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, kairomones, fall armyworm, parasitism.

INTRODUCTION

Research on kairomones is gaining momentum because they influence host finding in parasitoids. The beneficial manipulation of parasitoids with these

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semiochemicals may easily become another tool among many strategies regulating pest populations (Lewis et al., 1976). The development of reliable bioassays is paramount in understanding how these chemicals influence parasitoid behavior. In general, two kinds of bioassays exist, (1) an olfactometer for compounds that attract from a distance greater than several centimeters and, (2) an arena for short range, nonvolatile, contact chemicals (Vinson, 1977). The type of bioassay depends on parasitoid behavior and the kairomone under investigation. Since many kairomones only elicit responses over short distances (Quednau, 1967; Vinson, 1968), a small arena in which the parasitoid is comfortably confined, easily observed, and allowed to come into direct contact with the test chemical appears most appropriate for kairomones of low volatility.

The host-finding behavior of *Cotesia marginiventris* (Cresson), a larval parasitoid of the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), has been well defined and the presence of host related kairomones documented (Loke et al., 1983). Initially, different bioassay arenas were evaluated to select the most desirable method for assessing kairomone responses by *C. marginiventris*, and these responses were scored in relation to behavior and parasitization rates as functions of preconditioning, mating, diel activity, antennectomies, and host age. Our principal objective is to define and validate a reliable bioassay based upon parasitoid behavior relative to kairomones produced by FAW larvae.

METHODS AND MATERIALS

Bioassay Arenas. Glass vials (1, 2, and 4 drams) and Petri dishes (5, 10, and 15-cm diam) were evaluated as bioassay arenas. The vial bioassay was initiated by capturing the female parasitoid in the vial directly from the holding cage. The test substance was spotted onto the end of a strip of filter paper that was then inserted into the vial and held in place with the thumb. For the Petri dish arena, the test substance was spotted onto the center of a piece of No. 1 Whatman filter paper. The female parasitoid was removed from the holding cage in a No. 000 gelatin capsule and transferred to a covered Petri dish containing the filter paper. Parasitoids were allowed to acclimatize in both vials and Petri dishes and were then observed for 60 sec under two 20-W fluorescent bulbs at ambient laboratory conditions (26° C, 50% relative humidity, and normally lighted). The criteria used to evaluate arenas were the ease of parasitoid transfer to and from the arena, parasitoid behavior when confined in the arena, ease of administering the test substance and observing parasitoid response, parasitoid manipulation within the arena, and the amount of time required for a single replicate.

Conditioned Parasitoids. Sixty 3-day-old female parasitoids were divided into three equal groups. One group was not exposed to hosts or corn

plants; a second group was exposed to FAW-damaged corn leaves from which all FAW larvae had been removed; and a third group was exposed to FAW-damaged plants on which second-instar larvae remained. Exposure lasted 30 min, after which the parasitoids were held in a Plexiglas cage for 1 hr prior to starting the bioassay. Rearing procedures for parasitoids and corn plants are documented in Loke et al. (1983). Responses were quantified using a modification of a four-point system developed by Lewis and Jones (1971). An intense response (antennal palpation and/or ovipositor probing) when contacting the kairomone patch the first time was scored as a 3. Scores of 2 and 1 were given when a positive response occurred on the second or third contact, respectively, and a 0 was recorded if no response was elicited after three contacts. The number of visits within the initial 60 sec of actual observation and the duration of the first visit were recorded. A new Petri dish was used for every four parasitoids tested.

Virgin and Mated Females. Seventy-five parasitoid cocoons were isolated in a Plexiglas cage (24 cm^3) provisioned with honey and water and held for adult eclosion and mating. Virgin females were obtained by placing another 75 cocoons individually in 30-ml plastic cups. A small plug of cotton saturated daily with a 50:50 honey–water solution was inserted through a hole in each cup lid. Emergence was monitored until 15 or more females eclosed from each group within a 12-hr period, thereby providing a narrow age distribution for parasitoids used in the bioassays. Daily responses of each group to FAW-damaged corn leaves were collected for ca. 1 week.

Diel Activity. The response of 2- to 4-day old mated and conditioned female parasitoids at different times of the day was studied using FAW-damaged corn leaves. Twelve parasitoids were bioassayed starting at the beginning of the photophase (0800) and during the following time intervals: 0800–0830; 1000–1030; 1200–1230; 1400–1430; 1600–1630; and 1800–1830 hr.

Antennectomy Effects. Two- to 4-day-old female parasitoids were collected from the general emergence cage and anesthetized with CO_2. Subsequently their antennae were removed to form the following treatments: one antenna removed; half of both antennae removed; both antennae completely removed; and no antennae removed. At least 3 hr of recuperation were allowed prior to assaying in a Petri dish using FAW-damaged corn leaves.

Larval Damage and Parasitization. Sections of corn leaves damaged by selected instars of FAW larvae were evaluated to determine if these leaves and associated larval by-products would elicit antennation and ovipositor probing in 2- to 4-day-old parasitoids. The corn leaf sections (1.0 × 1.5 cm) with traces of frass were assayed in Petri dishes using ca. 20 individual female parasitoids.

Parasitization rates were determined by exposing individual groups of 30 larvae, with each group having a specified age, to a 2-day-old female parasitoid for 24 hr in a 7.5 × 10-cm-diam plastic cup. Subsequently, each larva

was transferred into a 30-ml diet cup to complete development. Each age was replicated four times and larvae were placed on 2-cm diet cubes within the plastic cup during the host-exposure period.

RESULTS AND DISCUSSION

Bioassay Arenas. Petri dishes were selected as the bioassay arena because they were easier to prepare, permitted more efficient handling of bioassay material, and allowed parasitoid movement to be watched and recorded. Also while in the dish, the parasitoid displayed more natural movements rather than attempting to escape as was the case with the vials. The 5-cm dishes were too confining and since no substantial differences could be discerned between the 10- and 15-cm dishes, the 10-cm Petri dish was selected as the bioassay unit.

Contacting a FAW-damaged corn leaf modified the pattern of movement in *C. marginiventris* from random to one consisting of intense antennal palpation, ovipositor probing, and klinokinesis (Figure 1). Not contacting damaged plant material resulted in the parasitoid examining about half of the dish during the observation period and exhibiting a substantial reduction in klinokinesis.

Conditioned Parasitoids. No significant differences were present between the scores of nonconditioned and conditioned parasitoids with and without previous ovipositional experience (Table 1). A significantly higher proportion of nonconditioned females made multiple visits to the corn leaf. However, these same females exhibited a marked reduction in the duration of their first visits. Strong responses to the damaged corn leaf included intense antennal palpation, ovipositor probing, and klinokinesis.

Virgin and Mated Females. The reactions of virgin and mated *C. marginiventris* females to FAW-damaged corn leaf sections (Figure 2) showed that mated females, ranging in age from 1 to 3 days, exhibited more intense bioassay responses (Figure 2A), had a higher percentage responding strongly (Figure 2B), and displayed a greater propensity to probe with their ovipositors (Figure 2C) than virgin females in the same age range. Mated and virgin females were most similar in response at 4–5 days old. Commencing on day 6 and continuing through day 8, the responses of virgin females declined more rapidly in all three bioassay categories than did those of mated females. In general, 2- to 4-day-old mated females appeared to be the most responsive. These results closely paralleled the observations of Kunnalaca and Mueller (1979) in which *C. marginiventris*, parasitizing *Plathypena scabra* (F.), were reported to be most active on the second and third days.

Diel Activity. The responses of *C. marginiventris* to kairomones at six different times throughout the photophase (0800–1800 hr) showed no significant differences. Therefore, conducting bioassays need not be restricted to

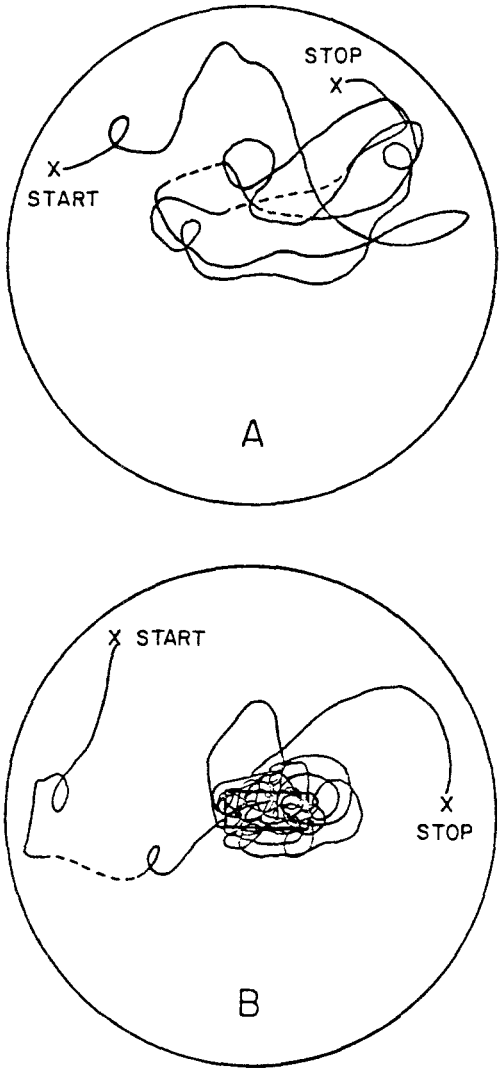

FIG. 1. Typical behavioral patterns in a Petri dish (10 cm diam) of *C. marginiventris* females not exposed (A) and exposed (B) to a section of corn leaf damaged by fall armyworm larvae.

a particular portion of the photophase. Parasitoids appeared to be most active around 1400 hr. *Cotesia marginiventris* is a day-adapted species and has been observed searching for hosts in bright sunlight in corn fields (Ashley, personal communication). Similar behavior has been noted in other parasitoids (Vinson, 1975; Kunnalaca and Mueller, 1979; Weseloh, 1980).

TABLE 1. RESPONSES OF NONCONDITIONED AND CONDITIONED (WITH AND WITHOUT PREVIOUS OVIPOSITIONAL EXPERIENCE) *C. marginiventris* FEMALES IN PETRI DISH BIOASSAY TO CORN LEAF SECTIONS DAMAGED BY FALL ARMYWORM LARVAE

Response	Nonconditioned	Conditioned	
		Oviposition	No oviposition
Mean (±SE)[a]			
Score	3.0 ± 0.2 a	2.9 ± 0.3 a	3.0 ± 0.0 a
Duration of first visit (sec)	34.8 ± 2.6 a	56.5 ± 4.5 b	58.8 ± 2.5 b
Number of visits	2.2 ± 0.9 a	1.3 ± 0.6 b	1.3 ± 0.4 b
Percent			
Responding strongly	95	90	100
Ovipositor probing	100	95	100
Multiple visits	70	36	20

[a]Means in the same row followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple range test. Means based on a four-point scoring system with values ranging from 0 to 3.

Antennectomies. The degree of antennectomy had a significant effect on the response of *C. marginiventris* to host kairomones (Table 2). Parasitoids with one antenna removed were affected primarily in their ability to respond strongly. Clipping off the terminal eight of the 16 flagellomeres on both antennae caused a significant reduction in all responses, and no response was elicited from parasitoids with both antennae removed. These results corrobo-

TABLE 2. EFFECT OF ANTENNECTOMY ON RESPONSE OF *C. marginiventris* TO CORN LEAF SECTIONS BY FALL ARMYWORM LARVAE

Degree of antennectomy	Mean (±SE) response[a]	Percent		
		Responding	Responding strongly	Ovipositor probing
None	2.9 ± 0.3 a	100.0	93.3	100.0
1 antenna	2.7 ± 0.4 a	100.0	73.3	100.0
1/2 of both antennae	1.3 ± 0.6 b	93.3	0.0	26.7
Both antennae	0.0 c	0.0	0.0	0.0

[a]Means in the same column followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple range test. Means based on a four-point scoring system with values ranging from 0 to 3.

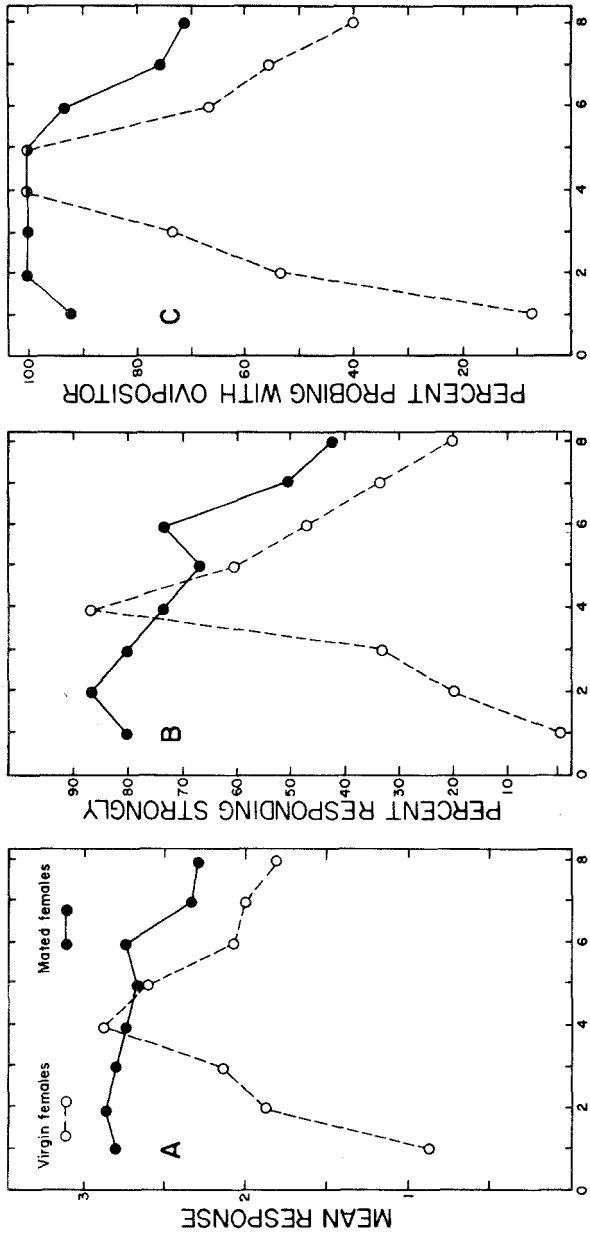

FIG. 2. Responses of virgin and mated *C. marginiventris* females to corn leaf sections damaged by fall armyworm larvae for (A) mean response, (B) percent parasitoids responding strongly, and (C) percent parasitoids probing with ovipositor.

TABLE 3. EFFECT OF HOST AGE ON PARASITIZATION AND LARVAL DEVELOPMENT IN *C. marginiventris*[a]

Age of FAW larva (hr)	Parasitoid larval duration (days)	Percent		
		Parasitization	Parasitoid eclosion	Multiple cocoons
12	7.7 ± 0.1 a	53.8 ± 18.7 a	81.0 ± 13.5 a	1.3
24	7.2 ± 0.3 a	79.1 ± 14.3 b	95.3 ± 4.1 a	0.0
48	6.9 ± 0.3 a	94.1 ± 4.2 c	94.8 ± 3.4 a	1.9
72	7.0 ± 0.0 a	81.7 ± 12.9 b	93.6 ± 8.5 a	0.0
96	6.8 ± 0.2 a	70.8 ± 5.2 b	94.1 ± 1.9 a	0.0
120	7.9 ± 0.1 a	45.0 ± 11.8 a	90.3 ± 2.9 a	12.7

[a]Means in the same column followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple range test.

rate other research demonstrating the importance of antennae in host finding (Greany and Oatman, 1971; Weseloh, 1971; Richerson et al., 1972).

Instar Damage and Parasitization. Female parasitoids did not exhibit a preference for corn leaves damaged by a particular FAW instar. Parasitoids responded strongly and equally to all test samples. This indicated that there was no difference, essentially, between kairomones of the various host instars produced in association with corn leaves. Therefore, larger and more easily handled FAW instars could be used in acquiring appreciable quantities of substances needed for testing as sources of kairomones.

Parasitization rates were highest in 48-hr-old larvae (Table 3). This was also shown to be the case for *C. marginiventris* parasitizing *Trichoplusia ni* (Hübner) (Boling and Pitre, 1970). However, the situation in the field may be different because Vickery (1929) reported that first instars are preferred since they can be stung before dispersing from the egg mass. Larvae younger than 24 hr and older than 96 hr were not as successfully parasitized as larvae between these two ages. This could be due to the ability of the older and larger hosts to physically defend themselves, encapsulate the developing parasitoid, or possess a cuticle too thick for successful oviposition.

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CHEMICAL COMPOSITION AND EFFICACY OF CEPHALIC GLAND SECRETION OF *Armitermes chagresi* (ISOPTERA: TERMITIDAE)

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Abstract—Soldiers of the neotropical humivorous termite *Armitermes chagresi* have large ice-tong-like mandibles for mechanical defense and a moderate length nasus from which cephalic gland defensive secretions are discharged. Soldiers do not eject secretion, but ooze droplets which are held at the nasus tip by hairs. The chemical secretion is composed of C_{22}–C_{26} macrocyclic lactones, including C_{24} and C_{26} and α- and β-hydroxylated lactones, with C_{24} macrolides predominating. The cephalic gland secretion has no pheromonal properties and does not induce alarm or attraction in soldiers or workers. Upon application, the secretion repels and is irritating to ants, but does not appear to act as an antihealant. The defensive behavior and chemistry of *A. chagresi* is discussed in light of termite ecology and evolution.

Key Words—*Armitermes chagresi*, Isoptera, Termitidae, termite, chemical defense, cephalic gland, macrocyclic lactones.

INTRODUCTION

Defensive specializations of termite soldiers are reflected in their limited behavioral repertory, diverse morphological modifications, and elaborate chemical weaponry (reviewed by Prestwich, 1979; Deligne et al., 1982). Primitive soldiers have well-developed mandibles, whereas in the highly modified nasute type the mandibles are vestigal and the head capsule has evolved into an ampule-shaped apparatus for discharging a viscous, glue-like secretion

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produced in the cephalic (frontal) gland. Defensive secretions may be applied to predators via biting, labral brushing, or squirting (Quennedey, 1975), and chemical functions include repellents, topical poisons, and entangling agents (Prestwich, 1979). Soldiers of different termite taxa differ in their synthetic capabilities. For example, *Nasutitermes* soldiers discharge a glue composed of tetracyclic and tricyclic diterpenes, whereas advanced rhinotermitids (e.g., *Schedorhinotermes*) utilize vinyl ketones, nitroolefins, and β-ketoaldehydes as contact poisons or to disrupt ant recruitment behavior (Prestwich et al., 1975; Prestwich and Collins, 1982; Kaib, 1982). *Macrotermes* soldiers ooze onto their mandibles a paraffin–olefin mixture from the frontal gland; it has been suggested that the secretion impairs wound healing (Prestwich et al., 1977).

Primitive mandibulate termites in the subfamily Nasutitermitinae (e.g., *Cornitermes*, *Armitermes*, and *Rhynchotermes*) have soldiers that appear to use a combined mechanical and chemical defense. Soldiers of these neotropical genera illustrate in part Emerson's (1961) hypothesis for the evolution of nasute termites in which ancestral species with mandibulate soldiers gave rise to nasute forms through an elongation of the rostrum and reduction in mandible size. Emerson's scheme is based on soldier morphology. Recently, Prestwich and Collins (1981a) proposed an alternative evolutionary sequence based on the chemistry of the frontal gland secretions and defensive behavior of soldiers. Chemical, ethological, and ecological aspects of defense in primitive nasutitermitines, however, are poorly documented. To further elucidate and evaluate mechanisms of termite defenses in the context of chemosystematic, phylogenetic, and ecological questions, we here present details of the defensive behavior of the soldier caste of *Armitermes chagresi* Snyder and describe the chemical composition and efficacy of its defensive secretion.

METHODS AND MATERIALS

Collection of Termites and Ants and Laboratory Arrangements. Termites used in all experiments were from portions of two colonies collected in the lowland rainforest of Barro Colorado Island, Panama (9° 09′ N, 79° 51′ W) collected on April 20, 1981. Transported north in an undisturbed block of nest carton, the termites were maintained in high humidity at 25°C in the laboratory in a 46 × 39 × 16-cm container. Ants used in studies of defensive behavior and cephalic gland secretion properties (*Odontomachus brunneus*, *O. bauri*, *Pachycondyla obscuricornis*, *P. apicalis*, *Ectatomma ruidum*, *Camponotus sericeiventris*) were collected on Barro Colorado Island and in mangroves in Coco Solo, Panama. Workers of *Camponotus planatus* and *Formica schaufussi* were obtained from colonies maintained in the laboratory, originally collected in Florida and Massachusetts, respectively.

Collection of Cephalic Gland Secretion. Termite secretions were obtained in three ways: (1) extraction of the extirpated soldier frontal gland with distilled hexane, (2) microcapillary collection of the secretion discharged from a soldier's gland in response to irritation, and (3) hexane extraction of crushed heads of soldiers, followed by filtration of the crude extract through a short Florisil column using 5% ether–hexane. The macrolides and hydroxy macrolides II and III were separated on a 230–400 mesh silica gel minicolumn by elution with 5% ethyl acetate in hexane. GC-MS and GC retention time comparisions with *A. neotenicus* compounds (Prestwich and Collins, 1981b; Prestwich, 1982) were thus performed on well-resolved peaks. The three techniques listed above gave secretions which were indistinguishable by TLC and GLC; consequently, the third method was employed for large-scale collections.

Instrumental Methods. Gas chromatography was performed on a Varian 3700 instrument equipped with a 2 m × 2 mm ID glass column packed with 1% SP-2100 on Supelcoport, temperature programed from $T_i = 200°$ (2 min delay), $T_p = 4°/\text{min}$, $T_f = 300°$ (hold 20 min). No lower molecular weight volatiles were observed when the secretion was examined from 50° to 200° on this column. Low-resolution electron-impact mass spectra were obtained using a Hewlett Packard model 5980A mass spectrometer interfaced to an HP5710A GC equipped with a 1% SP-2100-packed glass column. High-resolution mass spectra were obtained on an MS-30 instrument interfaced to an HP7210A GC and a DS-50 data system. NMR spectra were obtained on Varian Associates CFT-20 instruments operating at 20 MHz for ^{13}C and 80 MHz for ^{1}H. High-resolution [^{1}H]NMR spectra were obtained on a Bruker 360 spectrometer. Identifications were performed by comparisons of NMR, GC-MS, and GC retention time data to compounds isolated from *A. neotenicus* from Guyana.

SEM pictures were taken with an AMR 1000 A scanning electron microscope.

RESULTS

Soldier Defensive Behavior. The defensive specialization of *Armitermes* soldiers is evident in their morphology (Figure 1). Soldiers have well-developed mandibles and a substantial nasus to direct the secretion of the cephalic gland. To examine soldier defensive behavior, we perturbed a colony by breaking open a portion of a gallery or a nest, and subsequently recorded changes in the numbers of soldiers at the breach. Soldiers appeared in low numbers (~8) within the first few minutes of the perturbation and assumed defensive positions with their mandibles gaping wide. They soon left their guard positions in the absence of further stimulation. Mechanically agitating "guarding" soldiers did not induce recruitment of additional soldiers.

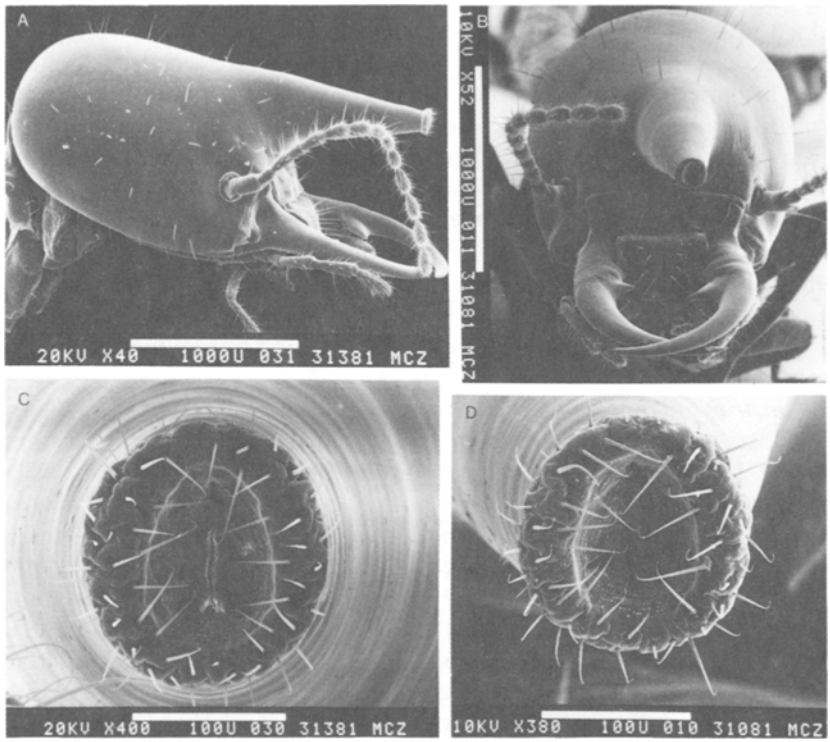

FIG. 1. Morphology of *Armitermes chagresi* soldier. (A) Lateral view of soldier. Note prolonged nasus and ice-tong-like mandibles. The cephalic gland fills approximately two thirds of the head capsule. (Magnification 40x.) (B) Head-on photo of soldier head showing nasal aperture and mandibles. Each mandible has a single marginal tooth (52x). (C) and (D) Nasal openings of two soldiers, showing slit for secretion discharge and hairs used to retain and daub the chemical (x400 and 360, respectively). Reference bars = 1000 μm. All photos by Edward Seling, MCZ SEM Facility, Harvard University.

We studied soldier defenses in response to ant predation by placing small portions of termite nests in the foraging arenas of various ant species. Ants used in the tests were neotropical species sympatric with *A. chagresi: Ectatomma ruidum*, *Odontomachus bauri*, *Pachycondyla obscuricornis*, *P. apicalis* (Ponerinae), and *Camponotus sericeiventris* (Formicinae). Soldiers guarding the entrances to broken galleries appeared firmly anchored in position even when moribund from injury incurred from attack. Ants were able to dislodge soldiers only after repeated attempts. When contact was made, soldiers would grip the mandibles or legs of ants and remain attached

after death. A droplet of frontal gland secretion, exuded at the nasus tip, would occasionally contact an ant, which responded by retreating and self-grooming. Occasionally the mandibles would inflict a wound on an ant, puncturing the cuticle. Frontal gland secretion flowed over the cuticle and into the wound. Some ant species were more effective predators. For example, workers of *Camponotus sericeiventris* easily attacked soldiers, lunging forward and recoiling quickly. This dislodged and disabled soldiers, which were then retrieved.

Chemical Composition of Frontal Gland Secretion. The cephalic secretion of *A. chagresi* was strikingly similar to that of the allopatric species *A. neotenicus* from Guyana (Figure 2). Both have C_{22}–C_{26} macrolides, including C_{24} and C_{26} α- and β-hydroxylactones (Figure 3). The *A. chagresi* secretion has a C_{22}:C_{24}:C_{26} ratio of nonoxygenated lactones (1) of 3.7: 51.3:6.5, and the *A. neotenicus* ratio is 2.5:47.8:5.0 For both species the α-hydroxy C_{24} lactone 2a is greater than the β-OH C_{24} lactone 2a, while for C_{26} lactones, the α:β ratio favors the β isomer 3b over the α isomer 2b. This interspecific similarity is particularly noteworthy in view of the variation among the sympatric species *A. neotenicus*, *A. holmgreni* (C_{32} macrolide), and *A. teevani* (spectrum of C_{22}–C_{38} macrolides (Prestwich and Collins, 1981b).

Behavioral Effects of Frontal Gland Secretion. Responses of workers and soldiers of *A. chagresi* were tested by offering soldier frontal gland secretion to termites traveling on an established trail. The secretion was collected by gently squeezing soldier heads with forceps, causing the gland to discharge its contents onto the tip of an applicator stick. The secretion, which is not heavy or viscous, flowed evenly from the nasus and did not become sticky after drying. Applicator sticks contaminated with fresh secretion were placed 1 cm from worker traffic on an established trial. We predicted that if the secretion had an alarm function, worker traffic would decrease upon exposure to the treated stick. We observed no apparent reaction to the secretion (Table 1), and in subsequent tests in which we offered secretion to individual soldiers or workers, we could not recognize any response, even when offered at close range (Table 2). There was no mandibular gaping, or investigation by antennal waving by soldiers or workers. When secretions were offered in defensive contexts (nest breaching, ant attacks), the results were similar. Synthetic preparations of secretion, offered in identical situations, did not produce any measurable response.

Role of Frontal Gland Secretion in Defensive Behavior. As described above, *A. chagresi* soldiers at times locked their mandibles onto ants, thereby piercing the cuticle. Either concomitant with or immediately following mandibular contact, the nasus tip would also make contact, and secretion would begin to flow from the frontal gland into the wound. We observed that the secretion had irritating and repellent properties. When sympatric ant species

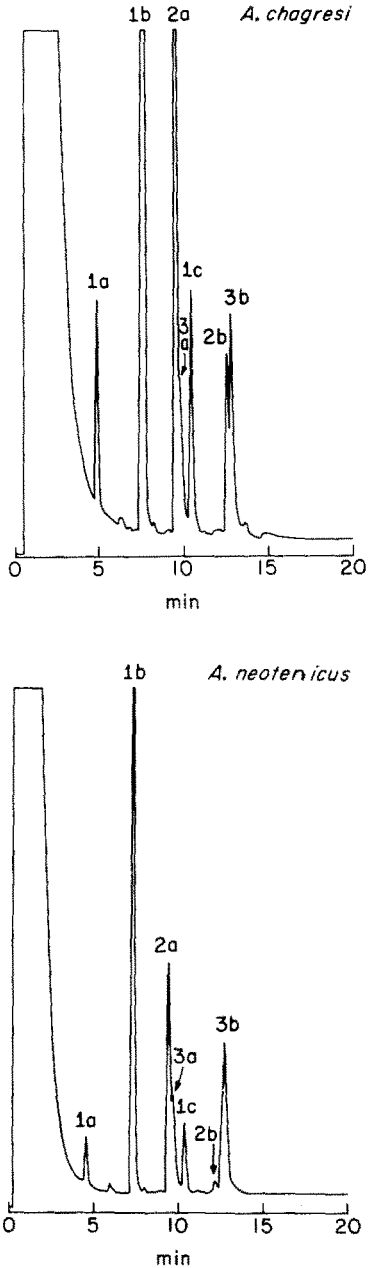

FIG. 2. GLC traces of *A. chagresi* (top) and *A. neotenicus* (bottom) soldier secretions (1% SP2100, 2 m × 2 mm ID, $T_i = 200°$, 2 min, $T_p = 40°$/min, $T_f = 280°$, 20 min). Compounds are identified by numbers (see Figure 3).

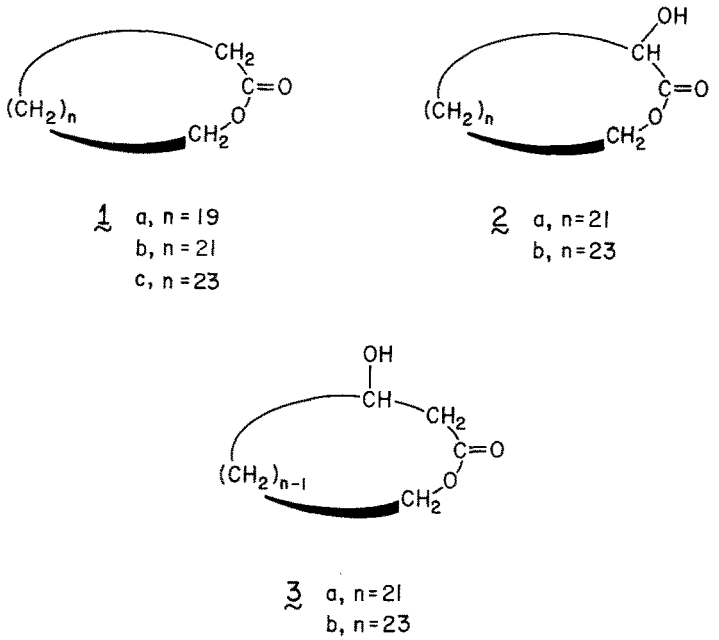

FIG. 3. Structures of macrocyclic lactones identified in *A. chagresi* cephalic secretion.

contacted secretion squeezed from a soldier's head onto the tip of an applicator stick, grooming occurred. Touching the ant's antennae, legs, or body in this way repelled individual *Pachycondyla obscuricornis*, *Odontomachus brunneus*, *O. bauri*, and *Camponotus sericeiventris* workers, confirming Emerson's (1961) results. Synthetic preparations of different macrolides pro-

TABLE 1. RESPONSES OF *A. chagresi* SOLDIERS AND WORKERS TO CEPHALIC GLAND SECRETION OF SOLDIERS[a]

	Caste tested			
	Workers		Soldiers	
	Secretion	Control	Secretion	Control
No response	37 (74)	40 (80)	36 (72)	38 (76)
Back away	13 (26)	10 (20)	2 (4)	4 (8)
Gape mandibles	0	0	12 (24)	8 (16)

[a]Fresh secretion was obtained from single glands by squeezing a soldier's head with forceps. The contents were discharged onto the tip of an applicator stick which was placed 2–3 mm from the antennae of individuals of either caste. Controls were untreated applicator sticks. Secretions were used for 5 min. Percentage response given in parentheses. $N = 50$ for each caste.

TABLE 2. CHANGES IN TRAFFIC OF *A. chagresi* WORKERS ON TRAIL IN RESPONSE TO SOLDIER CEPHALIC GLAND SECRETION[a]

	Trial 1 (workers/min)	Trial 2 (workers/min)
Background	14.4 ± 3.3	16.2 ± 4.4
Control	13.0 ± 1.7	25.6 ± 6.0
Secretion	10.2 ± 1.2	33.6 ± 5.7
10 minutes following secretion	9.4 ± 2.7	31.6 ± 3.2

[a]Methods as in Table 1. Secretion offered 3–5 mm on side of trail. Worker traffic was monitored before a control (untreated applicator stick) or secretion was offered and 10 min after secretion was removed. The mean and standard deviation of five consecutive 1-min counts are given for two separate trials. Worker traffic does not predictably change with the presence of secretion; comparisons of control and secretion tests are not significantly different (Student's *t* test).

duced similar behavior. Ants showed no apparent olfactory response to the secretion.

Because secretion might enter wounds inflicted by the mandibles, we hypothesized that it could function as a toxin, since it enters the hemolymph immediately, or as an antihealant that interferes with plasma coagulation and wound sealing, as suggested for *Macrotermes michaelseni* and *M. subhyalinus* by Prestwich et al. (1977). To distinguish between these two possible functions, we devised the following experiment which simulated injuries resulting from soldier defenses. We punctured the dorsal mesothorasic cuticle of workers of the various species of ants with an insect pin (size 0), thus creating an injury similar to that resulting from contact (treatment P). Pressure was applied with the pin to the cuticle only until the point of puncture; the pin tip never proceeded more than 0.5 mm into the thorax. In a second group, we likewise inflicted a wound and discharged into it the secretion of the cephalic gland of an *A. chagresi* soldier (treatment PS). A third group of ants received both punctures and secretion, but following application of the frontal gland material we sealed the wound with warm paraffin wax (treatment PSW). Control treatments involved isolated, untreated ants and ants receiving punctures sealed with paraffin (treatment PW). We then followed mortality in double-blind experiments over the next 48 hr, and predicted that differential mortality among treatments would permit us to distinguish if the secretion is a toxin or antihealant. If the secretion acts as a toxin, then there should be no difference in mortality among ants treated with secretion following wounding and ants treated similarly but also receiving an application of paraffin to seal the wound. High mortality should occur in these two treatments. However, if the secretion interferes with wound healing,

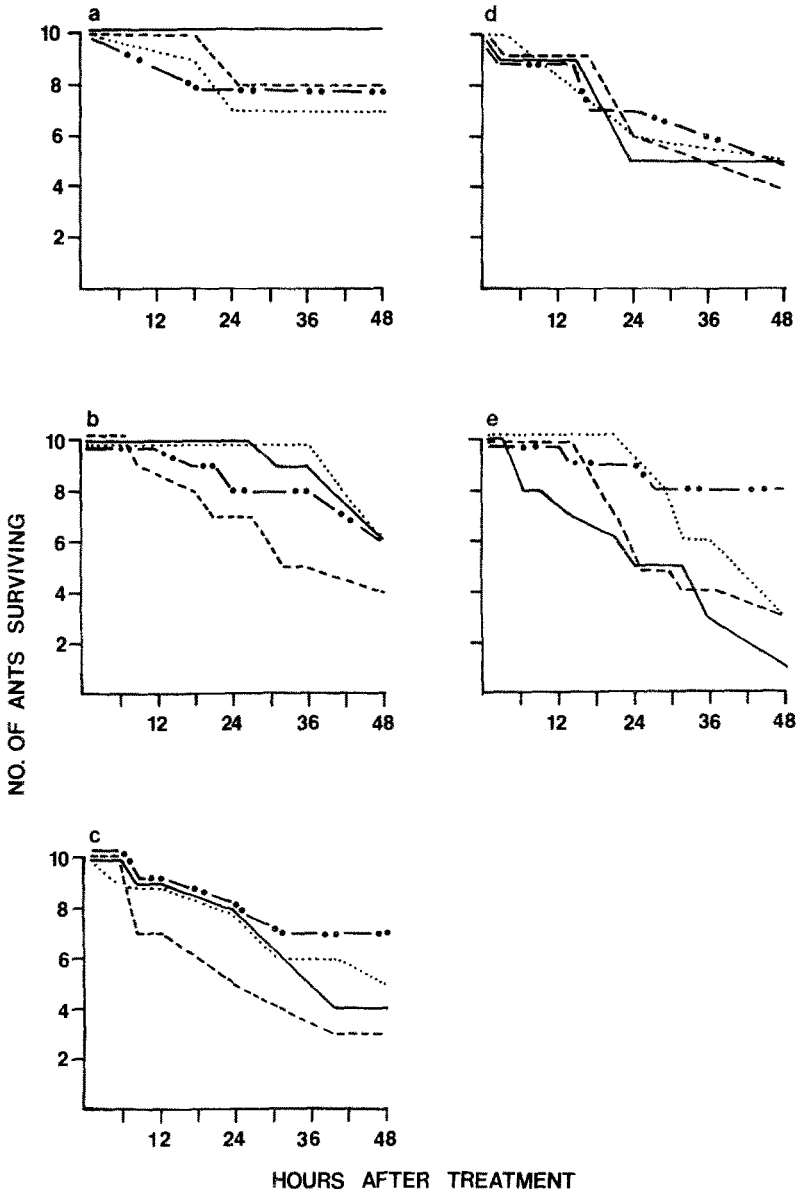

FIG. 4a–e. Survivorship of workers of *Camponotus planatus* receiving various treatments. ———, puncture only; . . . . . , puncture and wax; - - - - -, puncture and cephalic gland secretion; ——.——, puncture, cephalic gland secretion, and wax. Handling controls had a total of 18% mortality in all five replicates; 10 ants per treatment were used in each replicate.

then ants experiencing the PSW treatment should show lower mortality than those experiencing the PS treatment.

Although the effect of frontal gland secretion varied according to the species of ant tested, our results do not consistently indicate that it functions as an antihealant. In a series of tests with *Camponotus planatus*, no consistent pattern of mortality was evident in five separate trials (Fig. 4a–e).

Similar experiments involving application of C_{23} and C_{25} synthetic homologs of lactones (one treatment of each experimental procedure) produced different effects in ant species sympatric with *A. chagresi*. In the ponerine *Odontomachus brunneus*, PS, PSW, and topical applicaton (TA) all produced mortality (10 ants/treatment). The highest mortalities (90, 80, and 40%) resulted form PSW, PS, and TA treatments, respectively. In a second series, 70, 50, 40, and 30% mortality resulted from PSW, P, TA, and PS treatments, respectively (10 ants/treatment). These studies suggest the secretion acts as systemic toxin. In one set of experiments with *Camponotus sericeiventris* (5 ants/treatment), all treatments, including a handling control, yielded 80–100% mortality. There were no differences in mortality caused by C_{23} or C_{25} isomers. In a second series, we concentrated on examining the effect of topical application only. With 20 ants/treatment, TA produced 70% mortality, compared to 30% in a control group. In studies with *Odontomachus bauri* (10 ants/treatment), TA produced 30% mortality. In studies with a north temperate ant species, *Formica schaufussi*, no experimental treatments produced mortality patterns different from controls. Secretion toxicity, therefore, appears to be species dependent, but we are unable to statistically substantiate this hypothesis due to our small sample sizes.

DISCUSSION

There is considerable variability in the chemical composition of the cephalic gland secretion of *Armitermes* soldiers. Macrocyclic lactones have been described in the Guyanese species *Armitermes neotenicus*, *A. holmgreni*, and *A. teevani* (Prestwich and Collins, 1981b), in addition to *A. chagresi*. The secretion of *A. chagresi* strongly resembles that of *A. neotenicus* in the distribution of size (C_{22}–C_{26}) and the occurrence of C_{24} and C_{26} macrolides and the corresponding α- and β-hydroxylated lactones. This allopatric interspecific similarity contrasts with the marked interspecific differences seen among the three sympatric Guyanese species. Soldiers of the Brazilian species *A. euamignathus* possess only straight-chain hydrocarbons, primarily tri- and pentadecane (Coles, 1980; Prestwich, 1982). Mill (1982) found that macrocyclic lactones produced by soldiers of *A. teevani*, *A. neotenicus*, and *A. holmgreni* caused paralysis or moribundity in *Pseudomyrmex termitarius* and *Ectatomma* sp. 30 min after topical application.

Prestwich et al. (1977) reported that soldiers of *Macrotermes subhyalinus* and *M. michaelseni* produce a series of alkanes and olefins in the frontal gland that impair the healing of wounds inflicted by the mandibles. Our studies with *A. chagresi* do not suggest that macrocyclic lactones have an antihealant function. The effects of an antihealant are delayed in time, and producing mortality after an attack has occurred would seem to be an effective defensive strategy only against ants with small colony size, no recruitment communication, and forager site fidelity. It is possible that defensive secretions might secondarily act as antihealants, although their principle function is the direct repulsion of predators.

The frontal gland secretion of *A. chagresi* does not seem to have any communicative properties, eliciting neither alarm or attraction. Furthermore, soldiers do not appear to recruit additional soldiers to conflict areas along chemical trails, although our histological studies and behavioral bioassays shows that they do have a functional sternal gland (Thorne, unpublished; Traniello, unpublished). The absence of an alarm/recruitment response is in marked contrast to the highly coordinated defenses of other termite species (Eisner et al., 1976; Traniello, 1981; 1982; Stuart, 1982). Defensive behavior in *A. chagresi* seems to result from the summation of individual soldier responses to stimuli associated with defensive situations rather than a communication-mediated coordinated effort.

The defensive behavior of *A. chagresi* soldiers seems primitive, although in addition to a mechanical defense, they use a chemical irritant that is applied directly. They are capable of blocking breached galleries with their heavily sclerotized heads and mandibles but are unable to discharge defensive secretion over a distance, and therefore they must engage in direct contact with predators. Because soldiers lock onto opponents with their mandibles, defense may result in the expenditure of a soldier. As Emerson (1961) reported, mandibular movements alone, as well as frontal gland secretion, have a deterrent effect on ants. The defensive behavior of *A. chagresi* appears to resemble that of other humivorous termite species such as *Cubitermes* and *Noditermes* whose nests are composed of numerous chambers connected by galleries of a diameter that is only slightly larger than a soldier's head (Deligne and Pasteels, 1982). The soldiers' heads are phragmotic, possess slicing mandibles and a defensive secretion, and are effective in blocking the narrow nest galleries [the "static warfare" defense of Deligne and Pasteels (1982)]. In contrast to *A. chagresi* whose colonies are comprised of 7–8% soldiers (Thorne, 1982), *Cubitermes*, *Noditermes*, and other humivores have 0.02–3% soldiers (Haverty, 1977). These differences in investment in the soldier caste may be correlated with specific foraging habits and/or the relative importance of invertebrate and vertebrate predation. It appears that termite species that show the "static warfare" defense have soldiers that produce relatively large molecular weight, oily compounds of low volatility,

either alkanes, macrolides, or diterpenes. The use of macrolides as defensive secretions in *Armitermes* is striking because 16-, 18-, and 20-carbon lactones have been found in the Dufour's glands of solitary bees, where they are components of brood cell lining (Bergstrom, 1973; Albans et al., 1980; Cane, 1981). C_{18}–C_{24} lactones have been identified in halictids and are hypothesized to function as marking pheromones and polyester cell lining precursors (Duffield et al., 1981).

The synthesis of macrocyclic lactones (Prestwich and Collins, 1981b) and alkanes (Coles, 1980; Prestwich, 1982) by *Armitermes* soldiers does not support Emerson's (1961) hypothesis of the evolution of nasute termites. Also casting doubt on the diphyletic scheme is the apparent convergence in defensive tactics employed by *Armitermes* and *Rhynchotermes:* soldiers of these genera dab rather than squirt frontal gland secretions. Therefore, current evidence supports the hypothesis of monophyletic evolution of nasutes (Prestwich and Collins, 1981a).

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Journal of Chemical Ecology, Vol. 10, No. 3, 1984

Announcement

CURT P. RICHTER PRIZE IN PSYCHONEUROENDOCRINOLOGY

Through the generosity of the Institute of Clinical Pharmacology, Dublin, Ireland, an Annual Prize has been established for meritorious research in the area of Psychoneuroendocrinology. The sum of US $ 1000 will be awarded annually at the Congress for the best essay or manuscript (original research or review including original research) submitted by a scientist or physician under 40 years of age by January 1, 1984. The winning paper will automatically be considered for publication in the journal *Psychoneuroendocrinology* and should be prepared according to the journal's instructions to the authors. The aim of the prize is to encourage younger scientists to contribute to this interdisciplinary field.

Manuscripts should be submitted in quadruplicate to:

Gerhard Langer, MD.
Associate Professor of Psychiatry
Department of Psychiatry
University of Vienna
Lazarettgasse 14
A-1097 Vienna
AUSTRIA

Deadline for submission: March 31, 1984.

All submissions will be screened by a broad committee of established psychoneuroendocrinologists. The prize will be awarded at the 1984 meeting of the Society in Vienna, Austria (July 15–19, 1984).

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MULTICHEMICAL RESISTANCE OF THE CONIFER *Podocarpus gracilior* (PODOCARPACEAE) TO INSECT ATTACK

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Abstract—*Podocarpus gracilior* is resistant in nature to insect attack. Apparently, the resistance of *P. gracilior* is due to a multichemical defense mechanism. Chemicals identified as potential components of the multichemical defense are four norditerpenedilactones, including nagilactones, C, D, and F, which cause insect feeding deterrent activity ultimately coupled to an insecticidal activity, and podolide, an insecticide; two nonlethal growth-inhibiting biflavones, podocarpusflavone A and 7″,4‴-dimethylamentoflavone; and the ecdysis-inhibiting phytoecdysone, ponasterone A.

Key Words—*Podocarpus gracilior*, conifer, resistance, multichemical defense, norditerpene dilactones, phytoecdysone, biflavones, *Pectinophora gossypiella*, *Heliothis zea*, *Spodoptera frugiperda*, Lepidoptera, feeding deterrency, *Bombyx mori*.

INTRODUCTION

Host plant resistance to insect attack is largely due to chemical factors. In fact, it has long been speculated (Brues, 1946; Dethier, 1954) that phytophagous insects attack any available plant not containing repellent or toxic factors. The elucidation of these factors is not only important for understanding evolutionary and ecological aspects of plant-insect relationships, but may also be of a practical use. For example, plant breeding programs may genetically enhance the chemical defense of some plant species, while synthetic pesticide research may apply that defense artificially to another plant species.

Through this line of reasoning, potential insect control agents have been investigated in plants which are relatively free from insect attack. One such plant is the conifer *Podocarpus gracilior* Pilg. (Podocarpaceae).

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Podocarpus species in general are considered to be relatively immune to insect attack (Brown and Sanchez, 1974; Singh et al., 1978). At least some of this immunity may be attributed to ponasterones, compounds with molting hormone activity (Nakanishi, 1969; Brown and Sanchez, 1974; El-Ibrashy, 1976), which occur predominately in the Taxaceae and the Podocarpaceae. More recently, over 40 nor- and bisnorditerpene dilactones have been isolated from various tissues of 17 species of *Podocarpus* (Sassa et al., 1981). Some of these dilactones have a variety of biological effects, including insecticidal (Singh et al., 1978, 1979). The resistance of *P. nivalis* to insect attack is attributed to a high concentration of nagilactone C in the foliage (Russell et al., 1972). In addition, Brown and Sanchez (1974) suggested that the norditerpene dilactones, along with the phytoecdysones, may be important elements in the chemical ecology of *Podocarpus* species such that they offer potent lines of defense against insect attack.

This paper describes the isolation and characterization of three classes of chemicals from *P. gracilior* foliage which have three different effects on several species of nonadapted lepidopterans. These effects, all of which may contribute to the chemical defense of the plant, include a feeding deterrent activity ultimately coupled to an insecticidal activity due to nagilactones C, D, and F; an insecticidal activity due to podolide; a nontoxic growth inhibitory activity caused by podocarpusflavone A and 7″,4‴-dimethylamentoflavone, and ecdysis inhibitory activity induced by ponasterone A.

METHODS AND MATERIALS

Biossays. Extracts of *P. gracilior* were monitored for activity (including growth inhibition, insecticidal, and ecdysis inhibition) with an artificial diet assay. The extracts were dissolved in the appropriate solvent, applied to nonnutritive α-cellulose, evaporated to dryness, and added to the components of a meridic artificial diet, including solid nutrients (casein, sucrose, wheat germ, Wesson salts), vitamins (C and B-complex), and agar (Chan et al., 1978). Newly hatched larvae of four species of lepidopterous agricultural pests were placed singly on portions of the diet in plastic cups. For example, pink bollworm, *Pectinophora gossypiella*, was assayed in 2-dram scintillation vials; fall armyworm, *Spodoptera frugiperda*, in 4-dram scintillation vials; and cotton budworm, *Heliothis zea*, in 1-oz catchup cups. The larvae were placed in a dark incubator at 25°C and 60% relative humidity. Daily observations were made and larval weights were determined after 12 days (equivalent in time to fourth-instar control larvae).

The active constituents were isolated using the artificial diet bioassay. After purification and identification, this same bioassay was used to determine LD_{90} values, the doses causing 90% death, ED_{50} values, the doses causing 50% growth inhibition, and EI_{90} values, the doses causing 90% ecdysis

inhibition. All values were determined from log dose-probit curves (Finney, 1952).

A "choice" leaf disk bioassay was used to supplement the "no-choice" artificial diet bioassay in order to test for feeding deterrency as a cause for the observed growth inhibition. Leaf disks (1 cm^2) were punched out from a glandless cotton cultivar (Pima S-4 of *Gossypium barbadanse*), randomized and arranged (12 disks/dish) in a circle on moistened filter paper in polyethylene foam grids inside glass Petri dishes (100 × 15 mm). Alternating disks were treated on their upper surface with either 25 μl acetone or with nagilactones C or D dissolved in 25 μl acetone (applied with a 25-μl Oxford P-7000 pipettor). Three newly molted third-instar larvae of the cotton bollworm or of the fall armyworm were then placed in the dishes at 25°C and 60% relative humidity in a dark incubator. After 24–48 hr, the larvae were removed, and the disks were visually examined. The feeding deterrent activity of the nagilactones was reported as PC_{50} values, which were concentrations of compound with which less than 5% of the treated leaf disks were eaten, while over 50% of the untreated leaf disks were eaten.

An additional bioassay with silkworm, *Bombyx mori*, was used to investigate the effects of ponasterone A on insect ecdysis. This bioassay entailed orally injecting (Hamilton microsyringe) 10 μl of a solution prepared by solubilization of ponasterone A in 30% aqueous ethanol with 1 drop Triton X-100. All insects, including appropriate controls, were transferred to fresh control diet immediately following injection. This artificial control diet consisted of a fortified dried mulberry powder (Nihon Nohsan) to which a 4% agar solution was added. A 48 hr postinjection, the "old" head capsule was removed and a sketch (Figure 1) was drawn off the "new" head capsule.

Isolation and Identification of Bioactive Principles. A concentrated methanolic extract of dried leaves of *P. gracilior* grown in Nairobi, Kenya, was partitioned between ethyl acetate and water, the ethyl acetate fraction subsequently dried and washed with ether to remove pigments, and the ether-insoluble material subjected to column chromatography on silica gel 60 (30–70 mesh ASTM) in 10% methanol in chloroform. When bioassayed in artificial diet, the early eluted fractions were toxic, the later fractions caused a nontoxic growth inhibition, while still later fractions caused ecdysis inhibition.

Following further chromatography on silica gel 60 (30–70 mesh ASTM) in ethyl acetate–ethanol (9:1) the toxic fractions were combined, reduced in volume, and subjected to low-pressure liquid chromatography (Pharmacia peristaltic pump). Samples were injected (Altex slider injector) onto LiChroprep RP-18 (25–40 μm) reversed-phase packing material in a Pharmacia glass chromatographic column (SR 10/50, 1.0 × 50 cm). A prepacked Lobar LiChroprep RP 8 (size A, 240-10, 40–63 μm) column was placed in series with the Pharmacia RP-18 column. The injections were monitored at a wavelength of 280 nm (Pharmacia single-path monitor UV-1) under isocratic

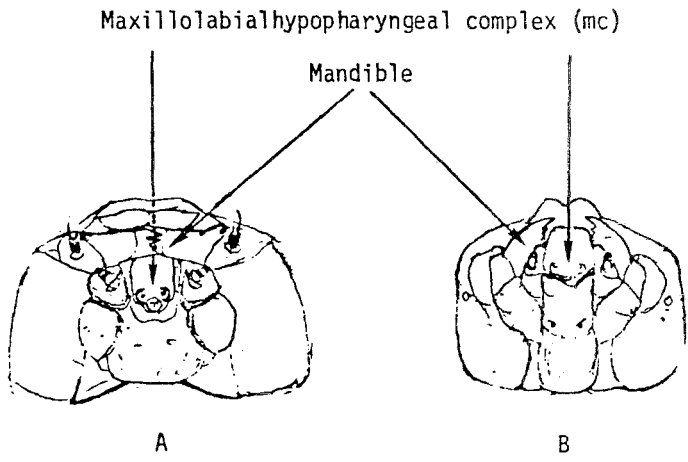


FIG. 1. Schematic depicting (ventral side) dysfunctional mouthparts in ecdysis-inhibited *Bombyx mori* larvae. (A) Normally ecdysed fifth-instar head capsule with fully closed mandibles. (B) Ecdysis-inhibited fifth-instar head capsule following artificial removal of fourth-instar head capsule. The adhering fourth instar head capsule prevented full expansion of the fifth instar head capsule resulting in the forward position of the maxillolabialhypopharyngeal complex (mc) such that the mandibles could not fully close.

conditions (water–methanol–acetonitrile, 5:4:1 v/v) at a flow rate of 50 ml/hr. The elution times for the active constituents, nagilactones C and D, were approximately 40 and 60 min, respectively.

The fractions causing a nontoxic growth inhibition were isolated by repeated thin-layer chromatography; followed by precipitation from acetone upon standing in a refrigerator. The precipitate was redissolved in acetone–methanol (1:1, v/v) and chromatographed on silica gel GF preparative TLC (Analtech) plates in ethyl acetate–petrol (3:1, v/v). Two yellow bands were cut from the plates, extracted with methanol, and rechromatographed on similar TLC plates in petrol–acetone (1:1 v/v). Subsequent extraction of the two resulting yellow bands from these latter TLC plates resulted in two pale yellow amorphous compounds, podocarpusflavone A and 7″,4‴-dimethylamentoflavone, both of which showed a nontoxic growth inhibitory activity against insects.

The fractions causing ecdysis inhibition were collected, dried, washed with small amounts of acetone, and extracted with methanol. Crystallization from methanol yielded needles of ponasterone A.

Additional insecticidal components, nagilactone F, podolide, and dihydropodolide were isolated from an ether soluble portion, less polar part than nagilactone C and D, by the aforementioned techniques including normal and reversed-phase column chromatograph.

HPLC Chromatography. A methanolic extract of *P. gracilior* foliage grown in Berkeley, California, was analyzed for the bioactive principles isolated from the Kenyan foliage with a DuPont, model 850 liquid chromatograph. A prepacked DuPont Zorbax ODS C_{18} (particle size 5–6 μm) stainless-steel column (25 cm $\times$ 4.6 mm ID) equipped with a stainless-steel guard column (7 cm $\times$ 2.1 mm ID) packed with pellicular Co: Pell ODS was used. The compounds were monitored by UV spectra using a DuPont variable-wavelength ultraviolet spectrophotometer detector. The samples were injected into the column using a Rheodyne rotary valve 7120 syringe-loading injector.

Prior to injection onto the HPLC, the methanolic extract was chromatographed on silica gel GF preparative TLC (Analtech) plates in 20% petrol-ethyl acetate. Bands corresponding to the reference dilactones and biflavones were cut, extracted (with methanol), and injected onto the HPLC. The injections were monitored at a wavelength of 305 nm under isocratic conditions of methanol–water (1 : 1, v/v) at a flow rate of 1.5 ml/min. The retention times of podocarpusflavone A, 7″,4‴-dimethylamentoflavone, nagilactone C, and nagilactone D were, respectively, 1.5, 2.2, 4.4, and 5.5 min.

The ether extract of the *P. gracilior* foliage collected in Berkeley was similarly prepurified on silica gel GF preparative TLC (Analtech) plates in 100% chloroform. The band corresponding to the reference podolide was cut, extracted with chloroform–methanol (1 : 1), and injected onto the HPLC. The injections were monitored at a wavelength of 222 nm under isocratic conditions of methanol–water (1 : 1 v/v) at a flow rate of 1.0 ml/min. The peak corresponding to podolide had a retention time of 6.3 min.

Ponasterone A was extracted from the methanolic extract with water. The water extract was reextracted with ethyl acetate, dried, washed with small amounts of ether, and subjected to TLC on silica gel (as above) plates in 20% methanol–water. The band corresponding to authentic ponasterone A was cut, extracted with methanol, and injected onto the HPLC. The injections were monitored at a wavelength of 242 nm under isocratic conditions of methanol–water (13 : 7, v/v) at a flow rate of 1.5 ml/min. The retention time of standard ponasterone A was 5.3 min.

Ponasterone A: mp 259–260°C, $C_{27}H_{44}O_6$, MS 465 (M^+), UV (EtOH) 244, 327 nm (ϵ = 12,000, 150), [^{1}H]NMR (C_5D_5N) δ 0.84 (6H, d, J = 6 Hz, 26-H, 27-H), 1.09 (3H, s, 19-H), 1.24 (3H, s, 18-H), 1.58 (3H, s, 21-H), 3.01 (1H, dd, 5-H), 3.78 (1H, s, 9-H), 3.83 (1H, s, 17-H), 4.23 (1H, s, 2-H), 5.04 (bs, OH), 6.26 ppm (1H, d, J = 2.2, 7-H).

Podolide: mp 296°C, $C_{19}H_{22}O_5$, MS 330 (M^+), 287, 271, 259, 243, 229, 215, 199, UV (EtOH) 218 nm (ϵ = 13,000), IR ($CHCl_3$) 1770, 1705 cm^{-1}, [^{1}H]NMR (C_5D_5N), δ 1.05 (3H, d, J = 7.0 Hz, CH_3), 1.16 (3H, s, CH_3), 1.18 (3H, d, J = 7.0 Hz, CH_3), 1.30 (3H, s, CH_3), 2.05 (2H, br d, H-1), 2.07 (1H, d, J = 5.0 Hz, H-5), 4.24 (1H, d, J = 1.5 Hz, H-7), 4.61 (1H, d, J = 4.0 Hz,

H-14), 5.16 (1H, dd, J = 1.5, 5.0 Hz, H-6), 5.80 (1H, dt, J = 3.0, 3.0, 10.0 Hz, H-2), 5.90 (1H, d, J = 10.0 Hz, H-3), 6.17 (1H, s, H-11).

2,3-Dihydropodolide: mp 248°C, $C_{19}H_{24}O_5$, Ms 332 (M^+), 304, 289, 273, 261, 245, 233, 216, UV (EtOH) 218 nm (ϵ = 10,400), IR (KBr) 1775, 1705 cm^{-1}, [^{1}H]NMR (C_5D_5N) δ 1.03 (3H, d, J = 7.0 Hz, CH_3), 1.17 (3H, d, J = 7.0 Hz, CH_3), 1.09 (3H, s, CH_3), 1.18 (3H, s, CH_3), 1.80 (1H, d, J = 4.5 Hz, H-5), 4.19 (1H, d, J = 1.5 Hz, H-7), 4.54 (1H, d, J = 4.5 Hz, H-14), 5.07 (1H, dd, J = 4.5, 1.5 Hz, H-6), 6.13 (1H, s, H-11).

Nagilactone F: mp 223°C, $C_{19}H_{24}O_6$, MS 316 (M^+), 288, 273, 245, 229, 227, UV (EtOH) 260 nm (ϵ = 15,000), IR (KBr) 1765, 1700, 1610 cm^{-1}, [^{1}H]NMR ($CDCl_3$) δ 0.98 (3H, d, J = 6.5 Hz, CH_3), 1.15 (3H, s, CH_3), 1.19 (3H, d, J = 6.5 Hz, CH_3), 1.33 (3H, s, CH_3), 1.93 (1H, d, J = 4.5 Hz, H-5), 4.85 (1H, q, J = 2.0 Hz, H-14), 5.04 (1H, td, J = 2.0, 4.5, 4.5 Hz, H-6), 5.74 (1H, d, j = 2.0 Hz, H-11), 6.18 (1H, dt, J = 2.0, 2.0, 4.5 Hz, H-7).

Nagilactone C: mp 290°C, $C_{19}H_{22}O_7$, MS 362 (M^+), UV (EtOH) 300 nm (ϵ = 3000), [^{1}H]NMR ($CDCl_3$) δ 1.19 (3H, d, J = 6.5 Hz, CH_3), 1.23 (3H, d, J = 6.8 Hz, CH_3), 1.29 (3H, s, CH_3), 1.52 (3H, s, CH_3), 1.88 (1H, d, J = 6.5 Hz, H-5), 3.30 (2H, m, H-2, H-15), 3.54 (1H, d, J = 4.0 Hz, H-1), 4.33 (1H, d, J = 6.0 Hz, H-3), 4.75 (1H, dd, J = 6.5, 8.5 Hz, H-6), 5.25 (1H, d, J = 8.5 Hz, H-7), 6.16 (1H, s, H-11), [^{13}C]NMR ($CDCl_3$) 19.2 (C-20), 20.1 (C-16), 20.7 (C-17), 26.2 (C-18), 29.6 (C-15), 38.1 (C-10), 50.1 (C-4), 51.2 (C-5), 51.5 (C-2), 58.1 (C-1), 60.1 (C-7), 67.9 (C-3), 107.2 (C-11), 111.7 (C-8), 162.0 (C-12), 165.1 (C-14), 117.7 (C-19).

Nagilactone D: mp 265–266°C (dec), $C_{18}H_{20}O_6$, CI-MS (NH_3) 332 (M^+), UV (EtOH) 303 nm (ϵ = 7100), IR (KBr) 3554, 1767, 1718, 1633, 1550 cm^{-1}, [^{1}H]NMR ($CDCl_3$) δ 1.24 (3H, t, J = 7.5 Hz, CH_3), 1.27 (3H, s, CH_3), 1.44 (3H, s, CH_3), 1.90 (1H, d, J = 6.5 Hz, H-5), 2.62 (1H, q, J = 7.5 Hz, H-15), 2.80 (1H, dd, J = 7.0, 16.0 Hz, H-7), 3.41 (1H, dd, J = 10.0, 16.0 Hz, H-7), 3.48 (1H, dd, J = 4.0, 6.0 Hz, H-2), 3.59 (1H, d, J = 4.0 Hz, H-1), 4.45 (1H, d, J = 6.0 Hz, H-3), 4.95 (1H, dt, J = 10.0, 7.0 Hz, 6-H), 6.30 (1H, s, H-11), [^{13}C]NMR ($CDCl_3$) 11.6 (C-16), 17.6 (C-20), 24.6 (C-15), 25.6 (C-7), 25.8 (C-18), 37.7 (C-10), 48.7 (C-4), 50.6 (C-2, C-5), 57.1 (C-1), 67.6 (C-3), 73.1 (C-6), 106.2 (C-11), 106.5 (C-8), 161.7 (C-9), 162.9 (C-12), 163.5 (C-14), 177.9 (C-19).

Podocarpusflavone A: mp 254–258°C (soften), 310°C (dec), $C_{31}H_{20}O_{10}$, FD-MS 552 (M^+), UV (MeOH) 215, 270, 335 nm (ϵ = 40,000, 35,000, 27,000), shifting in base to 218, 272, 370, respectively. FT-IR (KBr) 317, 1652, 1606, 1580, 1504, 1261, 1241, 1179, 1141, 834, 698 cm^{-1}, [^{1}H]NMR (C_5D_5N) δ 3.62 (3H, s, ArO-CH_3), 5.28 (OH), 6.73 (1H, d, J = 2.0 Hz, 6-H), 6.78 (1H, d, J = 2.0 Hz, 8-H), 6.89 (1H, s, 6″-H), 6.94 (1H, s, 3-H), 6.98 (2H, d, J = 9.0 Hz, 3‴-H, 5‴-H), 7.05 (1-H, s, 3″-H), 7.48 (1H, d, J = 9.0 Hz, 6′-H), 7.88 (2H, d, J = 9.0 Hz, 2‴-H, 6‴-H), 8.00 (1H, dd, J = 3.0, 9.0 Hz, 5′-H), 8.54 (1H, d, J = 3.0 Hz, 2′-H).

7″,4‴-Dimethyletheramentoflavone: mp 282–285°C, $C_{32}H_{22}O_{10}$, FD-MS 566 (M^+), UV (MeOH) 225, 270, 340 nm (ϵ = 53,000, 37,590, 29,800), shifting in base to 228, 272, 375, respectively. FT-IR (KBr) 3143, 1666, 1615, 1579, 1488, 1410, 1334, 1266, 1182, 697 cm^{-1}, [^{1}H]NMR (C_5D_5N) δ 3.62 (3H, s, ArO-CH_3), 3.75 (3H, s, ArO-CH_3), 5.28 (OH), 6.75 (1H, d, J = 2.0 Hz, 6-H), 6.78 (1H, s, 6″-H), 6.82 (1H, d, J = 2.0 Hz, 8-H), 6.96 (1H, s, 3-H), 7.00 (2H, d, J = 9.0 Hz, 3‴-H, 5‴-H), 7.07 (1H, s, 3″-H), 7.52 (1H, d, J = 9.0 Hz, 6′-H), 7.86 (2H, d, J = 9.0 Hz, 2‴-H, 6‴-H), 8.04 (1H, dd, J = 3.0, 9.0 Hz, 5′-H), 8.41 (1H, d, J = 3.0 Hz, 2′-H).

RESULTS

The methanolic extract of the leaves of a *P. gracilior* tree growing in Nairobi, Kenya, caused mortality within 12 days after incorporation into a meridic artificial diet of several lepidopterous pest species. Fractionation of this extract with concomitant biomonitoring (utilizing the aforementioned artificial diet feeding assay) separated the bioactivity into three components: the toxic and growth inhibitory action of nagilactones C, D, and F and podolide, the nontoxic growth inhibitory action of biflavone, and the toxic ecdysis inhibitory action of the phytoecdysone ponasterone A.

Although a trace amount of dihydropodolide was also isolated, a whole sample was used for characterization.

Table 1 shows that, although nagilactones C, D, and F and podolide

TABLE 1. ACTIVITIES OF FOUR NORDITERPENE DILACTONES ON SURVIVAL AND GROWTH RATE OF NEONATE LARVAE TREATED IN 12-DAY FEEDING BIOASSAY

Species	Test compound	LD_{90} (ppm)[a]	ED_{50} (ppm)[b]
Heliothis zea	Nagilactone C	1500	20
	Nagilactone D	800	4
	Nagilactone F		30
	Podolide		12
Spodoptera frugiperda	Nagilactone C	2000	18
	Nagilactone D	2000	6
	Nagilactone F		12
	Podolide	2000	7
Pectinophora gossypiella	Nagilactone C	1500	14
	Nagilactone D	200	4
	Nagilactone F		20
	Podolide	300	9

[a]LD_{90} values are the lethal doses for 90% death and are the means of five determinations.
[b]ED_{50} values are the effective doses for 50% growth inhibition and are the means of five determinations.

TABLE 2. TWENTY-FOUR-HOUR LEAF DISK "CHOICE" BIOASSAY WITH THIRD-INSTAR LARVAE OF *Heliothis zea* AND *Spodoptera frugiperda* AGAINST TWO NORDITERPENE DILACTONES[a]

Species	Test compound	PC_{50} (μg/disk)[b]
Heliothis zea	Nagilactone C	38
	Nagilactone D	15
Spodoptera frugiperda	Nagilactone C	38
	Nagilactone D	20

[a]Podolide and nagilactone F was not tested in the leaf disk bioassay, while podocarpusflavone A and 7″,4‴-dimethylamentoflavone were found inactive in this bioassay.
[b]PC_{50} values are concentrations of test compounds at which less than 5% of the treated leaves are eaten, while greater than 50% of the untreated leaves are eaten. The experiment was terminated when more than 50% but less than 80% of the control leaf disks were consumed.

were relatively potent growth inhibitors, the concentrations of the compounds that cause mortality were about two orders of magnitude higher. This may, at least in part, be due to a feeding deterrent effect of these compounds (Table 2). Nagilactone D was found more potent than nagilactone C in both the artificial diet and the leaf disk bioassays. Nagilactone D also proved more potent than podolide and nagilactone F in the artificial diet feeding test.

Possible metabolic effects of the nagilactones on the larvae were not tested for, although it was often observed in the leaf disk assay that after a few small bites out of the treated leaf disks, the larvae appeared to be less active and continued to feed on the untreated leaf disks more slowly.

Concentrations of either of the biflavonoids, podocarpusflavone A or 7″,4‴-dimethylamentoflavone, as high as 5000 ppm in artificial diet, resulted

TABLE 3. GROWTH INHIBITORY ACTIVITY[a] OF TWO BIFLAVONES FED IN ARTIFICIAL DIET

Species	Test compound	ED_{50} (mmol)	ED_{50} (ppm)[b]
Heliothis zea	Podocarpusflavone A	1.1	625
	7″,4‴-Dimethylamentoflavone	4.4	2500
Spodoptera frugiperda	Podocarpusflavone A	2.5	1400
	7″,4‴-Dimethylamentoflavone	5.8	3300
Pectinophora gossypiella	Podocarpusflavone A	4.2	2300
	7″,4‴-Dimethylamentoflavone	4.1	2300

[a]No deaths were recorded for either biflavone to 5000 ppm.
[b]ED_{50} values are the effective doses for 50% growth inhibition and are the means of five determinations.

TABLE 4. EFFECT OF PONASTERONE A ON GROWTH AND DEVELOPMENT OF NEONATE PINK BOLLWORM AND FOURTH-INSTAR (FIRST DAY) SILKWORM LARVAE[a]

Species	Amount in diet (ppm)	Amount orally injected (μg)	Effect
Pectinophora gossypiella	1		ED_{50}[b]
	2		EI_{95}[c]
Bombyx mori		5	EI_{95}[c]

[a]Extracts of *P. gracilior* containing approximately 500 ppm of ponasterone A were ineffective in the diet of *Heliothis zea* and *Spodoptera frugiperda.*
[b]ED_{50} values are the effective doses for 50% growth inhibition.
[c]EI_{95} values are the effective doses for 95% kill due to ecdysis inhibition.

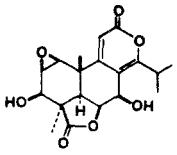
in tiny, but live, larvae. This nontoxic growth inhibitory effect may be due to some effect on larval metabolism, especially since they evoked no feeding deterrency response in the leaf disk assay (Table 3).

The increased activity of the monomethylether, podocarpusflavone A, vs. the dimethylether, 7″,4‴-dimethylamentoflavone, against *H. zea* (four-fold) and *S. frugiperda* (two-fold) is consistent with the generalization for monoflavonoids that increased growth inhibitory activity is positively correlated with an increased number of hydroxyl groups (Elliger et al., 1980). On a molal basis, the activities of these two biflavones as growth inhibitors were comparable to that activity reported for the monoflavonoids against *H. zea*, including those with a vicinal diol (Elliger et al., 1980).

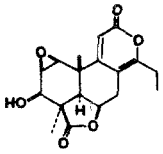
Ponasterone A had molting hormone activity when fed to *P. gossypiella* or when orally injected into *B. mori*. *H. zea* and *S. frugiperda* were apparently unaffected by dietary ponasterone A (Table 4). Dietary ponasterone A had earlier been reported to affect the molting of *Cecropia* silkworm by I.M. Riddiford (see Williams, 1970) and confused flour beetle (Robbins et al., 1970), in addition to inhibiting the growth of *B. mori* larvae (Shigematsu et al., 1974).

DISCUSSION

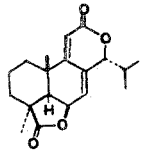
The first barrier to be overcome in an insect–plant relationship is a behavioral one (Dethier, 1970), dictated by both olfactory and gustatory cues sensed by the insect. In *P. gracilior*, the feeding deterrent action of the dilactones represents a behavioral defense barrier. When the insects are given a choice of food source, as in the leaf disk bioassay, they preferentially eat the source (leaf disks) containing no deterrent to the same source with deterrent. This deterrency can lead to severe growth inhibition and eventual death if no alternative food source is available. In the artificial diet bioassay, the test



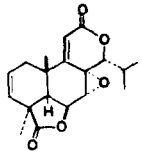
nagilactone C



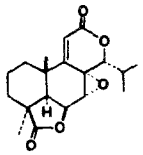
nagilactone D



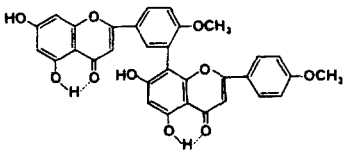
nagilactone F



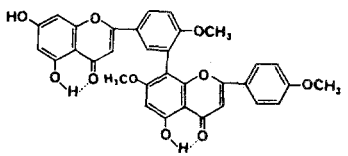
podolide



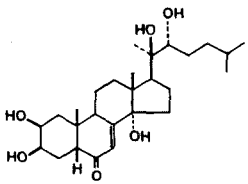
2, 3-dihydropodolide



podocarpusflavone A



7'', 4'''-dimethylamentoflavone



ponasterone A

SCHEME I

insects are not given a choice of food source, and their growth is severely retarded (Table 1). Eventually (within 12 days) the affected larvae die as very small first-instar larvae. Whether the insects died as a result of starvation or as a result of direct toxic action of nagilactones is unknown. Insects overcoming this behavioral barrier must still contend with at least two other chemical barriers in *P. gracilior*—a metabolic barrier due to biflavones and a developmental barrier due to ponasterone A.

Flavonoids are believed to contribute to the defense of several plant species against phytophagous insects (Feeny, 1968). For example, flavonoid tannin (Klocke and Chan, 1982) as well as a C-glycosyl-flavone (Elliger et

al., 1980), isolated from cotton and corn plants, respectively, inhibited growth of several lepidopterous larvae when incorporated into diet. Similarly, the activity of the biflavones manifests itself as a nontoxic growth inhibitory activity. The inhibited growth is probably not due to a feeding deterrent effect (Table 2), but occurs only after ingestion of the biflavones. The affected insects do not die, but remain smaller and are expected to be more amenable to increased mortality through longer exposure to natural predation, parasitization, and adverse physical phenomena. Also, the lowered consumption results in less damage per unit time inflicted upon the plant.

A third line of defense in *P. gracilior* is a developmental barrier which must be overcome by attacking insects. Due to its molting hormone activity, ponasterone A exemplifies this developmental barrier (Kubo et al., 1982). Ponasterone A not only promotes molting (apolysis) when orally injected into fourth-instar *B. mori*, but, perhaps more importantly, prevents the completion of the induced molt by inhibiting ecdysis, the final stages of the molting process, which consists of the splitting and shedding of the old cuticle and the stretching and expansion of the new cuticle. This inhibited ecdysis in *B. mori* was duplicated in *P. gossypiella* fed diet containing ponasterone A. The old head capsule(s) remained adhering to the newly synthesized head capsule, thus preventing the stretching of the new cuticle and resulting in impaired mandibular function (Figure 1). In addition, retention of the cuticular skin(s) resulted in a pharate larva unable to excrete or locomote. It is interesting, and unusual, that the pink bollworm larvae could synthesize a third head capsule even though continued feeding became impossible after synthesis of the second head capsule. Apparently, in a result similar to that found with other phytoecdysones (Kubo et al., 1981), ponasterone A either retained activity inside the insect through two molts before death occurred or induced effects were of sufficient stability. Moreover, the activity of ponasterone A was more than 10-fold greater when either orally injected into *B. mori* or fed in artificial diet to *P. gossypiella* than that of other phytoecdysones (Kubo et al., 1983).

The apparent diversity of protective strategies included in the chemical defense of *P. gracilior* is especially essential because of the wide differences in susceptibility of any insect species (or even strain) to any one defense. For instance, *H. zea* has overcome the developmental barrier of ponasterone A, but is still susceptible to the behavioral and metabolic barriers. Any insect species could develop resistance to any one of the barriers, but cross-resistance to the other barriers is unlikely since both the structures of the defensive chemicals and the activities that they induce widely differ.

The integrated chemical defenses of *P. gracilior* may be instructive to pest management programs striving to protect by artificial means economically-important plants from insect attack. For instance, chemical control by behavioral means (i.e., nagilactones) may be ecologically compatible and environmentally sound, integrating well with biological controls, since be-

havioral chemicals are specific for those organisms which can detect them. Chemical control by developmental means (i.e., ponasterone A) may also be specific, as ecdysis inhibition affects only chitin-bearing animals. Finally, chemical control by nontoxic growth inhibitory means (i.e., biflavones) may be useful, not by directly killing attacking insects, but by increasing the insects' susceptibility to adverse natural phenomena.

Since polymorphism of chemical defense in plant populations may result from herbivore pressure (Price, 1975), the multichemical defense mechanisms discussed from the Nairobi *P. gracilior* foliage were also analyzed for in *P. gracilior* foliage grown in Berkeley, California. By HPLC chromatography of the Berkeley foliage, peaks were separated with retention times corresponding to the four diterpene dilactones, two biflavones, and ponasterone A discussed from the Nairobi foliage. These retention times, as indicators of the defensive chemicals, were supported by cochromatography with authentic samples and by bioassay. Thus, the use of more than one chemical in the defensive strategy apparently conferred a potential for resistance in *P. gracilior* foliage, whether grown in Nairobi or in Berkeley.

The multicomponent defensive strategy is also employed by other plant species well-known for resistance to insects. Thus, ecdysis-inhibiting phytoecdysones (Kubo et al., 1981) as well as antifeedant and toxic neoclerodane terpenoids (Kubo et al., 1976; Kubo et al., 1982) have been isolated and identified from foliage of the mint plant, *Ajuga remota* (Labiatae) and other *Ajuga* species.

The evolution of the defensive strategies of plant species, which have contributed to their continued existence, include a novel chemistry which affords a variety of unique effects on phytophagous insects. The elucidation of these strategies is necessary for understanding the ecological, behavioral, metabolic, and developmental aspects of insect–plant relationships. In addition, the elucidation of these natural products and the effects they cause may be economically and ecologically important from a pest management standpoint.

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VOLATILE COMPOUNDS FROM THE PREDATORY INSECT *Podisus maculiventris* (HEMIPTERA: PENTATOMIDAE): Male and Female Metathoracic Scent Gland and Female Dorsal Abdominal Gland Secretions[1]

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Abstract—In the predatory spined soldier bug, *Podisus maculiventris,* the dorsal abdominal glands are much smaller in adult females than males. Females produce a mixture of (*E*)-2-hexenal, (*E*)-2-octenal, (*E*)-2-hexenoic acid, benzaldehyde, and nonanal in these glands. The female dorsal abdominal gland secretion may be a close-range pheromone since the dorsal abdominal gland secretion from males has been shown to be a long-range aggregation pheromone in this species. The metathoracic scent gland secretions of male and female spined soldier bugs are apparently identical, and similar to that of other pentatomids, except for the presence of the monoterpene alcohol, linalool.

Key Words—*Podisus maculiventris,* spined soldier bug, Hemiptera, Pentatomidae, allomone, pheromone, kairomone, linalool.

INTRODUCTION

Flying male and female spined soldier bugs, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), are attracted to a pheromone released from a pair of dorsal abdominal glands by calling males (Aldrich et al., 1984). Once sexually mature adults of this predaceous stink bug are in close proximity, the males readily recognize and court females. In addition, females of the egg

[1]Mention of a commercial product does not constitute an endorsement of this product by the USDA.

parasitoid, *Telenomus* n. sp. (Hymenoptera: Scelionidae), are phoretic on gravid female spined soldier bugs (Buschman and Whitcomb, 1980), but it is unknown precisely how this parasitoid locates mated female bugs.

In order to more fully understand the pheromonal system of *P. maculiventris* and the kairomonal cues of its parasitoids, we have identified the volatile components in the secretions from the small dorsal abdominal glands of adult females and the metathoracic scent gland of males and females.

METHODS AND MATERIALS

Insects and Gland Extracts. A culture of *P. maculiventris* was maintained in the laboratory on *Tenebrio molitor* L. larvae and pupae (Rainbow Mealworms, Compton, California) as previously described (Aldrich et al., 1978a). The culture was started with field-collected bugs from Geneva, New York, and augmented with field-collected bugs from Beltsville, Maryland. The insectary was kept at 28°C and 65% relative humidity, on a 16:8-hr light–dark cycle.

The dorsal abdominal glands opening between the third and fourth segments of mature adult females and the metathoracic scent glands of mature adult males and females were dissected from CO_2-anesthetized spined soldier bugs under tap water, dried with tissue paper, and immersed in 250 μl of triple-distilled CH_2Cl_2. After dissection of about 20 of each type of gland, they were macerated with a melting-point capillary tube and then removed from the solution. For one group of 15 ten-day-old female bugs, the lateral secretory tubules of the metathoracic scent gland (Everton et al., 1979) were removed and extracted separately from the median reservoir of the scent gland. Extracts of the lateral secretory tubules and the dorsal abdominal glands were concentrated under nitrogen to 25–50 μl for chemical analysis. Extracts of whole metathoracic scent glands and the sample of the median scent gland reservoir did not require concentration. Although the secretion from the dorsal abdominal glands of adult male *P. maculiventris* has been analyzed previously (Aldrich et al., 1978a), an extract of these glands was prepared as above and a gas chromatogram is included in Figure 1 for comparison to the dorsal abdominal gland secretion of adult females.

Chemical Analysis. Gas chromatography (GC) of the extracts was performed on two packed columns and one capillary column. A Packard 7400 GC equipped with a flame ionization detector was used with 1.8 m × 4 mm (ID) columns packed with either 3% SE-30 or 3% OV-275 on Chromosorb WAW (100/120). The temperature was held at 60°C for 2 min then programed to 250°C at 10°/min using nitrogen as the carrier gas. A 14-m

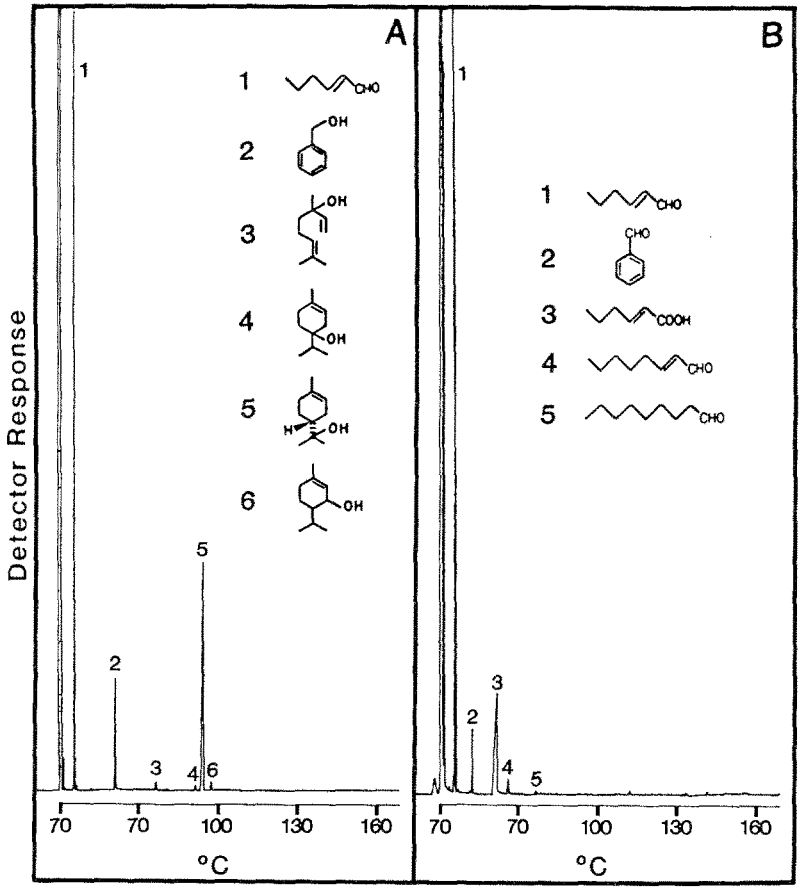

FIG. 1. Gas chromatograms of the dorsal abdominal gland secretions of *Podisus maculiventris* adult males (A) and females (B).

fused silica capillary column coated with a 0.25-μm film of DB-1™ phase (J & W Scientific, Rancho Cordova, California) was used in a Varian 3700 GC equipped with a flame ionization detector. Helium, at a linear flow velocity of 40 cm/sec, was the carrier. The dorsal abdominal gland extracts and the extracts of whole metathoracic glands were run at 70° C for 2 min to 240° C at 15°/min, with a precolumn split ratio of 50:1. The lateral secretory tubule extract was run at 80° C for 2 min to 260° C at 10°/min, with a precolumn split ratio of 2.2:1. The metathoracic gland reservoir sample was run identically to the secretory tubule sample except a precolumn split ratio of 14:1 was used.

Gas chromatographic–mass spectrometric (GC-MS) analyses were conducted using a Finnigan 4500 mass spectrometer. The GC-MS data for the metathoracic scent gland extracts were obtained using a 1.8 m × 0.315 mm

(ID) column packed with 3% SE-30 on Chromosorb WAW (100/120). The packed column temperature was held at 35°C for 1 min, then programed to 60°C at 25°/min, and finally increased at 5°/min to 250°C using helium as the carrier gas. Electron impact (EI) mass spectra were collected at 70 eV with the separator at 240°C and the source at 150°C. The GC-MS data for the dorsal abdominal gland extracts were obtained using a 15-m fused silica DB-1 capillary column held for 1 min at 40°C, then programed to 285°C at 90°/min with helium as the carrier gas. The EI spectra were collected as above and, in addition, methane chemical ionization (CI) spectra were collected for the components of the female dorsal abdominal gland secretion.

Each compound was identified by comparison of its mass spectrum with the published mass spectrum and/or the mass spectrum of the authentic standard (Heller and Milne, 1978; Stenhagen et al., 1969). Subsequently, all of 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 capillary column under isothermal conditions. Standards of (*E*)-2-hexenal, (*E*)-2-hexenoic acid, linalool, *n*-dodecane, *n*-tridecane, *n*-pentadecane, benzaldehyde, and 1-tridecanol were purchased from Aldrich Chemical Company (Milwaukee, Wisconsin). (*E*)-2-Decenal and (*E*)-2-decenyl acetate were purchased from Bedoukian Research Inc., (Danbury, Connecticut). Nonanal and (*E*)-2-octenal were synthesized by oxidation of the corresponding alcohols purchased from Aldrich Chemical Company. (*E*)-4-Keto-2-hexenal was synthesized by oxidation of (*E*)-2-hexenal with SeO_2 in dioxane/water.

RESULTS

Dorsal Abdominal Glands. (*E*)-2-Hexenal (1) is a major component in the secretion of both male and female dorsal abdominal glands (Figure 1). The other components in the male secretion (Figure 1A) are benzyl alcohol (2), linalool (3), terpinen-4-ol (4), (+)-α-terpineol (5), and *trans*-piperitol (6) (Aldrich et al., 1978a; Aldrich et al., 1984). Females (Figure 1B) produce benzaldehyde (2), (*E*)-2-hexenoic acid (3), (*E*)-2-octenal (4), and nonanal (5) in addition to (*E*)-2-hexenal.

The dorsal abdominal glands are much larger in males (>500 μg secretion/individual) than females (<10 μg secretion/individual) (Aldrich et al., 1978a). This sexual dimorphism is substantiated by the GC traces shown in Figure 1 in that the female dorsal abdominal gland extract was first concentrated and then run at 32 × greater detector sensitivity than the unconcentrated male gland extract in order to obtain equivalent peak sizes for the two extracts.

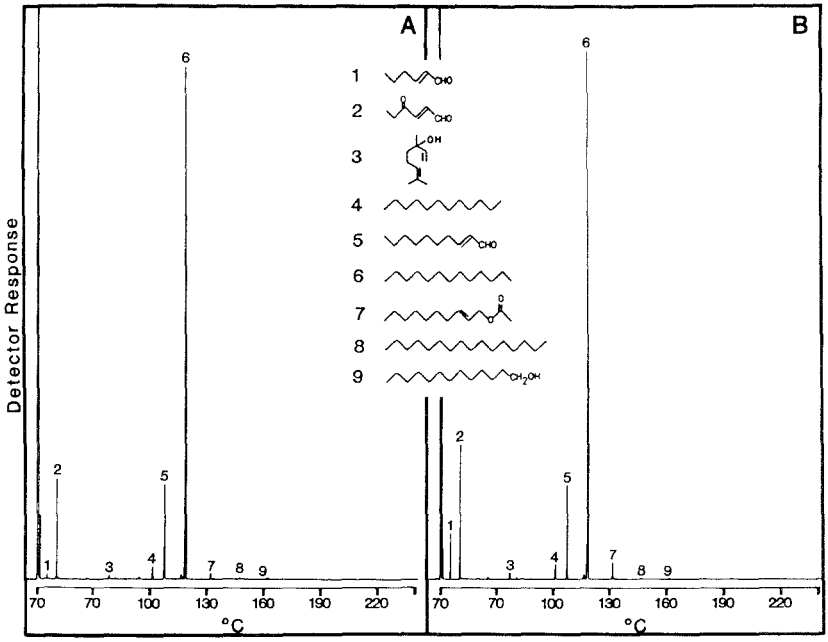

FIG. 2. Gas chromatograms of the metathoracic scent gland secretions of *Podisus maculiventris* males (A) and females (B).

Metathoracic Scent Glands. The metathoracic scent gland secretions of male and female spined soldier bugs are qualitatively and quantitatively alike (Figure 2). (*E*)-2-Hexenal appears to be somewhat more abundant in females' secretion (Figure 2B) than males' (Figure 2A), but this difference may simply be due to individual or age variations. The outstanding feature of this metathoracic scent gland secretion (Figure 2) is the presence of the monoterpene alcohol, linalool (3), in a secretion otherwise dominated by straight-chain alkanes [*n*-dodecane (4), *n*-tridecane (6), and *n*-pentadecane (8)] and α,β-unsaturated carbonyl compounds [(*E*)-2-hexenal (1), (*E*)-4-keto-2-hexenal (2), (*E*)-2-decenal (5), (*E*)-2-decenyl acetate (7)]. 1-Tridecanol (9) is also a minor component in this secretion in males and females.

The lateral secretory tubules were dissected from the median reservoir of the metathoracic scent gland for one group of sexually mature female *P. maculiventris* and the two extracts were analyzed separately by GC (Figure 3). Linalool was undetectable in the lateral secretory tubules of *P.* maculiventris (Figure 3B). On the other hand, (*E*)-2-decenyl acetate was relatively much more concentrated in the lateral secretory tubules (Figure 3B) than in the median reservoir (Figure 3A).

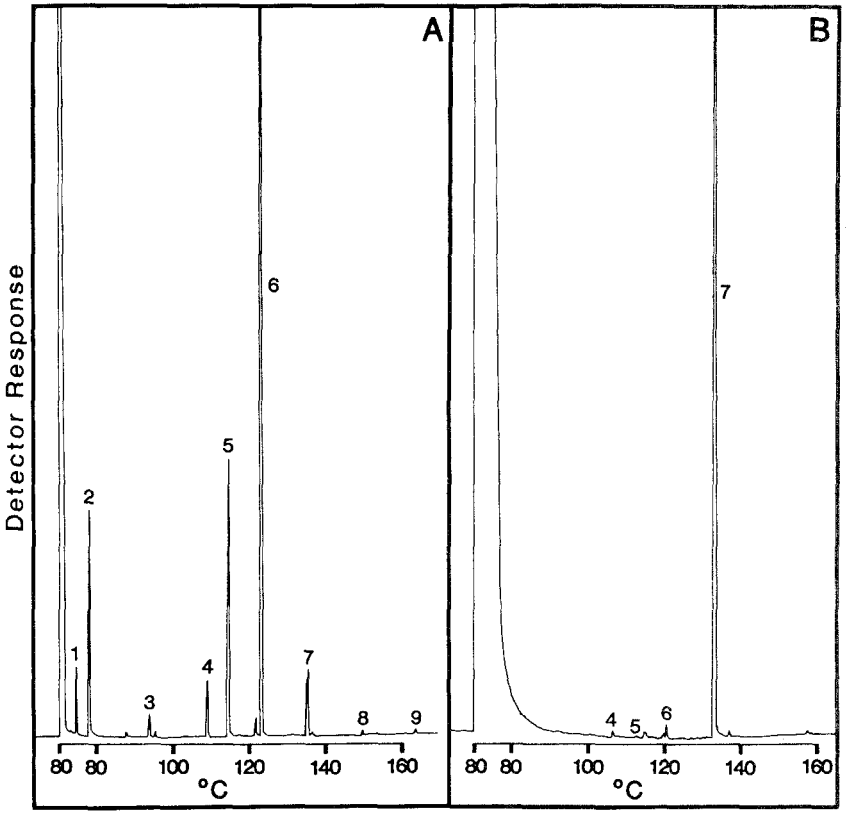

FIG. 3. Gas chromatograms of the secretions in the median reservoir of the metathoracic scent gland from female *Podisus maculiventris* (A) and the secretion in the lateral secretory tubules of these metathoracic scent glands (B). The compound numbers correspond to the structures listed in Figure 2.

DISCUSSION

Adult male and female spined soldier bugs both have synthetically active dorsal abdominal glands whose secretions are sex specific, but in males these glands contain enormously more secretion than they do in females. (*E*)-2-Hexenal occurs in the dorsal abdominal gland secretion of each sex, while all other components are unique to one or the other sex—benzaldehyde, (*E*)-2-hexenoic acid, (*E*)-2-octenal, and nonanal in the female secretion; benzyl alcohol, linalool, terpinen-4-ol, (+)-α-terpineol, and *trans*-piperitol in the male secretion (Aldrich et al., 1978a; Aldrich et al., 1984). Nonanal has not been previously found in true bugs (Staddon, 1979).

In contrast, the metathoracic scent gland secretions of male and female *P.*

maculiventis appear to be identical. The composition of this glandular secretion is similar to that of other pentatomids (e.g., Gilby and Waterhouse, 1965), except for the presence of the monoterpene alcohol, linalool. Cotton stainer bugs in the genus *Dysdercus* produce and store linalool in the lateral secretory tubules of their metathoracic scent glands (Everton et al., 1979; Daroogheh and Olagbemiro, 1982). Apparently linalool enhances the attractiveness of *Dysdercus* females to courting males (Osmani and Naidu, 1966; Hebbalkar and Sharma, 1982). In *P. maculiventris,* (*E*)-2-decenyl acetate is relatively more concentrated in the lateral secretory tubules than in the median reservoir of the metathoracic scent gland, confirming that the tubules are the site of biosynthesis for the esters in the secretion (Aldrich et al., 1978b; Everton and Staddon, 1979), but linalool does not accumulate in the lateral secretory tubules. It seems likely that linalool plays some communicative role in *P. maculiventris.* This compound is known to be a pheromonal component of certain beetles (Young et al., 1973; Hedin et al., 1974), bees (Bergstrom and Tengo, 1978; Hefetz et al., 1979), and butterflies (Hayashi et al., 1978; Honda, 1980).

Spined soldier bug males only sporadically release their aggregation pheromone and, when silent, they are essentially invisible to their parasitoids (Aldrich et al., 1984). The females produces a much fainter odor than male bugs. We suggest that this mating system may have evolved because *P. maculiventris* adults are long-lived and vulnerable to parasitism (Warren and Wallis, 1971). Evolution has favored males that endure the risk of discovery by parasitoids for the potential bonanza of mating with more and healthier females.

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SPECIFICITY OF HERMIT CRAB ATTRACTION TO GASTROPOD PREDATION SITES

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Abstract—Chemical cues are important to hermit crabs in location of shells. The type of gastropod flesh at a predation site (area where a gastropod is being consumed) influences species of crabs attracted to the site. Additionally, amount of flesh at a site is an important factor influencing relative size of the site attendants. Chemical information broadcast from natural predation sites appears highly specific and may serve to separate shell searching of various sizes and species of hermit crabs in time and space.

Key Words—Hermit crabs, predation, gastropods, attraction, discrimination, interspecific attraction, *Pagurus longicarpus*, *P. pollicaris*, *P. maclaughlinae*, *Clibanarius vittatus*.

INTRODUCTION

Gastropod shells are vital for the survival of most hermit crabs. Shells must be replaced periodically owing to crab growth or shell damage (Fotheringham, 1976a; Markham, 1968; Spight, 1977). New shells are obtained by chance encounter of an empty shell, by shell exchange with another individual, and by attending gastropod predation sites. McLean (1974) described the predation site as an area where a predator consumes its gastropod prey, leaving a shell available for hermit crab occupation. McLean suggested that the sites act as a source pool of shells for hermit crabs. Evidence from the northeast Gulf of Mexico suggests that predation sites are a primary source of new shells in soft-bottom communities (Hazlett and Herrnkind, 1980; Wilber and Herrnkind, 1982). The relative importance of predation sites as shell sources in hard-bottom communities appears to vary with wave action. Such variability in predation site importance may constitute a major difference between

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soft-bottom and hard-bottom hermit crab shell acquisition potential (Gilchrist, in review).

McLean (1974, 1975, 1980) recognized the importance of predation sites as a shell source for hermit crabs. Rittschof (1980a,b) presented observations characterizing chemically mediated shell-searching behavior by hermit crabs. While both McLean (1975) and Rittschof (1980b) demonstrated that some hermit crabs attended predation sites in disproportionately high numbers relative to background densities, Rittschof argued that the observations could result from different densities of hermit crabs concentrated in various habitats. However, Rittschof (1980b) and Hazlett (1982) have established that some hermit crab species are attracted to specific prey items. Here additional evidence is provided that attraction to a predation site is specific for some hermit crab species and is dependent, to some extent, upon the genus of gastropod consumed at the predation site. Data to support this inference are taken from natural and simulated predation sites. Further, there is positive correlation between prey size (flesh wet weight) at a simulated predation site and both size and number of hermit crabs attracted to the site.

METHODS AND MATERIALS

Study sites were situated in the Alligator Harbor region of Florida on the northeastern Gulf of Mexico. Sites were randomly selected within sandflat-grassbed areas and each site was at least 10 m from its nearest neighbor. Duplicate samples were taken simultaneously in similar habitats. Water depth ranged from 2 cm to 4 m over the sampling areas, and the water temperature varied by 3° C over the sampling period (average water temperature 22.7° C).

Although 10 species of hermit crabs occur in the study areas, four species account for more than 90% of the individuals (Gilchrist, 1982): *Clibanarius vittatus* (35.2%), *Pagurus longicarpus* (29.5%), *P. pollicaris* (16.7%), and *P. maclaughlinae* (3.6%). The relative abundance of the species and diversity of shells occupied by crabs varies among sites as a function of microhabitat differences (see Hazlett, 1981, for a review of such differences). However, general patterns of crab abundance and shell use are discernible. *Clibanarius vittatus* and *Pagurus pollicaris* are ubiquitous throughout the habitats surveyed. However, frequency of *P. pollicaris* occurrence decreases dramatically toward the shoreline (depths less than 7 cm). *Pagurus maclaughlinae* is found only in areas of dense seagrass cover (pure and mixed stands of *Thalassia testudinum, Syringodium filiforme,* and *Halodoule wrightii*), although specimens can be collected from floating algal clumps (*Gracilaria* and *Sargassum*) tumbling through grassbeds. *Pagurus longicarpus* is predominantly in sandy areas.

Natural Predation Sites. Thirty-seven natural predation sites were

monitored over the sampling period. Upon discovery, each site was marked with a weighted float adjacent to the area. Several characteristics of the sites were taken, including major predator, numbers of hermit crabs attending, types of shells occupied by attending crabs, predators attending which were not involved in the predation event, and the identity of the prey item. The sites were monitored continuously for 20 min and subsequently at 30-min intervals for a period of 200 minutes.

Simulated Predation Sites. Simulated predation sites were established to examine hermit crab responses to various gastropod fleshes. A simulated site consisted of a cage (0.063 m^2 with 100-mm mesh), a perforated clear plastic box beneath each cage to contain the attractant, and the attractant (flesh and shell). Previous experiments indicated that the cage and the plastic box alone were not sufficient to attract a significant number of hermit crabs (Gilchrist, 1982). Series of simulated predation sites were conducted at high, low, and changing tides during May through August of 1980. Duplicate samples were taken simultaneously in similar habitats, at least 3 m apart. Statistical analyses revealed no significant differences in results from duplicates ($P > 0.3$, all χ^2 tests) relative to attendance frequencies, shell-type numbers, number of hermit crabs attending, and size or frequency of damaged shells. Thus, duplicates were combined and treated as one sample in the analyses.

Flesh attractants were frozen 24 hr and thawed 30 min prior to field placement. Rittschof (1980b) and Gilchrist (1982) have shown that the freeze-thaw treatment can approximate the rate of natural attraction in the field. Different quantities (3, 5, 10, 20, and 30 g wet weight) of 12 common gastropods were used along with shell from which flesh was extracted as attractants; all of the gastropod genera used produce shells occupied by at least one of the hermit crab species studied. When the flesh of the largest single gastropod was not sufficient to constitute a sample (i.e., did not weigh 30 g), flesh of conspecific individuals were added until the desired weight was obtained. Gastropods in excess of 30 g were bisected to include foot, gonadal, and digestive tissues. Gastropod fleshes were finely chopped with a razor blade before field placement. Sites were sampled at 30-min intervals, which was found to optimize number of sites and distance between sites while minimizing observer disturbances of the sites (Gilchrist and Abele, in review). Simulated sites were initiated between civil dawn and dusk and were monitored for four hours each.

A respondent was scored as positive if the animal was inside the cage or touching the outside of the cage at the time of collection. Any organism meeting these criteria was considered a respondent, including gastropods, crustaceans, and fishes. This estimate should be conservative in that many animals clustered around the cage, but not touching the cage, were not included in this analysis. All crustacean and gastropod respondents were removed at the time of the census and were bagged for later identification and

morphometric analyses. Fish predators were not taken from the area because disturbance to the sites was considerable when early attempts were made to remove fishes. However, as noted previously by Rittschof (1980a), fish remain in the vicinity of predation sites only for a short period of time. The total number of samples collected for this portion of the study was 240 (12 fleshes × 5 weights × 2 duplicates × 2 replicates). Replicates were performed within 20 days of each duplicate.

Randomly directed transects (64) were taken in the sampling areas to determine presence of hermit crab species not collected at predation sites. Full descriptions of sampling techniques are presented elsewhere (Gilchrist, 1982).

Shell Switching. During each month, 20 simulated predation sites were conducted using flesh of one gastropod genus and the shell of another gastropod genus. This experiment was designed to examine effects of the visual stimulus of shell on predation site attendance by hermit crabs. The size of the shell used in the experiment was equivalent to the size of the shell from which the bait was extracted (shell length and shell width equivalent). Five of the shell genera more commonly worn by hermit crabs and flesh of five common gastropods found in the area were used in the experiments. Four duplicates were made during each of the four sampling months. Pairings for the flesh–shell groups were as follows: *Melongena* (flesh)/ *Littorina* (shell), *Fasciolaria* (f)/ *Cantharus* (s), *Littorina* (f)/ *Nassarius* (s), *Thais* (f)/ *Busycon* (s), and *Nassarius* (f)/ *Terebra* (s).

RESULTS

Natural Predation Sites. Table 1 summarizes the observations from 37 natural predation sites. Three primary predators accounted for more than 90% of the predation events: *Callinectes sapidus* (37.2%), *Melongena corona* (24.3%), and *Thais haemostoma* (16%). *Callinectes sapidus* crushes or peels the shells of prey gastropods, *M. corona* engulfs the prey and bypasses the operculum with its long radula, and *T. haemostoma* engulfs the prey and bores past the operculum and into the flesh. Completion of the predation event occurred after an average of 30 min for the gastropod predators, while consumption of prey by the crustacean predator ended after an average of 8 min. Up to 30 shell exchanges occurred per minute after the shells were liberated by the predators. Subsequent to a 4- to 10-min exchange period, the crabs dispersed from the area. When the shell had been removed from the area, hermit crabs continued to come to the site of the predation event for approximately 20 min.

In the study, blue crabs (*C. sapidus*), xanthid crabs (*Panopeus* spp.), box crabs (*Calappa flamea*), killifish (*Fundulus* spp.), pufferfish (*Spherodon* sp.), and the spiny boxfish (*Diodon sp.*) were all observed attending predation

TABLE 1. PREDATORS, NUMBER OF HERMIT CRABS ATTENDING, TYPES OF PREY, AND SHELL OCCUPIED BY HERMIT CRABS FROM 37 NATURAL PREDATION SITES

Predators	*N*	*N* of hermit crabs (total)	Prey	Shells used
Blue crab[a]	15	538	*Littorina*	*Littorina*
Blue crab			*Polinices*	*Nassarius*
Boxfish				*Cantharus*
Killifish				*Terebra*
xanthids				*Polinices*
				Melongena
Crown concl[a]	10	291	*Littorina*	*Littorina*
Killifish			*Fasciolaria*	*Nassarius*
Pufferfish			*Melongena*	*Cantharus*
Blue crab				*Terebra*
Crown conch				*Melongena*
				Fasciolaria
				Busycon
Dog whelk[a]	8	240	*Littorina*	*Littorina*
Blue crab				*Polinices*
xanthids				*Melongena*
Killifish				*Cantharus*
				Busycon
				Fasciolaria

[a] Predator consuming gastropod at predation site.

sites where hermit crabs were congregated. Blue crabs and boxfishes were observed feeding on hermit crabs gathered at the predation sites.

Hermit crabs attending the natural predation sites inhabited primarily *Littorina irrorata* shells (>75%). The next most common shell inhabited by the hermit crabs at these sites was *Nassarius vibex* (12%). Eight other shell genera were represented by less than 2% of the population at the natural sites. The most common hermit crab at the natural sites was *Pagurus longicarpus* (67%), while the least common of the four hermit crab species observed in this study was *P. maclaughlinae* (<2%).

No hermit crabs were observed attempting to feed on the prey snail while the predator was present. When the predator was another gastropod, hermit crabs would climb onto the shell of the predator. If remnants of flesh remained in the shell, hermit crabs seizing the shell would attempt to either take the shell and move out of the area or would pull the flesh from the shell without feeding and enter the newly liberated shell.

Discrimination of Gastropod Genera. Table 2 summarizes responses of the four major hermit crab species to shells and fleshes of 12 common

TABLE 2. NUMBER OF CRABS ATTRACTED TO VARIOUS WEIGHTS OF FLESH FROM 12 GASTROPOD GENERA[a]

Bait	Wet weight (g)	*C. vittatus*	*P. pollicaris*	*P. longicarpus*	*P. maclaughlinae*
Melongena	3	11 (8.5)	0 (1.7)	3 (3.8)	0 (0)
	5	13 (13.9)	1 (2.8)	9 (6.3)	0 (0)
	10	7 (16.4)**	5 (3.3)	16 (7.4)**	0 (0)
	20	22 (20.0)	4 (4.0)	7 (9.0)	0 (0)
	30	28 (21.2)*	6 (4.2)	1 (9.5)**	0 (0)
Fasciolaria	3	3 (10.5)	1 (2.8)	13 (6.8)	0 (0.6)
	5	1 (4.1)*	4 (1.1)*	8 (2.7)**	2 (0.3)
	10	10 (13.3)*	6 (3.7)	8 (8.8)	2 (0.8)
	20	22 (21.5)	8 (5.9)	11 (14.3)*	1 (1.3)
	30	44 (30.8)**	3 (8.5)**	13 (20.4)**	0 (1.9)
Littorina	3	11 (8.1)*	0 (0.4)	13 (23.0)**	11 (5.9)**
	5	5 (18.8)**	3 (0.8)	55 (52.4)	16 (13.4)*
	10	15 (20.9)**	1 (1.0)	61 (60.0)	21 (15.4)**
	20	19 (17.8)	0 (0.8)	54 (51.2)	13 (13.1)
	30	37 (22.0)**	0 (1.0)	67 (63.1)*	2 (16.1)**
Nassarius	3	0 (0.0)	0 (0.0)	15 (13.2)	8 (12.6)*
	5	0 (0.0)	0 (0.0)	10 (17.1)**	23 (16.4)**
	10	0 (0.0)	0 (0.0)	14 (14.2)	15 (13.6)
	20	0 (0.0)	0 (0.0)	18 (16.1)	15 (15.4)
	30	0 (0.0)	0 (0.0)	12 (8.3)*	5 (8.0)*
Thais	3	0 (3.3)*	0 (0.8)	6 (1.8)**	0 (0)
	5	1 (6.7)**	7 (1.6)**	4 (3.7)	0 (0)
	10	10 (10.6)	4 (2.6)	5 (5.9)	0 (0)
	20	28 (24.5)	4 (6.0)	12 (13.6)	0 (0)
	30	35 (28.9)**	3 (7.0)*	14 (16.0)	0 (0)
Busycon	3	3 (11.9)**	10 (4.2)**	4 (0.9)*	0 (0)
	5	21 (16.8)	3 (5.9)8	0 (1.3)	0 (0)
	10	8 (8.4)	4 (3.0)	0 (0.7)	0 (0)
	20	17 (12.6)*	1 (4.4)*	0 (1.0)	0 (0)
	30	2 (1.3)	0 (0.5)	0 (0.1)	0 (0)
Collumbella	3	0 (0.0)	0 (0.2)	0 (2.0)	3 (.9)
	5	0 (0.0)	0 (0.4)	5 (4.5)	2 (2.0)
	10	0 (0.0)	0 (0.2)	4 (2.6)	0 (1.2)
	20	0 (0.0)	0 (0.1)	2 (1.3)	0 (0.6)
	30	0 (0.0)	0 (0.1)	0 (0.6)	0 (0.3)
Cantharus	3	7 (12.5)**	0 (0.0)	19 (13.5)**	0 (0)
	5	4 (10.6)**	0 (0.0)	18 (11.4)**	0 (0)
	10	24 (16.4)**	0 (0.0)	10 (17.6)**	0 (0)
	20	11 (10.1)	0 (0.0)	10 (10.9)	0 (0)
	30	9 (5.3)*	0 (0.0)	2 (5.7)*	0 (0)
Turbo	3	0 (0.2)	11 (7.4)	0 (2.4)	0 (0)
	5	2 (0.6)	26 (23.6)	7 (7.6)	0 (0)
	10	0 (0.5)	15 (17.6)	11 (5.6)**	0 (0)
	20	0 (0.5)	10 (16.9)**	5 (5.4)	0 (0)
	30	0 (0.3)	13 (9.5)	1 (3.0)	0 (0)

TABLE 2. Continued

Bait	Wet weight (g)	*C. vittatus*	*P. pollicaris*	*P. longicarpus*	*P. maclaughlinae*
Polinices	3	3 (1.6)	5 (5.7)	2 (2.6)	0 (0)
	5	3 (4.2)	9 (14.9)**	14 (6.9)**	0 (0)
	10	0 (5.1)**	23 (18.4)*	9 (8.5)	0 (0)
	20	3 (6.3)*	25 (22.4)	11 (10.3)	0 (0)
	30	13 (4.7)**	16 (16.6)	0 (7.7)**	0 (0)
Prunum	3	0 (0.0)	0 (0.0)	5 (0.4)*	3 (4.1)
	5	0 (0.0)	0 (0.0)	18 (16.6)	8 (9.2)
	10	0 (0.0)	0 (0.0)	8 (12.3)	12 (6.8)**
	20	0 (0.0)	0 (0.0)	12 (14.7)	12 (8.2)**
	30	0 (0.0)	0 (0.0)	22 (16.1)**	1 (7.8)**
Terebra	3	0 (0.0)	0 (0.0)	4 (3.9)	0 (0.2)
	5	0 (0.0)	0 (0.0)	14 (13.6)	0 (0.6)
	10	0 (0.0)	0 (0.0)	4 (4.8)	4 (0.2)
	20	0 (0.0)	0 (0.0)	1 (1.0)	0 (0)
	30	0 (0.0)	0 (0.0)	1 (1.0)	0 (0)

[a]Numbers in parentheses are individuals expected at the sites by chance alone, using contingency analyses. * and ** are used to indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

gastropod genera. If there is no discrimination between the various gastropod genera, relative abundances of the hermit crab species at the simulated predation sites should be proportional to abundances found in the surrounding areas. *Clibanarius vittatus* was found in proportions greater than expected by chance alone at sites containing *Melogena, Fasciolaria, Thais, Busycon,* and *Cantharus. Pagurus pollicaris* appeared in greater than expected numbers at sites containing *Turbo* and *Polinices* attractants. *Pagurus longicarpus* was collected in numbers greater than expected by chance alone at sites with *Littorina, Nassarius, Cantharus, Collumbella, Prunum,* and *Terebra* simulated predation sites. *Pagurus maclaughlinae* was found at sites containing *Nassarius, Collumbella, Prunum,* and *Terebra* in numbers greater than expected by chance alone.

Chemical discrimination of the gastropod genera by the hermit crabs was also examined using the shell switching experiments. If discrimination of the gastropod genera is visual (from the presence of the shell) alone, one would expect proportions of hermit crabs attracted to simulated predation sites containing both the flesh and shell of one species to be similar to simulated predation sites with the shell of that species, regardless of the flesh present. This does not occur in most cases (Table 3). Proportions of hermit crabs at the shell-switch sites were not significantly different from simulated predation sites where fleshes, but not shells, were the same. This does not imply that

TABLE 3. EFFECTS OF VISUAL VS. CHEMICAL CUES ON SHELL SELECTION BY FOUR HERMIT CRAB SPECIES.

Flesh/Shell[a]	*C. vittatus*	*P. pollicaris*	*P. longicarpus*	*P. maclaughlinae*
Melongena/Littorina	165	58	17	0
Fasciolaria/Cantharus	88	39	68	16
Littorina/Nassarius	67	30	137	71
Nassarius/Terebra	0	0	54	83
Thais/Busycon	44	19	29	1

[a]All values for each crab species not significantly different from numbers of crabs expected at fleshes presented. As long as a shell stimulus was present, hermit crabs remained at the flesh site.

visual cues are not important in shell selection, but rather it indicates that chemical cues are more important in location of newly liberated shells.

Table 4 summarizes R × C contingency analyses (Haberman, 1973; Everitt, 1977) of hermit crab responses to five flesh weight-shell combinations of 12 gastropod genera. Such analyses allow for testing of independence between row (flesh weight–shell combinations) and column (crab species) effects using standardized residuals (d_{ij}). The null hypothesis in each case was that no discrimination between flesh weight–shell combinations occurs for the crab species.

Clibanarius vittatus was collected from sites containing *Melongena* (30 g), *Littorina* (3 g, 30 g), *Thais* (30 g), *Fasciolaria* (30 g), *Busycon* (20 g), *Cantharus* (10 g, 30 g), and *Polinices* attractants in numbers greater than expected by chance alone.

Pagurus pollicaris was found at sites containing *Thais* (5 g), *Fasciolaria* (5 g), *Busycon* (3 g), and *Polinices* (10 g). Other amounts of these fleshes did not elicit a significant positive response.

Pagurus longicarpus attended sites containing *Melongena* (5 g, 10 g), *Littorina* (3 g, 5 g, 10 g), *Turbo* (10 g), *Prunum* (30 g), *Fasciolaria* (3 g, 5 g), *Nassarius* (30 g), *Busycon* (3 g), *Cantharus* (3 g, 5 g), and *Polinices* (5 g).

Pagurus maclaughlinae was found at simulated predation sites containing *Littorina* (3 g, 5 g, 10 g), *Prunum* (10 g, 20 g), and *Nassarius* (5 g).

In Figure 1, size frequencies of *C. vittatus* (hard carapace length) collected at simulated sites using fleshes of four gastropod genera are plotted against flesh wet weight. When all data for each hermit crab species responding to a bait were combined, there was a significant positive correlation between crab size and the amount of bait at the simulated predation site ($r = 0.82$ for *P. maclaughlinae*, $r = 0.71$ for *P. longicarpus*, $r = 0.80$ for *P. pollicaris*, and $r = 0.63$ for *C. vittatus*; overall r for all hermit crab species combined was 0.69). Thus, mean carapace length for each hermit crab species increases with increasing amounts of flesh at the simulated

TABLE 4. FLESH-WEIGHT PREFERENCES OF FOUR COMMON HERMIT CRAB SPECIES FROM 12 GASTROPOD GENERA AS A FUNCTION OF STANDARD d_{ij} AND FLESH WEIGHT CLASS[a]

Flesh	Wt	C.v.	P.p.	P.l.	P.m.	Flesh	Wt	C.v.	P.p.	P.l.	P.m.
Melongena	3	0	0	0	0	*Fasciolaria*	3	−	0	++	0
	5	0	0	+	0		5	−	+	++	0
	10	−−	0	++	0		10	−	0	0	0
	20	0	0	0	0		20	0	0	−	0
	30	+	0	−−	0		30	++	−−	−−	0
Littorina	3	−	0	−−	++	*Nassarius*	3	0	0	0	−
	5	−−	0	0	+		5	0	0	−−	++
	10	−−	0	0	++		10	0	0	0	0
	20	0	0	0	0		20	0	0	0	0
	30	++	0	+	−−		30	0	0	+	−
Thais	3	−	0	0	0	*Busycon*	3	−−	++	−	0
	5	−−	++	0	0		5	0	−	0	0
	10	0	0	0	0		10	0	0	0	0
	20	0	0	0	0		20	+	−	0	0
	30	++	−	0	0		30	0	0	0	0
Columbella	3	0	0	0	0	*Cantharus*	3	−−	0	++	0
	5	0	0	0	0		5	−−	0	++	0
	10	0	0	0	0		10	++	0	−−	0
	20	0	0	0	0		20	0	0	0	0
	30	0	0	0	0		30	+	0	−	0
Turbo	3	0	0	0	0	*Polinices*	3	0	0	0	0
	5	0	0	0	0		5	0	−−	++	0
	10	0	0	++	0		10	−−	+	0	0
	20	0	−−	0	0		20	−	0	0	0
	30	0	0	0	0		30	++	0	−−	0
Prunum	3	0	0	0	0	*Terebra*	3	0	0	0	0
	5	0	0	0	0		5	0	0	0	0
	10	0	0	−	++		10	0	0	0	0
	20	0	0	0	+		20	0	0	0	0
	30	0	0	++	−−		30	0	0	0	0

[a] An 0 indicates no preference for the flesh-weight combination, a + (or + +) indicates a significant preference for the combination, and a − (or − −) indicates that significantly fewer crabs attended the sites than would be expected by chance alone. *C.v. is *Clibanarius vittatus*, P.p. is *Pagurus pollicaris*, P.l. is *Pagurus longicarpus*, and P.m. is *Pagarus maclaughlinae*.

predation sites. Additionally, the size range of the hermit crabs attending the simulated predation sites with larger amounts of flesh is greater than at simulated sites containing smaller amounts of attractant.

Sizes of Attracted Hermit Crabs. Small, immature *C. vittatus* were commonly collected at simulated predation sites containing 3 g and 5 g of gastropod flesh, while mature females and males were collected frequently at sites containing 30 g of flesh (Table 5). Overall, the smallest crabs (immature

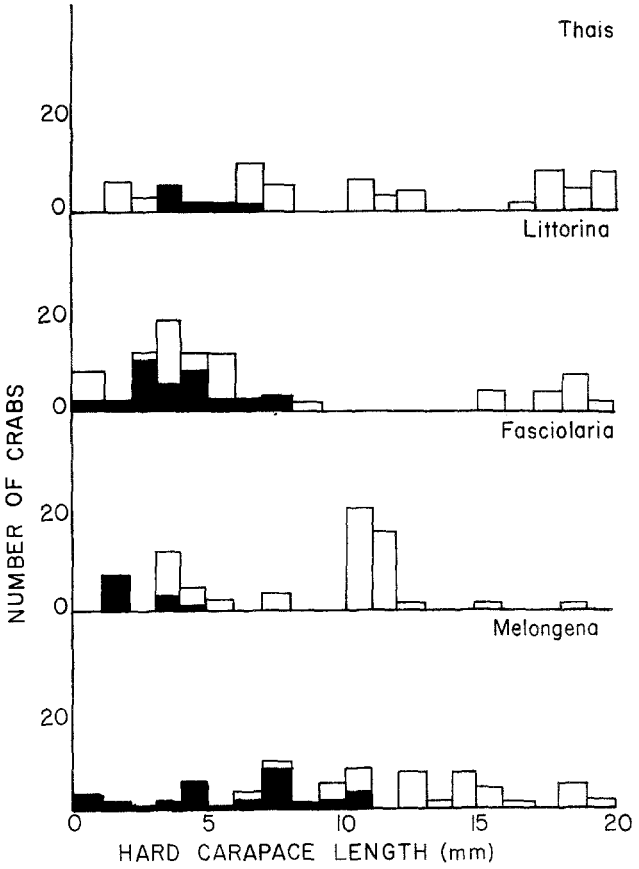

FIG. 1. Numbers of crabs attracted to various types of gastropod flesh as a function of crab size. Solid blocks represent crabs attracted to 3, 5, and 10 g of flesh while the open blocks represent crabs attracted to 20 and 30 g of flesh.

large-bodied crabs and all size ranges of small-bodied crabs) attended sites with smaller amounts of bait more frequently than sites with larger amounts of bait, while large-bodied crabs had a tendency to frequent sites containing only relatively large amounts of baits. When large crabs were collected from sites containing 3 g or 5 g of flesh, the numbers of individuals at the sites were not greater than expected by chance alone.

DISCUSSION

Data presented herein strongly support the idea that hermit crabs are acutely responsive to chemical signals which can lead to location of new shells. Rittschof (1980a, b) suggested that such shell location behaviors evolved from

TABLE 5. FEMALE HERMIT CRAB SIZE DISTRIBUTION IN RELATION TO FLESH WEIGHT AT TWO SIMULATED GASTROPOD PREDATION SITES

Hermit crab	*Melongena* (wt in g)	$\bar{X}$ size (mm)	(HCL)[e] ± SD	*Littorina* (wt in g)	$\bar{X}$ size (mm)	(HCL) ± SD
Clibanarius vittatus[a]	3	3.2	0.7	3	2.4	0.4
	5	6.5	1.1	5	4.7	1.5
	10	6.3	1.3	10	4.5	1.8
	20	7.7	1.3	20	5.6	1.7
	30	8.1	2.1	30	6.4	2.3
N Total	51			32		
Pagurus pollicaris[b]	3			3		
	5	2.9	0.0	5	2.4	0.6
	10	5.7	0.9	10	3.9	0.0
	20	4.3	1.1	20		
	30	6.1	2.4	30		
N total	9			3		
P. longicarpus[c]	3	2.7	0.5	3	3.1	1.5
	5	3.3	1.4	5	3.7	0.9
	10	5.3	1.8	10	5.0	1.4
	20	5.1	0.2	20	6.1	2.3
	30	7.3	0.0	30	6.8	2.0
N total	20			161		
P. maclaughlinae[d]	3			3	1.3	0.6
	5			5	1.5	1.0
	10			10	2.7	1.1
	20			20	3.4	0.7
	30			30	3.1	0.2
N total	0			29		

[a] Female with eggs 5.2 mm.
[b] Female with eggs 4.7 mm.
[c] Female with eggs 2.6 mm.
[d] Female with eggs 1.2 mm (minimum values).
[e] Hard carapace length.

specific chemoreceptive capacities of the crabs for distinctive small peptides. While responses to purified chemicals were not examined, varying amounts of flesh from 12 gastropods genera were used to evaluate information potentially conveyed in chemical signals broadcast from simulated predation sites. The data suggest that the chemical signal not only provides information about the identity of the shell, supplementing observations made by McLean (1974, 1975), Rittschof (1980a,b), and Hazlett (1982), but that the signal also may relay some information about the relative size of the available resource.

The general question addressed by the data was whether different gastropod fleshes attract hermit crabs in proportions significantly different from those found in nature. Overall, the relative proportions of all four hermit

crab species observed in this study at the natural predation sites were significantly greater than background levels (χ^2; $P < 0.05$). Results from the simulated predation sites were analogous, supporting the hypothesis that the freeze–thaw method of bait preparation elicits results comparable to natural predation events (Rittschof, 1980a). The relative proportions of the four hermit crabs at the natural sites were not significantly different from proportions obtained at comparable (i.e., same gastropod species) simulated predation sites (all χ^2 P values > 0.5). However, the relative numbers of hermit crabs at the natural sites were variable in terms of predator (Table 1). Fewer hermit crabs attended natural sites where blue crabs were the predators than where gastropods were the predators (Mann-Whitney U; $P < 0.05$). This difference may be related to the variable duration of the predation event relative to the type of predator and/or to the fact that blue crabs are hermit crab predators. Rittschof (1980a) suggested that loss of visual, chemical, and tactile stimuli at predation sites would cause dispersal of the aggregation.

Rittschof (1980a) raised another line of inquiry as to why hermit crabs should show species-specific responses to certain gastropod fleshes when almost any site would supply a selection of potentially occupiable resources. Analyses of results shown in Table 2 and Figure 1 confirmed the observation that certain hermit crabs are attracted to particular gastropod fleshes. Rittschof suggested that a possible answer to this specificity was the reduction of interspecific competition. However, as pointed out by many hermit crab workers (see Abrams, 1981), intraspecific competition for shells may be more intense than interspecific competition. I suggest that information provided to the hermit crab from the chemical signal may indicate the relative size of the available resource, thus allowing some intraspecific spatial separation.

The rationale for inferring spatial separation is based upon the observation that the mean size of hermit crabs attending simulated predation sites and the size range of individuals at the sites generally increase as a function of the attractant weight. While it is not critical to the final interpretation of the patterns observed, I suggest two alternative (not necessarily exclusive) scenarios for the development of the patterns. First, hermit crabs rarely trade down in shell size (Bertness, 1981; Hazlett, 1981). Therefore, smaller hermit crabs attending predation sites with large amounts of flesh should be released from attempts of larger hermit crabs to confiscate their shells. Second, larger hermit crabs, in acquiring a new shell, liberate a shell potentially usable by a smaller hermit crab.

The overall increase in hermit crab size with increasing amounts of bait could be attributable only to abundance and movement patterns of the different sizes of hermit crabs. Hazlett (1981) and others have suggested that smaller crabs are less mobile than larger crabs. An earlier study in Florida (Gilchrist, 1982) confirmed this observation for large-scale movements. However, the study also suggested that smaller hermit crabs were highly active

and mobile in intertidal areas. The x- and y-axis movement patterns on a daily basis for smaller crabs were well within the predicted active envelope of the attractants (Gilchrist, unpublished data). Thus, although smaller crabs may be less mobile than larger crabs over longer distances, the predicted sphere of influence for the chemical attractant may decrease the advantage of such differences. Differential patterns should lead to unequal probabilities of contacting a chemical signal. For example, at a site containing 3 g of flesh, there may be a relatively small active envelope (space within which concentration of behaviorally active chemicals are at or above a threshold level). If there are more small than large hermit crabs within an area, and the active envelope of a site with 3 g of flesh is small, one would expect to find large numbers of smaller hermit crabs attending these sites.

Similarly, large numbers of small crabs would be expected to attend sites containing 30 g of flesh since the active envelope of the greater amount of flesh should theoretically be larger than for the small amount of flesh. Since the active envelope should be relatively larger, larger hermit crabs, which are less abundant over time (Gilchrist, 1982) would have a higher probability of encountering the chemical signal. If movement and abundance patterns alone explained size differences attracted to the various weights of flesh, one would expect to find at least as many smaller crabs at 30-g sites as found at 3-g sites. Overall, more crabs attended 30-g sites (356) than 3-g sites (175). For each crab species separately summed over all baits to which they were responsive, there were fewer small crabs at the 30-g sites than expected by chance alone (all χ^2 values, $P < 0.10$).

Alternatively, the increase in both size range and the mean size of the crabs at the sites with increasing bait weight may be an ontogenetic response. The evidence presented here indirectly suggests a size- or age-related shift in response to various gastropod fleshes, or more accurately, the chemicals released from the various gastropod fleshes. Such a change, in combination with acute bait discrimination, could serve to separate adult crabs spatially at predation sites, while smaller, more immature crabs may have less discriminatory capabilities, but require smaller amounts of chemical stimulus to elicit a response. Thus, juvenile crabs may be attracted to a wider range of genera than would adults of the same species.

As suggested by Rittschof (1980a), questions yet to be asked of this system relate to the roles of chemical signals in the ecology of hermit crabs in different environments. One such role proposed in this study may be the reduction of both inter- and intraspecific interactions of the hermit crabs relative to an overlapping resource, the gastropod shell.

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SINGLE CELL RESPONSES OF THE DOUGLAS-FIR BEETLE, *Dendroctonus pseudotsugae* HOPKINS (COLEOPTERA: SCOLYTIDAE), TO PHEROMONES AND HOST ODORS[1]

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Abstract—Olfactory perception of pheromones and host odors by *Dendroctonus pseudotsugae* males and females was investigated through single cell recordings. Responses of 71 cells (35 cells in males; 36 cells in females) were recorded to 1 μg of 10 pheromones and three host terpenes. The olfactory cells were classified into four types based on sensitivity and specificity for the various odors: (1) 3,2-MCHone (3-methyl-2-cyclohexenone) cells; (2) 3,2-MCHol (3-methyl-2-cyclohexenol) cells; (3) frontalin cells; and (4) synergist cells. 3,2-MCHone and 3,2-MCHol cells in both males and females were the most sensitive and specific of all cells recorded with a threshold ca. 0.001–0.01 μg on filter paper. Frontalin cells were less specialized than the two aforementioned cell types and had a somewhat higher threshold of ca. 0.01 μg. A larger percentage of these three cell types were recorded in males than in females. The synergist cells had the highest threshold of all recorded cell types and were the least specific. Each of these cells was most responsive to either *cis*-verbenol, *trans*-verbenol, 1,2-

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MCHol, verbenone, α-pinene, or limonene; however, responses could be elicited by stimulation with other pheromones or host terpenes at higher dosages. Instances of inhibition of nerve impulse activity from spontaneously active cells were observed during 3,2-MCHol and 3,2-MCHone stimulation, thus suggesting multifunctional effects for these compounds. The results help explain the role of chemicals in host selection, aggregation, and colonization behavior of the beetle and further elucidate hypotheses put forth in an earlier electroantennogram study.

Key Words—Olfaction, nerve cells, bark beetle, *Dendroctonus pseudotsugae*, behavioral physiology, Coleoptera, Scolytidae, neurobiology.

INTRODUCTION

The behavior of bark beetles (Coleoptera: Scolytidae) in host selection and species propagation is complex and involves a series of visual, olfactory, and sonic stimuli (for a review see Borden, 1982). This behavior is especially complex and highly orchestrated for aggressive *Dendroctonus* beetles which must overcome the copious resin flow of a prospective host. Many *Dendroctonus* species utilize both insect-produced compounds (i.e., pheromones) and resin odors (e.g., terpenes) as olfactory cues to facilitate mass attack. The Douglas-fir beetle, *Dendroctonus pseudotsugae*, offers a good example for the study of the olfactory mechanisms involved in this genus, since much is known about the specific compounds involved in these behavioral sequences (Rudinsky and Ryker, 1977; Ryker et al., 1979).

D. pseudotsugae females attracted to potential host trees by certain terpenes, e.g., α-pinene, limonene, and camphene (Rudinsky, 1966a), initiate host attack and release at least seven pheromones which, along with host resin odors, attract additional males and females for mass attack (Ryker et al., 1979). Predominantly males respond to the odors released by pioneering females (Rudinsky, 1969) which include: 3,2-MCHone (3-methyl-2-cyclohexenone) (Kinzer et al., 1971), 3,2-MCHol (3-methyl-2-cyclohexenol) (Vité et al., 1972, Rudinsky et al., 1974), 1-methyl-2-cyclohexenol (Libbey et al., 1983), frontalin (1,5-dimethyl-6,8-dioxabicyclo[3.2.1]octane) (Kinzer et al., 1969; Pitman and Vité, 1970; Rudinsky et al., 1974), verbenone (Rudinsky et al., 1974) and *trans*-verbenol (Rudinsky et al., 1972). Of these odors, the tripartite mixture of 3,2-MCHone in low concentrations, 3,2-MCHol, and frontalin was found to be most attractive in field tests (Rudinsky et al., 1974). The other pheromones and host terpenes function in various combinations to synergize response to this three-component mixture.

Upon orientation to a potential host tree and location of the female gallery by the male, sonic interactions between males and females stimulate changes in both the quality and quantity of pheromone release (Rudinsky, 1969; Rudinsky and Michael, 1972; Rudinsky and Ryker, 1977). Intersexual

sonic and chemical communication leads to increased pheromone release by the female and release of various pheromones by the male (Rudinsky et al., 1976a,b; Libbey et al., 1976). Increased release of the multifunctional pheromone, 3,2-MCHone (an attractant at low concentrations, but inhibitory at high concentrations) (Rudinsky, 1973) by females, along with its release by males, leads to inhibition of further aggregation by both sexes (Rudinsky, 1969; Rudinsky and Michael, 1972; Rudinsky et al., 1976a).

The purpose of this study was to investigate the olfactory receptor system in *D. pseudotsugae* through single-cell recordings and to further elucidate receptor mechanisms suggested in an earlier electroantennogram (EAG) study (Dickens et al., 1983).

METHODS AND MATERIALS

Insects. Adult *D. pseudotsugae* used in this study emerged from naturally infested bolts of Douglas-fir, *Pseudotsuga menziesii* (Mirb.) Franco, from the McDonald Forest of Oregon State University, Corvallis, Oregon. Following emergence, beetles were sexed (Jantz and Johnsey, 1964) and maintained on moist filter paper in Petri dishes at 6°C until use within 1–3 weeks.

Electrophysiology. Single-cell recording techniques used are described in detail elsewhere (Boeckh, 1962; Dickens, 1979). In general, recordings were made with 50.8-μm-diameter tungsten wire electrolytically sharpened to a tip diameter of ca. 1–2 μm. The recording electrode was positioned under optical control (320×) with a Leitz high-power micromanipulator near the base of one of the three sensory bands encircling the antennal club. The indifferent electrode was implanted in the body of the beetle through the oral cavity.

The signal passed through a Bioelectric NF1 preamplifier to a Tektronix 122 low-level preamplifier capacitance coupled to a Tektronix 3A74 amplifier and displayed on a Tektronix 561B oscilloscope. In some instances, electrical activity passed through an active 60-Hz notch filter. Records of electrical activity were made on Polaroid film with a Tektronix 105A oscilloscope camera.

Chemical Stimuli and Experimental Protocol. Odorous stimuli used included eight compounds isolated from *Dendroctonus* species, two compounds specific for the genus *Ips*, and three terpenes identified from host Douglas-fir, *P. menziesii* (von Rudloff, 1972) and purported to be attractants of adult *D. pseudotsugae* (Rudinsky, 1966; Rudinsky et al., 1977). The source and purity of the various compounds tested are summarized in Table 1.

Pheromone and host odors used as test stimuli were prepared as serial dilutions in nanograde pentane. Odorous stimuli were presented as 10-μl

TABLE 1. SOURCE AND PURITY OF STIMULUS COMPOUNDS USED IN EXPERIMENTS

Compound	Compound purity (%)	Source
3-Methyl-2-cyclohexenone	98	A[a]
3-Methyl-2-cyclohexenol	97	B
1-Methyl-2-cyclohexenol	98.6	C
Frontalin	99	B
endo-Brevicomin	99	B
trans-Verbenol	99	B
cis-Verbenol	99	B
Verbenone	99	B
Ipsenol	99	D
Ipsdienol	99	D
α-Pinene	98	B
Limonene	98	E
Camphene	98	E

[a](A) Aldrich Chemical Co., Milwaukee, Wisconsin; (B) Chem Samp Co., Columbus, Ohio; (C) A.C. Oehlschlager, Department of Chemistry, Simon Fraser University; (D) Borregaard; and (E) K&K Laboratories.

samples placed on filter paper (20 mm × 7 mm) inserted into glass cartridges (75 mm; 5 mm ID) oriented toward the preparation from ca. 1 cm. Pentane served as the control and elicited no change in impulse frequency from the spontaneous firing rate. Stimulus duration was 1–2 sec. Air flow was ca. 2 liters/min.

In order to examine qualitatively the relative specificity of the cells recorded, each preparation was initially exposed to the volatiles of 1 μg of each of the stimulus compounds on filter paper listed in Table 1. At this dosage only one or a few compounds elicited activity above the spontaneous firing rate (generally ca. 0–9) spikes/sec). The preparation was then stimulated by serial dilutions of the compounds found to be active at 1 μg.

Stimulus dilutions in nanograde pentane were standardly presented in order from the lowest to the highest dosage of a given compound. At least 3 min were allowed between each stimulus except at higher dosages when 5 min were allowed between successive stimuli. These intervals allowed for complete recovery of single-cell activity. Responses of 71 cells (35 cells in 12 males; 36 cells in 14 females) were recorded to 1 μg of the various odorants.

RESULTS

General Electrophysiology. Generally one or two spike heights occurred in each recording of the 71 cells to 1 μg of selected bark beetle pheromones and host terpenes (Figure 1). At this dosage each cell was generally

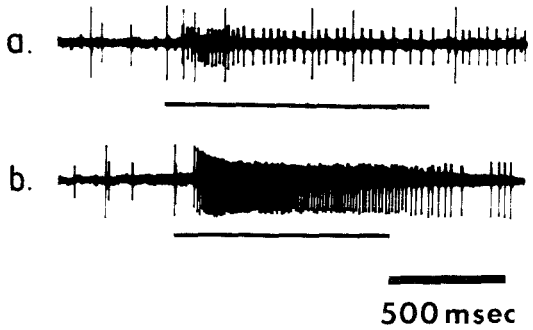

FIG. 1. Responses of two cells in one preparaton to 1 μg of limonene (a) (small-amplitude spike) and 1μg of 3,2-MCHone (b) (large-amplitde spike). Horizontal bar represents duration of stimulus. Data from a *D. pseudotsugae* female.

activated, i.e., a phasic change in spike frequency, by only one or at most a few odors. A considerably larger number of cells in both sexes was activated by the pheromones than the host compounds (Table 2). More cells were activated by pheromones in males than in females, whereas host compounds,

TABLE 2. NUMBER OF OLFACTORY CELLS WITH PRIMARY RESPONSES TO VAPORS OF 1 μg ON FILTER PAPER OF PHEROMONES AND HOST ODORS TESTED

		Number of cells		
	Compound	Male	Female	Male and female
Dendroctonus compounds	3,2-MCHone	6	3	9
♂ 24	3,2-MCHol	7	4	11
♀ 19	1,2-MCHol	[a]	6	6
Σ ♂ + ♀ = 43	Frontalin	4	2	6
	endo-Brevicomin	0	0	0
	trans-Verbenol	0	1	1
	cis-Verbenol	6	2	8
	Verbenone	1	1	2
Host tree compounds	α-Pinene	3	1	4
♂ 7	Limonene	4	8	12
♀ 9	Camphene	0	0	0
Σ ♂ + ♀ = 16				
Ips compounds	Ipsenol	0	1	1
	Ipsdienol	0	0	0
	Not activated	4	7	11
		Σ ♂ = 35	Σ ♀ = 36	Σ ♂ + ♀ = 71

[a]Odorant unavailable for testing on males.

especially limonene, activated more cells in females than males. Only one cell was stimulated by ipsenol, a pheromone specific for *Ips* bark beetles.

Classification of Cell Types. The olfactory cells could be classified into four types based on sensitivity and relative specificity for the various odors tested.

Cells responsive to 3,2-MCHone or 3,2-MCHol represent two of the cell types and were the most sensitive and specific of all cells recorded (e.g., Figure 2). Cells responsive to 3,2-MCHone and 3,2-MCHol at 1 μg in females responded with 38.0 (SE = 3.51) and 36.3 (SE = 0.9) impulses/initial 500 msec, respectively. Similarly, in males 3,2-MCHone cells responded to 1 μg of the stimulus with 36.2 (SE = 5.6) impulses/initial 500 msec. 3,2-MCHol cells in males were slightly more responsive than in females with 45.0 (SE = 3.0) impulses/initial 500 msec. These cells in both sexes had a threshold from 0.001 μg to 0.01 μg with a gradual increase in the initial phasic response pattern over several log steps (Figure 3). At stimulus dosages near threshold levels, these cells seldom responded to any other odor tested. Cells specialized to the ketone never responded to the alcohol and vice versa (Figures 2 and 4). In fact, in one preparation, mutual inhibition occurred. That is, increasing dosages of 3,2-MCHol or 3,2-MCHone stimulated their specific cells while causing a concomitant decrease in spike activity below the spontaneous rate for the cell specific for the other substance (Figure 4).

Although cells primarily sensitive to 3,2-MCHol and 3,2-MCHone were the most specific of the cells recorded, these cells could occasionally be activated by the vapors of other odorants, e.g., *cis*-verbenol and α-pinene (e.g., Figure 2).

A third cell type was most highly activated by frontalin. At the 1 μg level these cells responded with 30.5 (SE = 8.5) impulses/initial 500 msec in females and 35.3 (SE = 9.5) impulses/initial 500 msec in males. The frontalin cells had a threshold near 0.01 μg and showed increased phasic responses over several log steps in stimulus intensity (Figure 5). This cell type occasionally responded to other compounds, e.g., *endo*-brevicomin, verbenone, and *trans*-verbenol.

A fourth cell type was most responsive to either *cis*-verbenol, *trans*-verbenol, 1,2-MCHol, verbenone, α-pinene, or limonene (Figure 6). These cells responded to 1 μg of the most stimulatory compounds with 35.7 (SE = 16.7) impulses/initial 500 msec and 36.3 (SE = 15.6) impulses/initial 500 msec in females and males, respectively. These cells were the least specific of the cells recorded and responded to other host terpenes and pheromones at higher dosages (Figures 6 and 7).

Inhibition. On numerous instances cells spontaneously active at ca. 3–10 impulses/sec were completely inhibited by stimulation with 1 μg of 3,2-MCHone and 3,2-MCHol (Figures 4, 6, and 8). For example, spontaneous activity by a cell sensitive to 0.01 μg of limonene was inhibited by 1.0 μg of

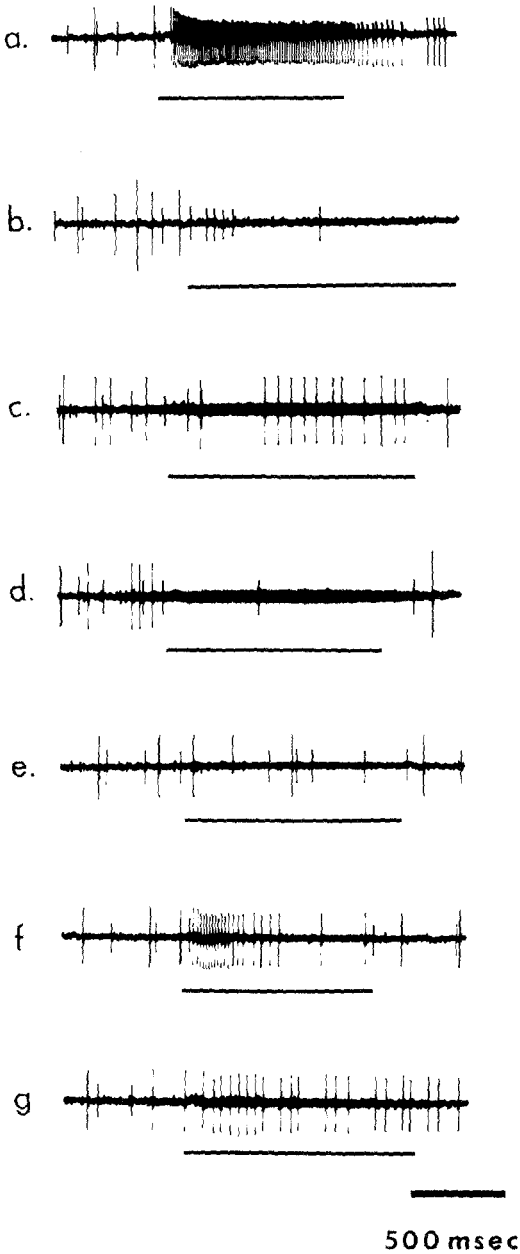

FIG. 2. Responses of 3,2-MCHone cell (large-amplitude spike) to 1 μg of various other odorants: (a) 3,2-MCHone; (b) 3,2-MCHol; (c) 1,2-MCHol; (d) (±)-frontalin; (e) camphene; (f) α-pinene; (g) *cis*-verbenol. Horizontal bar represents duration of stimulus. Data from a *D. pseudotsugae* female.

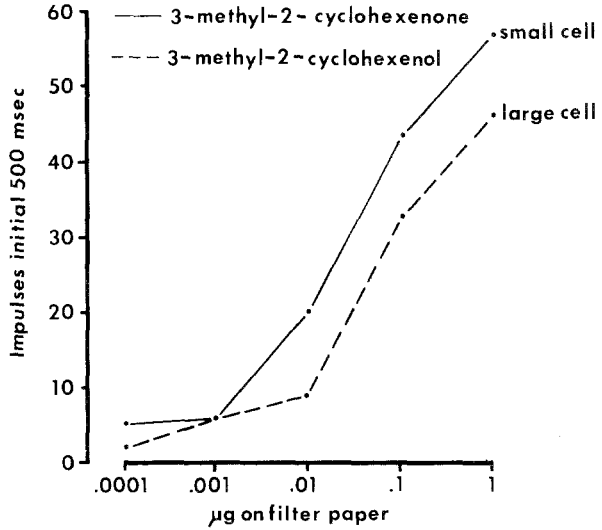

FIG. 3. Dosage–response curves constructed from responses of two simultaneously recorded cells, one sensitive to 3,2-MCHone, the other to 3,2-MCHol. Data from a *D. pseudotsugae* male.

3,2-MCHol (Figure 8). Furthermore, when the cell was presented simultaneously with the previously activating 0.01 μg limonene stimulus and 1.0 μg 3,2-MCHol, response to limonene was completely inhibited.

DISCUSSION

Characteristics of Olfactory Receptor Types. Pheromone and host odor/synergist receptor cells in *D. pseudotsugae* males and females show both similarities and differences when compared to receptor cells observed for other bark beetle species. For example, antennal olfactory cells sensitive to the pheromones, 3,2-MCHone, 3,2-MCHol, and frontalin in *D. pseudotsugae* had thresholds of response (0.001–0.01 μg on filter paper) similar to the threshold observed for cells in *Ips pini* females sensitive to its major aggregation pheromone, ipsdienol (0.005–0.05 μg on filter paper) (Mustaparta et al., 1979). A slightly higher threshold (0.01–0.1 μg on filter paper) was found for cells sensitive to the active stereoisomers of 4-methyl-3-heptanol in male and female *Scolytus scolytus* (Wadhams et al., 1982).

Similarly, cells activated in *D. pseudotsugae* males and females by *trans*-verbenol, *cis*-verbenol, and verbenone had a threshold (0.1 μg) near that found for *I. pini* cells activated by the same compounds (0.05–0.5 μg) (Mustaparta et al., 1979). However, these cells in *D. pseudotsugae* were also highly

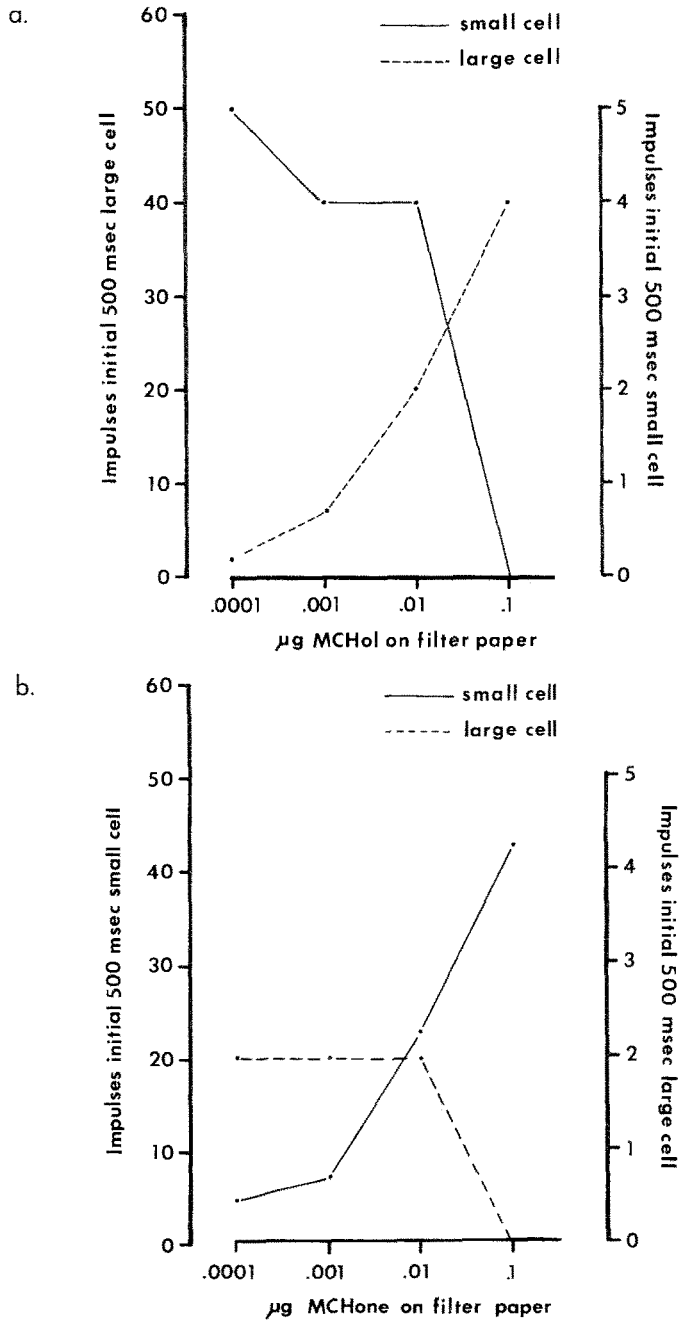

FIG. 4. Dosage–response curves constructed from responses of two cells in the same preparation, one sensitive to 3,2-MCHol (a) the other to 3,2-MCHone (b). Note decrease in concomitant spontaneous activity in cell specialized for the odorant not being tested. Data from a *D. pseudotsugae* male.

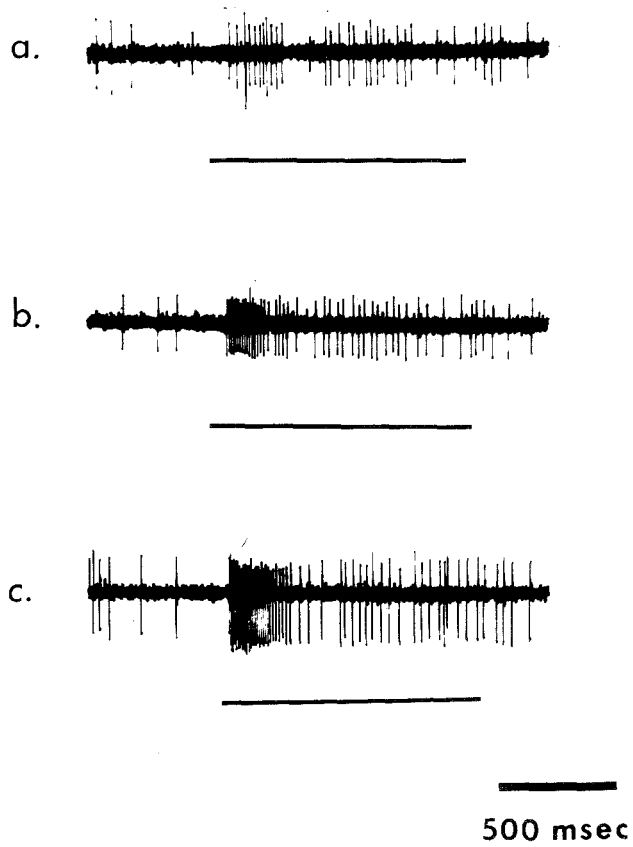

FIG. 5. Response of frontalin cell to increasing dosages of racemic frontalin. (a) 0.01 μg; (b) 0.1 μg; (c) 1.0 μg. Horizontal bar represents duration of stimulus. Data from a *D. pseudotsugae* female.

activated by one or more host terpenes (i.e., limonene, camphene, and/or α-pinene). Each of these compounds has been found to function as attractive synergists when presented with frontalin and low concentratoins of 3,2-MCHone and 3,2-MCHol (Rudinsky et al., 1974; Rudinsky and Ryker, 1977).

Both the large percentage of cells sensitive to host odors and other synergists (40% in males; 52.8% in females) (Table 3) and their high level activation (>70 impulses/initial 500 msec) is in contrast with the characteristics of cells responsive to host odors in *I. pini* (Mustaparta et al., 1979). In *I. pini* females, only two of 95 cells recorded were responsive to a host odor, i.e., myrcene, and even at 500 μg on filter paper less than 40 impulses/initial 500 msec were elicited.

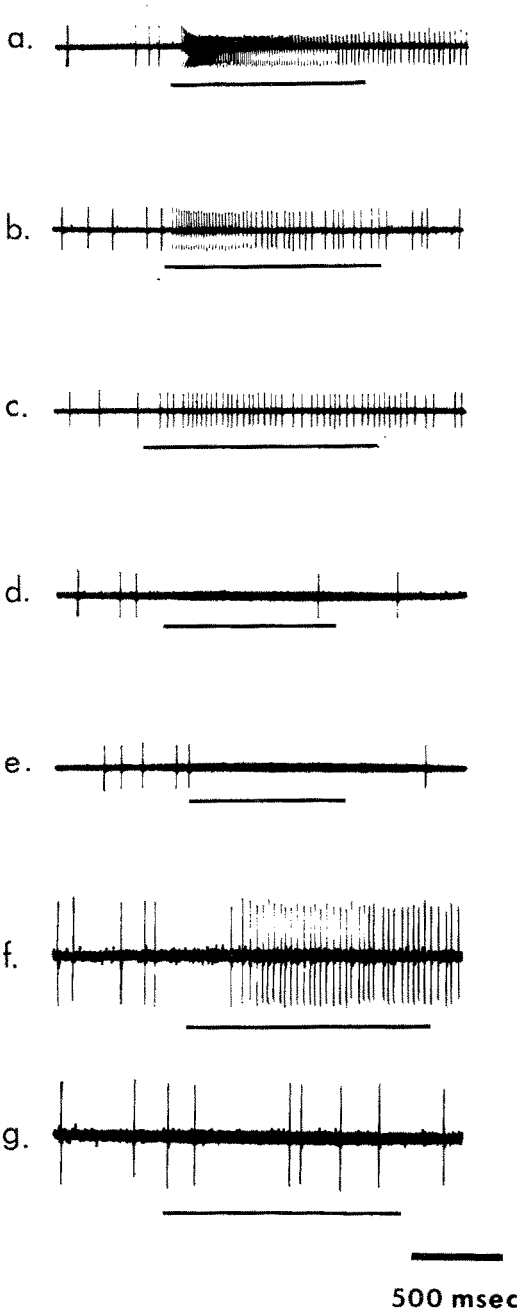

FIG. 6. Response of a synergist cell highly activated by limonene at 1.0 μg to other host terpenes and pheromones at the same dosage. (a) limonene; (b) camphene; (c) *cis*-verbenol; (d) 3,2-MCHone; (e) 3,2-MCHol; (f) 1,2-MCHol; (g) (±)-frontalin. Gain was increased in (f) and (g). Horizontal bar represents duration of stimulus. Data from a *D. pseudotsugae* female.

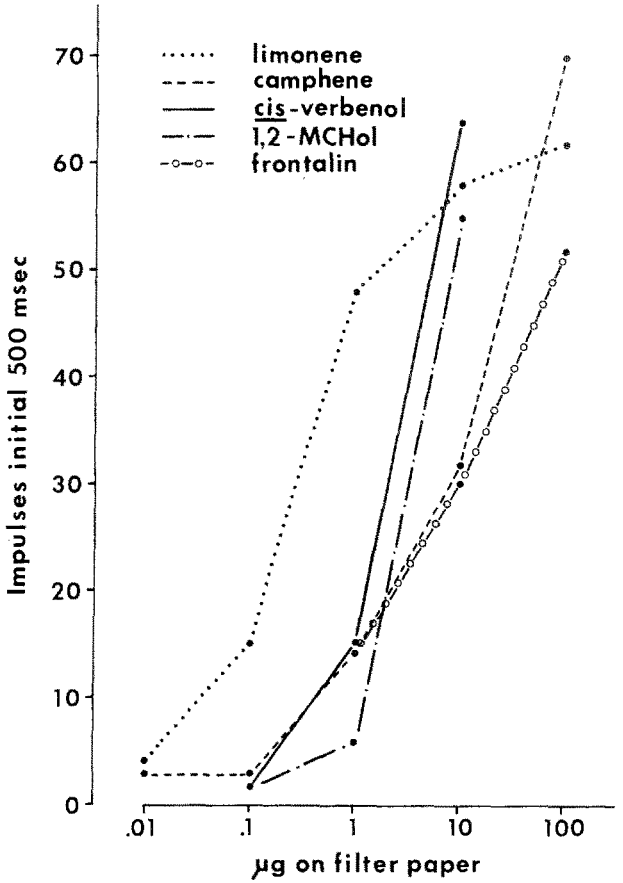

FIG. 7. Dosage-response curves for a synergist cell highly sensitive to limonene but responsive to other host terpenes and pheromones at higher dosages. Data from a *D. pseudotsugae* female.

In general, the specificity of the olfactory cell types recorded in *D. pseudotsugae* males and females is not as great as that observed for other bark beetle species (Mustaparta et al., 1979; Mustaparta 1979). The most specialized cells recorded in *D. pseudotsugae* were sensitive to 3,2-MCHone and 3,2-MCHol and responded with a phasic change in impulse frequency to such compounds as α-pinene and *cis*-verbenol at 1 μg on filter paper (Figure 2). In *I. pini* some cells sensitive to ipsdienol failed to respond to any other odor tested even at 500 μg on filter paper (Mustaparta et al., 1979).

Each of the four receptor cell types recorded gave an impulse frequency of 30–45 impulses/initial 500 msec for their primary odorant at the 1-μg dosage. Even at higher dosages, responses of the various cell types were simi-

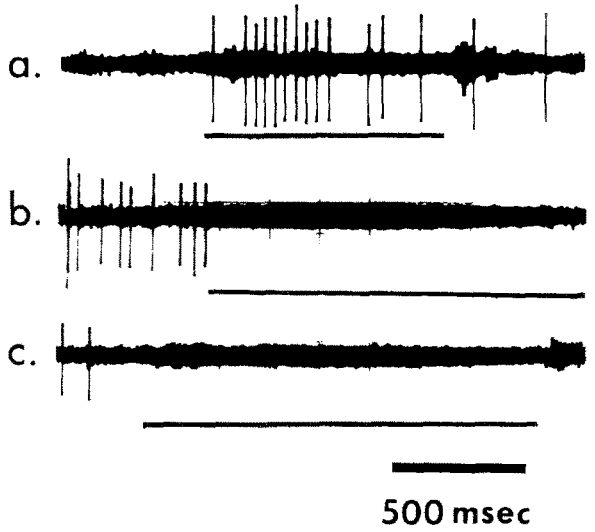

FIG. 8. Response of a single olfactory cell to 0.01 μg of limonene (a). Inhibition of spontaneous activity of limonene cell by 1.0 μg 3.2-MCHol (b). When stimuli in (a) and (b) were presented simultaneously, response to (a) was inhibited (c). Horizontal bar represents duration of stimulus. Data from a *D. pseudotsugae* male.

lar (48–60 impulses/initial 500 msec for 10 μg on filter paper). Since impulse frequency is considered to reflect the number of stimulus acceptor interactions (Kaissling and Priesner, 1970), similar numbers of impulses to the same dosage of stimulus would be indicative of similar numbers of receptor

TABLE 3. THRESHOLD OF FOUR CELL TYPES OBTAINED FROM EAG AND SINGLE-CELL STUDIES OF ADULT *D. pseudotsugae* AND PERCENT OF EACH CELL TYPE RECORDED IN SINGLE-CELL STUDIES

	Threshold (μg)			% of cells recorded[b]	
	EAG[a]				
Cell type	♂	♀	Single cell	♂	♀
1. 3,2-MCHone	0.001	0.01	0.001–0.01	17.1	8.3
2. 3,2-MCHol	0.001	0.01	0.001–0.01	20	11.1
3. Frontalin	1.0	0.1	0.01	11.4	5.6
4. Synergist	1.0–10.0		0.1	40	52.8

[a]Data from Dickens et al. (1983).
[b]Of cells recorded from males and females, 11.5% and 19.4%, respectively, not activated by odorants tested; 2.8% (1 cell) of cells recorded in female activated by ipsenol.

sites on the various cell types. Thus, the lower thresholds observed for 3,2-MCHone and 3,2-MCHol cells suggest a greater affinity of these odor molecules for their respective receptor sites rather than simply a larger population of responsive receptor sites on these cells which would increase the probability of odor molecule–receptor site interaction.

The mechanisms involved in the inhibition of spontaneous activity and the blocking of cellular response by 3,2-MCHone and 3,2-MCHol to a previously activating stimulus are unclear (Figures 4, 6, and 8). However, since stimulation of one cell occurred with concomitant inhibition of the second cell in the same preparation and most probably the same sensillum, the mechanism of inhibition likely occurs at the cellular level rather than because of mechanical restriction at the cuticular pore (Figure 4). The mechanism of inhibition could be that receptor sites are blocked by antagonists, i.e., competitive inhibition (Katz and Miledi 1973; Cotman and McGaugh 1980), or that the antagonists act on separate receptor sites to maintain the membrane potential steady or drive it farther away from threshold (Wilson 1979).

EAG Correlations. In general, results from single-cell experiments presented here are in accordance with results obtained from an earlier EAG study (Dickens et al., 1983). Dosage–response curves constructed from EAGs of *D. pseudotsugae* males and females to serial dilutions of four pheromones and three host terpenes were indicative of at least four receptor types in each sex. The two most sensitive receptor types responded to 3,2-MCHone and 3,2-MCHol. A receptor type with intermediate sensitivity responded to frontalin, while a fourth receptor type with the highest threshold responded to the synergists *trans*-verbenol, verbenone, limonene, and camphene (Table 3). Although males and females had differing thresholds and levels of response to the highest dosage tested, similarities in shapes of dosage–response curves indicated similar receptor mechanisms for each compound in both sexes. Lower thresholds and differing responses at dosages above threshold observed for EAGs were thought to be indicative of differences in the size of receptor site populations for each sex to a particular odor. Therefore, the likelihood of a molecule of a given odorant interacting with its receptor site to produce a response would be greater in the sex with the larger receptor site population for that particular odor.

Single-cell studies, especially with regard to the more specialized 3,2-MCHone and 3,2-MCHol cells, verify the hypothesis put forth in relation to the EAG studies (Dickens et al., 1983) (Table 3). Cells primarily responsive to 3,2-MCHone and 3,2-MCHol in both males and females had similar thresholds between 0.001 μg and 0.01 μg. However, of the cells recorded, a much larger percentage of these two cell types were recorded in male beetles than in females which had a 10$\times$ higher EAG threshold for both odors than did males (Table 3). Therefore, the lower threshold for these two odors observed in EAG studies was a reflection of the greater percentage of cells

responsive to these odors in males than in females and not due to any difference in sensitivity of responsive receptor sites.

The relationship between EAG responsiveness and single-cell responses for the other two cell types recorded is less clear. The fact that females were more sensitive than males to frontalin at the EAG level is difficult to reconcile, since more cells sensitive to frontalin were recorded in males than females and frontalin cells had similar thresholds in both sexes. This apparent enigma might be answered by the fact that relatively few cells recorded were responsive to this compound and frontalin not only stimulated cells primarily sensitive to it but also interacted with receptor sites on the synergist cells.

Behavioral Correlations. Information provided by previously reported EAG experiments (Dickens et al., 1983) and single-cell experiments presented here elucidate olfactory mechanisms involved in the aggregation and colonization of Douglas-fir by *D. pseudotsugae.* 3,2-MCHol, 3,2-MCHone at low concentrations, and frontalin have been demonstrated to be a strong three-component attractant for *D. pseudotsugae* in field tests (Rudinsky et al., 1974). This correlates well with data presented here indicating the presence of three cell types each primarily responsive to one of these compounds. The fact that various host odors, e.g., α-pinene, limonene, and camphene (Furniss et al., 1972; Rudinsky et al., 1972a), and insect-produced compounds, e.g., *trans*-verbenol, verbenone, and 1,2-MCHone (Rudinsky et al. 1974; Ryker et al., 1979), may act to synergize response to the tripartite attractant is corroborated by the responses of synergist cells to these compounds.

Although both male and female *D. pseudotsugae* have been shown to be responsive to host odors (Rudinsky 1966a; 1966b), females initiate the colonization of prospective host trees and are more responsive to host odors than males (Rudinsky 1966). Accordingly, more cells responsive to host odors, especially limonene, were recorded in females than males (Table 2). Thus, this heightened sensitivity for host odors would better equip the female for host selection.

In field tests, *D. pseudotsugae* males have been shown to be more responsive to both the aggregative and antiaggregative effects of the pheromones 3,2-MCHone, 3,2-MCHol, and frontalin, than females (Rudinsky, 1966b, 1973; Rudinsky et al., 1972a, 1974). The greater responsiveness of males to the pheromone may be explained by the fact that 48.5% of the cells recorded were keyed to the pheromones 3,2-MCHone, 3,2-MCHol, and frontalin in the males, whereas only 25% of the females' cells were keyed to components of this three-component mixture (Table 3).

Conclusions. The olfactory receptor system of *D. pseudotsugae* males and females is primarily composed of four receptor types. Three of the receptor types occur more frequently in males and are each maximally sensitive to one of the three major components of the *D. pseudotsugae* aggrega-

tion pheromone, i.e., 3,2-MCHone, 3,2-MCHol, and frontalin. The fourth receptor type was most sensitive to compounds found to synergize response of the beetles to the three major pheromone components and aid in orientation to host trees and host selection by females. More cells of this latter cell type were responsive to host odors, especially limonene in females than in males. The specificity of each cell type in *D. pseudotsugae* was not nearly as restricted as the specificity of cells reported in *Ips* species of bark beetles (Mustaparta et al., 1979; Mustaparta, 1979). The cells recorded in *D. pseudotsugae* more closely resembled those recorded in *D. frontalis* which were responsive to both pheromones and host odors at high dosages (Dickens and Payne, 1977, 1978; Dickens, 1979).

Pheromonal synergists activated a large percentage of cells recorded in both male and female *D. pseudotsugae* (40% and 52.8% in males and females, respectively). Additionally, 22.5% of all the cells recorded were most responsive to one of three host terpenes tested. This is in contrast to *I. pini* females in which only 6.3% of the cells recorded responded to host odors (myrcene) and then only mildly (<40 impulses/initial 500 msec) (Mustaparta et al., 1979). This difference in perception of host odors between *Dendroctonus* and *Ips* species could be explained by the relative importance of host odors in their basic biology and behavior. *Dendroctonus* species are aggressive and rely upon host odors possibly for primary attraction and/or as pheromonal synergists to orient them to suitable hosts for mass attack in order to overcome a copious host resin flow (Rudinsky, 1966; Cates and Alexander, 1982). Less aggressive *Ips* species may rely more on pheromones since they attack weakened trees with reduced resin pressure (Cates and Alexander, 1982).

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PLANT-DETERMINED VARIATION IN THE CARDENOLIDE CONTENT, THIN-LAYER CHROMATOGRAPHY PROFILES, AND EMETIC POTENCY OF MONARCH BUTTERFLIES, *Danaus plexippus* L.[1] REARED ON MILKWEED PLANTS IN CALIFORNIA: 2. *Asclepias speciosa*[2,3]

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Abstract—The pattern of variation in gross cardenolide concentration of 111 *Asclepias speciosa* plants collected in six different areas of California is a positively skewed distribution which ranges from 19 to 344 μg of cardenolide per 0.1 g dry weight with a mean of 90 μg per 0.1 g. Butterflies reared individually on these plants in their native habitats ranged from 41 to 547 μg of cardenolide per 0.1 g dry weight with a mean of 179 μg. Total cardenolide per butterfly ranged from 54 to 1279 μg with a mean of 319 μg. Differences in concentrations and total cardenolide contents in the butterflies from the six geographic areas appeared minor, and there were no

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[2]Apocynales: Asclepiadaceae.
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differences between the males and the females, although the males did weigh significantly more than females. The uptake of cardenolide by the butterflies was found to be a logarithmic function of the plant concentration. This results in regulation: larvae which feed on low-concentration plants produce butterflies with increased cardenolide concentrations relative to those of the plants, and those which feed on high-concentration plants produce butterflies with decreased concentrations. No evidence was adduced that high concentrations of cardenolides in the plants affected the fitness of the butterflies. The mean emetic potencies of the powdered plant and butterfly material were 5.62 and 5.25 blue jay emetic dose fifty units per milligram of cardenolide and the number of ED_{50} units per butterfly ranged from 0.28 to 6.7 with a mean of 1.67. Monarchs reared on *A. speciosa,* on average, are only about one tenth as emetic as those reared on *A. eriocarpa.* Unlike *A. eriocarpa* which is limited to California, *A. speciosa* ranges from California to the Great Plains and is replaced eastwards by *A. syriaca* L. These two latter milkweed species appear to have a similar array of chemically identical cardenolides, and therefore both must produce butterflies of relatively low emetic potency to birds, with important ecological implications. About 80% of the lower emetic potency of monarchs reared on *A. speciosa* compared to those reared on *A. eriocarpa* appears attributable to the higher polarity of the cardenolides in *A. speciosa.* Thin-layer chromatographic separation of the cardenolides in two different solvent systems showed that there are 23 cardenolides in the *A. speciosa* plants of which 20 are stored by the butterflies. There were no differences in the cardenolide spot patterns due either to geographic origin or the sex of the butterflies. As when reared on *A. eriocarpa,* the butterflies did not store the plant cardenolides with R_f values greater than digitoxigenin. However, metabolic transformation of the cardenolides by the larvae appeared minor in comparison to when they were reared on *A. eriocarpa.* Although *A. eriocarpa* and *A. speciosa* contain similar numbers of cardenolides and both contain desglucosyrioside, the cardenolides of *A. speciosa* overall are more polar. Thus *A. speciosa* has no or only small amounts of the nonpolar labriformin and labriformidin, whereas both occur in high concentrations in *A. eriocarpa. A. speciosa* plants and butterflies also contain uzarigen, syriogenin, and possibly other polar cardenolides with R_f values lower than digitoxin. The cardenolide concentration in the leaves is not only considerably less than in *A. eriocarpa,* but the latex has little to immeasurable cardenolide, whereas that of *A. eriocarpa* has very high concentrations of several cardenolides. Quantitative analysis of R_f values of the cardenolide spots, their intensities, and their probabilities of occurrence in the chloroform–methanol–formamide TLC system produced a cardenolide fingerprint pattern very different from that previously established for monarchs reared on *A. eriocarpa.* This dispels recently published skepticism about the predictibility of chemical fingerprints based upon ingested secondary plant chemicals.

Key Words—*Danaus plexippus,* Lepidoptera, Danaidae, monarch butterflies, *Asclepias speciosa,* Asclepiadaceae, milkweeds, ecological chemistry, plant–insect interactions, chemical ecology, chemical defense, coevolution, thin-layer chromatography, cardenolide fingerprints, cardenolides, cardiac glycosides, desglucosyrioside, labriformin, labriformidin, syriogenin, uzarigenin, emetic potency, emesis.

INTRODUCTION

This paper is the second in a series in which we present a multifaceted analysis of cardenolides in monarch butterflies sequestered from seven species of larval foodplants of the genus *Asclepias* (Asclepiadaceae) in California. Our purposes, explained in more detail in our initial study on *Asclepias eriocarpa* Benth. (Brower et al., 1982), are to investigate quantitative variation and emetic potencies in the cardenolide contents of the butterflies as they relate to the milkweed plants growing in their natural habitats, to adduce evidence about the chemistry of the cardenolides, and, by means of thin-layer chromatography, to establish plant-determined cardenolide fingerprints of the butterflies. We now report on cardenolides in monarchs reared on *Asclepias speciosa* Torr. from six localities which we selected to represent a broad variety of California environments (Figure 1).

METHODS AND MATERIALS

Geographic and Ecological Distribution of Asclepias speciosa.

Although *Asclepias speciosa* is absent from the arid southwestern United States, it appears highly tolerant to habitat and elevation differences and ranges along the Pacific Coast northward to British Columbia and eastward to the Great Plains from Manitoba to Texas (Woodson, 1954). The species occurs abundantly as a weed along roadsides, railways, cultivated fields, and waterways (including irrigation ditches) and has the northernmost distribution of the western North American *Asclepias* species. In northern California *A. speciosa* ranges from Contra Costa County through the North Coast Ranges, northward from Fresno County along the western slopes of the Sierra Nevada and Cascade Ranges, and along the eastern margins of the same mountains northwards from Inyo County (Woodson, 1954; Lynch, unpublished data from herbarium collections as cited in Brower et al., 1982). Isolated weedy patches are also found widely in the northern Central Valley.

Locations, Methods, and Dates of Sample Collections.

Immature monarchs (*Danaus plexippus plexippus* L, western U.S.A. population, Urquhart, 1960) were collected from milkweeds over an extensive area of California during the summers of 1975 and 1976. Collections of the individually matched plant–butterfly samples followed the procedures established in Brower et al. (1982). As we found in that study, larvae reared on *A. speciosa* nearly always form their chrysalids on the individual plants upon which they feed. This contrasts sharply with larvae in the eastern U.S. population which often wander off their plants, pupate on adjacent vegta-

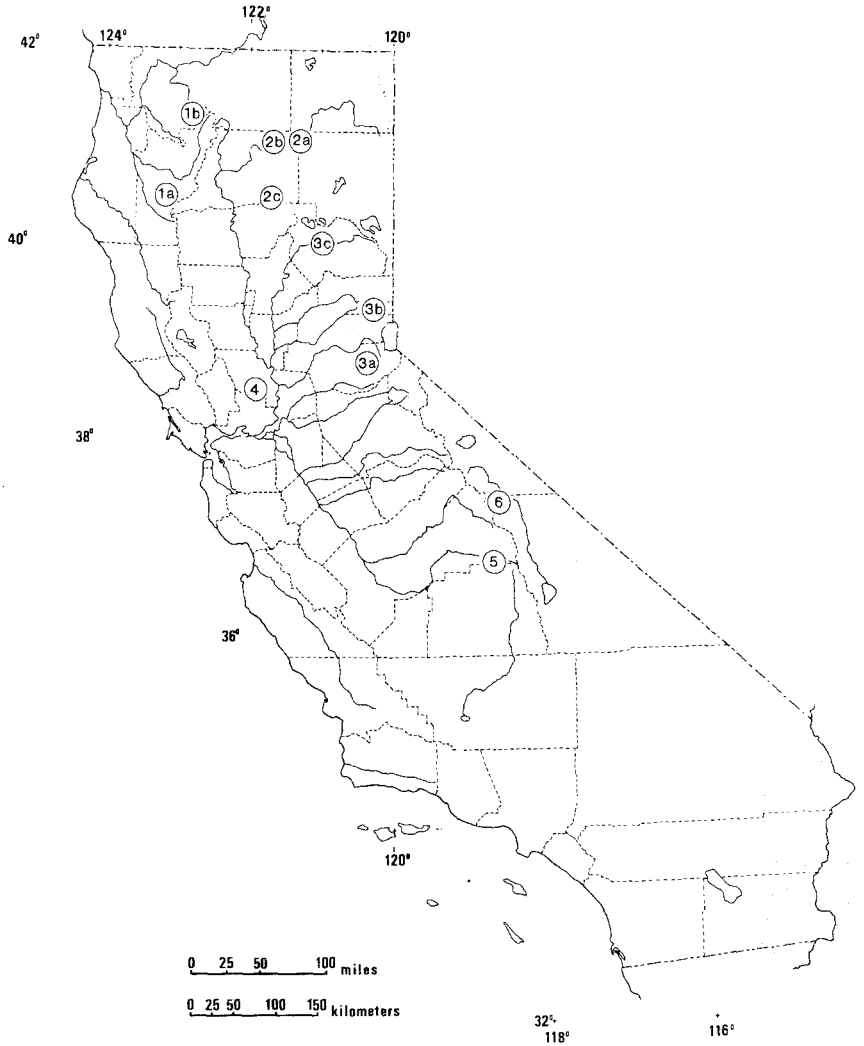

FIG. 1. Collection sites of monarch butterflies and their individual *Asclepias speciosa* foodplants analyzed in the study. The numbered open circles identify 11 sites within six general areas from which we collected a total of 111 pairwise butterfly and plant samples. Area 1 is in the North Coast Ranges, area 2 is in the montane zone of the southern Cascades, area 3 is in the montane zone of the northern Sierra Nevada, area 4 is in the northern Central Valley, area 5 is in the montane southern Sierra Nevada, and area 6 is along the eastern base of the Sierra Nevada in the Owen's River Valley. See text for details.

tion, and generally show low fidelity to individual milkweed plants (Urquhart, 1960; Rawlins and Lederhouse, 1981; Borkin, 1982; Brower and Lynch, personal observations). By selectively collecting larvae or chrysalids from plants which showed clear evidence of having been eaten, we are confident that nearly all larvae ate only the individual plants upon which they were collected. In some instances we enclosed early instar larvae on individual plants with nylon net bags and subsequently collected them as late fifth-instar larvae or chrysalids.

Figure 1 indicates 11 sites within the six general areas from which we collected a total of 111 pairwise butterfly and plant samples. We chose these six areas to represent a wide range of habitats encompassing most of the known distribution of *A. speciosa* in California. In the forested montane regions the plants were widely scattered in clearings and along roadsides, but in the more arid Central Valley and Owen's River Valley they were more restricted to areas with moist soils. The six areas, numbered 1–6 in Figure 1, included sites in the North Coast Ranges (Figure 1, 1), the volcanic southern Cascade Ranges (2), the primarily granitic northern Sierra Nevada (3), the northern Central Valley (4), the southern Sierra Nevada (5), and the eastern base of the Sierra Nevada along the Owen's River Valley (6). Samples from five of the six areas were collected in 1975 between July 2 and 14 September, while those from area 4 (northern Central Valley, Davis) were collected in the following year during late May and early June 1976. Geographic and altitudinal information was confirmed using California state regional maps (Anon., 1972, 1975a–e).

Area 1 (North Coast Ranges) is represented by two collection sites, one near Hayfork (Figure 1, 1a) in Trinity County and one near Callahan in Siskiyou County (Figure 1, 1b). Although *A. speciosa* is common in this region along roadsides, waterways, in fields, and in partially cleared mixed conifer woodlands, we observed few adult monarchs and careful searching of hundreds of individual plants yielded only six pairwise samples. These were Nos. 417–420, collected on August 10, 1975, on the roadside of California State Highway 3 (CA3) at 732 m elevation south of Hayfork, and Nos. 421 and 424 collected on August 11, along the roadside of CA3 above Big Mill Creek east of Callahan at 975 m.

Area 2 (southern Cascades, Montane) is just south of Lassen Volcanic National Park and includes three collection sites between Nubeiber in Lassen County and Childs Meadow in Tehama County. We found numerous plants along the roadsides and in partially cleared mixed conifer forests and collected 22 pairwise samples. These were: No. 304 collected in Lassen County on July 28, 1975, along CA 299 at Nubeiber (Figure 1, 2a) at an elevation of 1280 m; on July 28, No. 321, on July 29, Nos. 338–348 and 350, and on September 14, Nos. 662 and 673–675 in Shasta County, along the roadside of CA 299 near McArthur (Figure 1, 2b) at 1005 m; and No. 334 on July 29, and Nos. 427, 429–

430 on August 12, along the roadside of CA 89 at Child's Meadows (Figure 1, 2c) in Tehama County at an elevation of 1465 m.

Area 3 (northern Sierra Nevada, Montane) included collection sites from the southern edge of Lake Almanor in Plumas County in the Sierra Nevada–Cascade transition zone to Eldorado County, southwest of Lake Tahoe. Plants were numerous along the roadsides and in partially shaded meadows. We collected a total of 34 pairwise samples including Nos. 148 and 151 on July 2 along the roadside of U.S. Highway 50 (US 50) in Eldorado County between Riverton and Whitehall (Figure 1, 3a) at approximately 1000 m; Nos. 213–215 and 218–224 on July 13, and Nos. 302 and 303 on July 27, in Sierra County in open pastureland and along the roadside of CA 89 between Sierraville and Randolph (Figure 1, 3b) at 1510 m; Nos. 351–356 on July 30, and Nos. 436–438 on August 12, in Plumas County on CA 89 between Canyon Dam and Greenville (Figure 1, 3c) at 1200–1500 m; and Nos. 646, 649, and 651–656 on September 13, in Plumas County along the roadside of CA 89 between Crescent Mills and Quincy at 1000–1100 m.

Area 4 (northern Central Valley, Davis) was represented by a single population of plants located in a disturbed, open field within the city limits of Davis, Yolo County, at an elevation of 14 m (Figure 1, 4). We obtained 23 pairwise samples during May and June 1976 by bagging first and early second-instar larvae on individual plants. (These larvae had been collected in the wild in Fresno as eggs on *A. fascicularis* Dcne.) These were Nos. 833–852 collected on May 21, No. 856 on May 26, and Nos. 972 and 973 on June 11.

Area 5 (southern Sierra Nevada, Montane) included a single locality in Cedar Grove, Kings Canyon National Park (Figure 1, 5). We located several small stands of *A. speciosa* in a sparse pine forest and along a dirt road 1 mile northeast of the ranger station at 1420 m, about 200 m up the road from Area 5 in Brower et al. (1982). Five pairwise samples were obtained including No. 287 collected on July 19, 1975, and Nos. 628–631 which we bagged as fourth-instar larvae on August 4 and later collected on August 28.

Area 6 (eastern base, Sierra Nevada, Owen's River Valley) was located near Bishop, Inyo County, at an elevation of 1280 m (Figure 1, 6). We collected 21 pairwise samples from plants found growing abundantly in moist open meadows, along irrigation ditches, and in moist roadside depressions. The samples were Nos. 152 and 155–164 collected on July 3, 1975, Nos. 445, 446, and 448–450 on August 18, and Nos. 500 and 505 on August 19.

Analytical Procedures

Gross Cardenolide Content. During the fall of 1976, we determined the gross cardenolide content (equivalent to digitoxin) of 111 butterflies (66 males and 45 females) and the leaves from the respective individual plants upon which they had been reared. All the butterfly samples were from separate plants except for one male and one female in area 3 which were

both collected on one plant. The individual samples were spectroassayed by the same procedures used in Brower et al. (1982) which gives results in micrograms (equivalent to digitoxin) per 1.0 g dry weight of material analyzed.

Cleanup Prior to Thin-Layer Chromatography. The cleanup of *A. speciosa* plant and butterfly extracts utilized a lead acetate precipitation procedure (Nelson et al., 1981; Brower et al., 1982) to remove pigments and other interfering substances. The following minor modifications of those procedures were made because of the lower concentrations and greater polarity of the cardenolides from *A. speciosa* compared to *A. eriocarpa.* (1) For the plant samples, 7 ml (instead of 3 ml) of ethanol extract was carried through the lead acetate cleanup. To compensate for this greater volume, 10% lead acetate (rather than 5%) was used for precipitation, and the excess lead was removed with 1.0 ml of granular ammonium sulfate (rather than 0.5 ml). (2) The depigmented aqueous supernatant was extracted 2 × with 2 ml of 2:1 $CHCl_3$-ETOH rather than 2 × with 2 ml of $CHCl_3$. (3) After the first transfer and evaporation of the $CHCl_3$-ETOH extract to dryness (with N_2 as previously described), the residue was again dissolved in 0.5 ml of 2:1 $CHCl_3$-ETOH, the supernatant pipetted from a small residue of salts, and again evaporated to dryness. This salt removal process was repeated until a negligible salt precipitate was observed, i.e., 3 × for the plants, and 4 × for the butterflies.

Prior to cleanup, the *A. speciosa* plant extracts had a mean cardenolide concentration of 7.94×10^{-5} M (95% confidence limits = 0.33, $N = 5$, SD = 0.26), and the cleaned extracts 9.86×10^{-5} M (95% confidence limits = 0.34, $N = 5$, SD = 0.27). After correcting for volume differences, concentrations in the uncleaned and cleaned samples were, respectively, 304 and 269 μg/0.1 g, giving an average loss of cardenolides of only 12%. Respective values for the butterfly samples (which did not require volume corrections) were 6.38×10^{-5} M (95% confidence limits = 0.38, $N = 5$, SD = 0.30) and 5.93×10^{-5} M (95% confidence limits = 0.58, $N = 5$, SD = 0.46), giving an average loss of cardenolides of only 7%.

Thin-Layer Chromatography (TLC). The conditions for TLC development in the chloroform–methanol–formamide and the ethyl acetate–methanol solvent systems, visualization, and photography of developed chromatograms were the same as previously described in Brower et al. (1982). We carried out the TLC analyses in April and May 1977 on 58 individual plants and their corresponding 58 butterfly samples representing all six areas. The rationale for selecting the samples and the methods of quantifying the $R_{\text{digitoxin}}$ and spot intensity values also are as described previuosly.

As will be seen, the overall spot intensities in the plants were consistently less than anticipated from the spectroassay data. The most likely reason for this was interference which appears to have given higher absorbancies and therefore overestimations of the gross plant cardenolide concentrations by

about 30%. It is possible that noncardenolide components in the plants were responsible. For example, we recently determined that *cis*-polyisoprene (rubber) and α- and β-amyrin and their acetates are major constituents of the latex of *A. speciosa* (Van Emon and Seiber, in press). The α- and β-amyrin were identified perviously in *A. syriaca* latex (Schmid and Ludwig, 1927), as was polyisoprene in several milkweed species (Whiting, 1943). None of these materials gives a TNDP-positive response on TLC, but they may contribute to the difficulty we experienced in obtaining accurate absorbancies as well as in resolving cardenolides from noncardenolides during the cleanup and TLC analyses.

Another problem emerged in the course of the TLC analyses: the mean $R_{\text{digitoxin}}$ value for digitoxigenin was 1.89 (see Results), whereas in the *A. eriocarpa* study, this value was 2.67, i.e., a 1.41-fold difference occurred in the relative separation of digitoxin and digitoxigenin in the two studies. As a consequence, direct quantitative comparisons of the R_d values for the cardenolides are not possible. In the future, it will be necessary to implement more rigid control on the conditions under which the chromatographic separations are done, including such factors as temperature, humidity, plate preparation and handling, etc. (see Stahl, 1965). Nevertheless, this problem does not affect the relative positions of the cardenolides in three TLC regions, and valid comparisons of TLC patterns are possible for the spots based on the mean and standard deviations of each spot's probability of occurrence and intensity values as described in the results.

Statistical Analyses

Statistical analyses were performed via the Univeristy of Florida Northeast Regional Data Center utilizing SAS, Release 79.6 (Anon., 1982a,b). We analyzed dry weights, cardenolide concentrations, and the total cardenolide contents of the butterflies, as well as the cardenoiide concentrations of the plants. PROC UNIVARIATE and FREQ PLOT NORMAL tests were used to examine frequency distributions and to test for normality (Kolmogorov D statistic).These statistics provide stem-leaf, box, and normal probability plots which allow a thorough examination of the degree of skewness or kurtosis in the data. If the data were not normal ($P < 0.05$), they were transformed ($\log_{10}$) and reexamined for normality prior to further testing.

We carried out several two-way-analyses of variance (two-way ANOVAs) separately on the plant and butterfly data to determine the significance of the influence of sex, area, and their interaction. We used the general linear models procedure (PROC GLM) because of unequal numbers in the various cells. The minimum number of observations in any cell was two.

Type IV sum of squares statistics were used if there was one or more significant interactions in the data, whereas type II sum of squares were used if there was none. We also used Duncan's multiple-range tests to compare the

significances of the differences between mean values for the geographic areas or the sexes with $P \leqslant 0.05$.

To relate cardenolide concentrations and total cardenolide contents of individual butterflies to the individual plants which the larvae had eaten, regressions of the butterfly data (Y, the dependent variable) were run against the plant concentrations (X, the independent variable) using PROC GLM. Plots of residual values were also done to examine the data for possible relationships not explained by the linear regression model. We used the $\log_{10}$ transformation of the plant concentration data based on an analysis by C. Nelson (unpublished) of the data of Sieber et al. (1980). In that study monarch larvae were fed a range of dosages of pure cardenolides, and it was found that both concentrations and total amounts stored best conformed to the relationship $Y = b\ (\log X) + a$. Examination of our total data set on the butterfly–plant concentration relationships for the seven Californian *Asclepias* species (to be published subsequently) has confirmed this logarithmic relationship. This indicates regulation of uptake and is considered further in the discussion.

We carried out each regression in two steps. The first considered the overall relationship of Y to X and calculated r^2, the slope, and the intercept as in Table 3A and as plotted in Figure 3. The significance of the difference of the slope and Y intercept values from zero is based on the t statistic. The second set of regressions broke down the dependence of the butterfly cardenolide concentrations (and also total cardenolide contents and dry weights) on the cardenolide concentrations in the plants by sex, by area, and by their interactions.

The significance of the regressions in the TLC analyses was limited to testing by type I sum of squares because of insufficient repetetitions of the area, sex, and plate variables.

Blue Jay Emetic-Dose Fifty (ED_{50}/Assay)

The ED_{50} assays and statistical analyses of the butterfly and plant materials are also as described in Brower et al. (1982) and were carried out in March 1978 utilizing a total of 38 blue jays (*Cyanocitta cristata bromia* Oberholser, Corvidae) which we captured in Hampshire County, Massachusetts, between December 12, 1977 and March 2, 1978. The mean and standard deviation of the weights of the 38 birds when force fed were 91.04 and 6.28 g, respectively. The numbers of birds force fed the powdered butterfly and plant material were, respectively, 20 and 18. Of these, respectively, 10 and 8 birds fell within the range necessary to estimate the ED_{50} values (Dixon and Massey, 1957).

The test powder was made by combining the residual powders of 24 butterflies remaining after the individual spectroassays and consisted of 1.47 g of material. This included two males and two females from area 1, four males

and four females from area 2, four males and four females from area 3, and two males and two females from area 5. Residual plant materials corresponding to the 24 butterflies were combined in 4 × the respective residual weights of the butterfly powders. Gross cardenolide concentrations of aliquots from these plant and butterfly pools were, respectively, 128 and 263 μg/0.1g.

RESULTS

Gross Cardenolide Concentrations (μg/0.1 g Dry Weight)

Quantitative Variation in Plants. The pattern of variation is plotted as a histogram in Figure 2. All 111 plants contained cardenolide and the concentration range in the six geographic areas was 19–344 μg/0.1 g with a grand mean of 90.3 μg and a grand standard deviation of 64.6 μg (Table 1, Figure 2). This variation encompasses approximately one order of magnitude, and in terms of cardenolide per gram dry weight represents from 0.02% to 0.34% (mean = 0.09%).

The curve in Figure 2 displays the expected normal distribution calculated by the z statistic (Steel and Torrie, 1960), and it can be seen that the variation is positively skewed (moment of skewness = 1.43), i.e., the majority of plants contain less than the average concentration of cardenolide. The distribution departs significantly from normality (mean and median = 90.3 and 67.0; $D = 0.155$; $P < 0.01$). Although the $\log_{10}$ transformed data also depart from normality (moment of skewness = 0.20; mean and median = 1.857 and 1.826; $N = 111$, $D = 0.091$, $P < 0.023$), the normal probability, box, and stem-leaf plots indicated that the $\log_{10}$ data are sufficiently close to normality so that the F statistic is still appropriate (Sokal and Rohlf, 1969, p. 377).

Inspection of the means in Table 1 suggests differences both in the cardenolide concentrations of the plants from the six geographic areas and in the plants fed on by the males vs. the females. The two-way ANOVA (Table 2A) indicates that the area difference is highly significant but that the difference in the plants according to the sex of butterfly that fed upon them is not, and that the interaction is also not significant. The lowest concentrations are in the area 4 (Davis) samples. When area 4 is removed from the two-way ANOVA, neither the area nor sex differences are significant (Table 2B). The grand variance of the cardenolide concentration of the plants upon which the males were reared is slightly higher than those on which the females were reared (for s^2 male/s^2 female, $\log_{10}$ data, $F = 1.659$; $df = 65, 44$; $0.05 > P > 0.025$), but when the area 4 data are removed, this difference again is insignificant ($F = 1.322$, $df = 53, 33$; $0.25 > P > 0.10$). Since the area 4 samples were collected in the second year, the difference in areas per se cannot be distinguished from a difference due to the two years of collection.

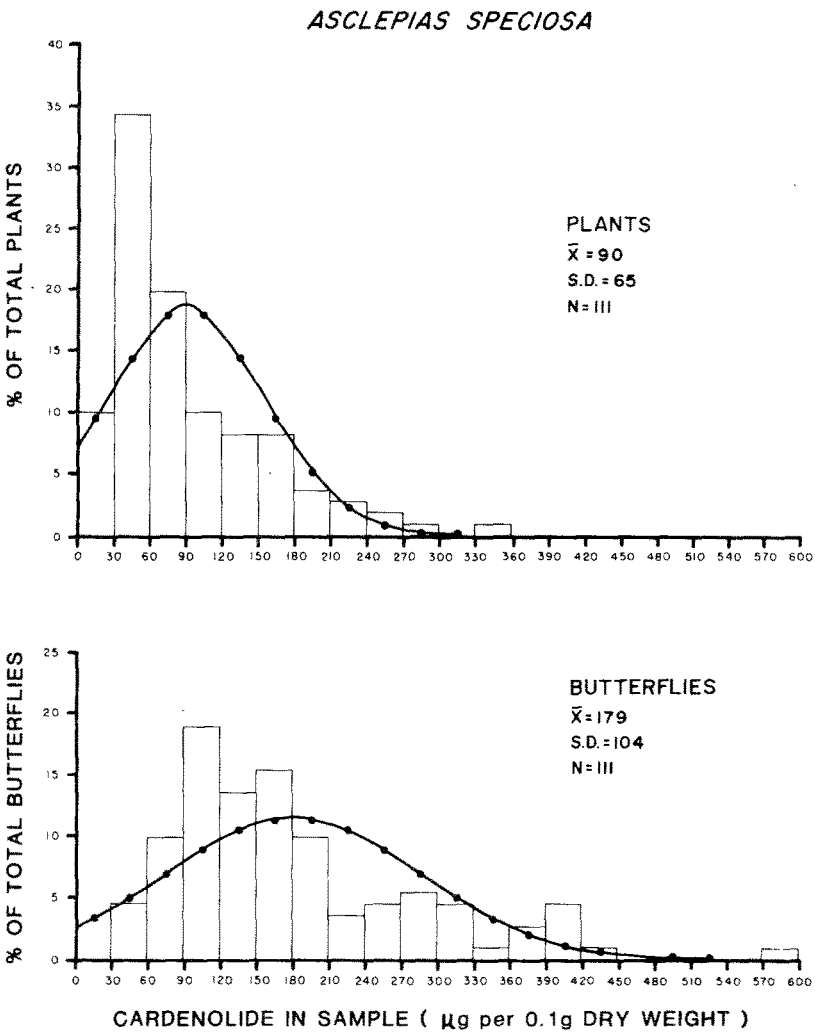

FIG. 2. Frequency distributions of the gross cardenolide content (as μg 0.1 g dry weight, equivalent to digitoxin) of 111 *Asclepias speciosa* plant samples and 111 adult monarch butterflies reared thereon. All individual butterflies were collected as 5th-instar larvae or chrysalids on the respective individual milkweed plants growing in their natural environments from the six geographic areas shown in Figure 1. The curves are the calculated normal distributions.

The evidence thus indicates that geographic variation in the gross cardenolide concentrations of individual *Asclepias speciosa* plants is relatively minor in these diverse California habitats. We previously reached the same conclusion in studying *Asclepias eriocarpa* samples collected in many of the same areas and at the same times (Brower et al., 1982).

TABLE 1. SUMMARY OF MEANS AND GRAND STANDARD DEVIATIONS OF CARDENOLIDE CONCENTRATIONS OF 111 WILD-COLLECTED INDIVIDUAL *Asclepias speciosa* PLANTS[a] AND MONARCH BUTTERFLIES REARED THEREON[b]

	Plant material[c]			Butterfly material[c]		
	Males	Females	Means[d]	Males	Females	Means[d]
Area 1: north Coast Ranges (N = 4, 2)	156.5	63.5	110.0	197.5	266.0	231.8
Area 2: southern Cascades, Montane (N = 13, 9)	133.3	96.3	114.8	275.6	265.9	270.8
Area 3: northern Sierra Nevada, Montane (N=22, 12)	110.2	98.5	104.4	201.9	153.5	177.7
Area 4: north Central Valley, Davis (N = 12, 11)	36.3	56.3	46.3	96.6	117.7	107.2
Area 5: southern Sierra Nevada, Montana (N = 3, 2)	145.3	59.5	102.4	177.0	177.0	177.0
Area 6: eastern base, Sierra Nevada, Owens River Valley (N = 12, 9)	73.9	63.4	68.7	127.9	161.8	144.9
Grand means[e]	99.1	77.4	90.3	182.4	174.9	179.4
Grand SDs[e]	73.1	47.4	64.6	103.0	106.5	104.0
Grand Ns	66	45	111	66	45	111
Ranges	19–344			41–547		

[a]Plant 356 from area 3 was fed upon by one male and one female butterfly so that statistics for this plant include one duplicate cardenolide determination.
[b]Collections are from the six areas shown in Figure 1. Data are μg (equivalent to digitoxin) per 0.1 g dry weight of butterfly or plant material. All samples were collected in the summer of 1975 except for those from area 4 which were collected in May–June 1976.
[c]The plant material corresponds to the butterflies according to the sex of the butterfly; the butterfly material represents the butterflies reared on their respective plants.
[d]The means above the grand means are the mean male value added to the mean female value divided by 2.
[e]Based on all 66 males, 45 females, and their respective plants.

Quantitative Variation in Butterflies. All 111 butterflies contained cardenolide. The concentration range throughout all geographic areas was 41–574 μg/0.1 g with a grand mean of 179.4 μg/0.1 g (Table 1). This range spans approximately one order of magnitude and in terms of cardenolide per gram dry weight represents from 0.04% to 0.57% (mean = 0.18%), i.e., about twice that in the plants.

As seen in Figure 2, the pattern of variation in cardenolide concentration in the butterflies is also positively skewed (moment of skewness = 1.20), but to a lesser extent than the plants. This skewness departs to a highly significant

TABLE 2. TWO-WAY ANOVA OF CARDENOLIDE CONCENTRATION DATA IN TABLE 1[a]

Source of variation	*df*	*SS*	*MS*	*F*	*P*
A. Plant material (all areas, $N = 111$)					
Model	11	2.84431	0.25857	3.88	< 0.0001
Error	99	6.59059	0.06657		
Corrected total	110	9.43490			
Area	5	2.16676		6.51	< 0.0001
Sex	1	0.04766		0.72	> 0.39
Sex × area	5	0.56135		1.69	> 0.14
B. Plant material (excluding area 4, $N = 88$)					
Model	9	1.03468	0.11496	1.50	> 0.16
Error	78	5.97301	0.07658		
Corrected total	87	7.00769			
Area	4	0.57422		1.87	> 0.12
Sex	1	0.21861		2.85	> 0.09
Sex × area	4	0.21387		0.70	> 0.59
C. Butterfly material (all areas, $N = 111$)					
Model	11	2.09812	0.19074	4.22	< 0.0001
Error	99	4.47431	0.04519		
Corrected total	110	6.57243			
Area	5	1.92916		8.54	< 0.0001
Sex	1	0.00000		0.00	> 0.99
Sex × area	5	0.16179		0.72	> 0.61
D. Butterfly material (excluding area 4, $N = 88$)					
Model	9	1.11425	0.12381	2.39	< 0.020
Error	78	4.04682	0.05188		
Corrected total	87	5.16107			
Area	4	0.98394		4.74	< 0.002
Sex	1	0.00951		0.18	> 0.66
Sex × area	4	0.11853		0.57	> 0.68

[a]Type II sum of squares, all data are $\log_{10}$ of μg/0.1 g.

degree from a normal distribution (mean and median = 179 and 153, respectively; $N = 111$, $D = 0.150$; $P < 0.01$). However, the $\log_{10}$ transformed data do not depart from normality (moment of skewness = 0.02, mean and median = 2.186 and 2.185; $N = 111$, $D = 0.056$, $P > 0.15$). Therefore, we again based the ANOVAs on the transformed data.

The grand variance of the cardenolide concentrations of the 66 males does not differ significantly from that of the 45 females ($\log_{10}$ data, $F = 1.007$, $df = 65, 44, 0.50 > P > 0.25$), nor does it when the area 4 data are removed ($F = 1.011$, $df = 54, 34, 0.50 > P > 0.25$). The grand butterfly standard deviation is 104 μg, about 1.6 times that of the plants they were reared upon (Table 1, Figure 2).

The two-way ANOVA of all six areas indicates that the area difference is highly significant but that the sex difference is not (Table 2C). Duncan's test indicates that area 4 has the lowest concentrations of all six samples (107 μg/0.1 g). When area 4 is removed from the analysis, the area difference is still significant (Table 2D), and a second Duncan's test indicates that area 2 (271 μg/0.1 g) and area 6 (145 μg/0.1 g) differ significantly. The data thus suggest an overall geographic trend of high concentrations in butterflies to the north, intermediate concentrations in the Sierras, and low concentrations in Owen's River Valley.

Cardenolide Concentrations of Butterflies as a Function of Cardenolide Concentrations of Plants

The mean cardenolide concentration in the butterflies is twice that of their respective plants (the grand mean ratio = 1.99), the overall range in the butterflies (41–547 μg/0.1 g) is shifted substantially upwards from that of the plants (19–344 μg/0.1 g), and the untransformed grand variance of the butterflies is 2.6 times that of their respective plants (Table 1; $F = 0.697$, $df = 110$; $0.05 > P > 0.025$, $\log_{10}$ data). Thus the butterflies increase both the mean and the variance of their cardenolide concentrations compared to their respective plants.

Figure 3 is a plot of butterfly concentrations against the respective plant concentrations for both males and females. The main model regression (Table 3A) confirms that the butterfly concentrations are highly dependent on the overall plant concentrations ($Y = 199.95 \log_{10} X - 191.93$; $r^2 = 0.317$; $P < 0.0001$), and both the Y intercept and the slope are significantly different from 0 at the $P < 0.0004$ level. This regression equation predicts that butterflies which have fed upon plants containing as little as 20 μg/0.1 g would contain approximately 68 μg/0.1 g of cardenolide, those feeding on 100-μg plants would contain 208 μg/0.1 g, and those feeding on 300-μg plants would contain 303 μg/0.1 g. Thus the butterflies significantly increase their body concentrations above that of their plants when feeding on low concentration plants, about equal them at 300 μg, and level off thereafter. The wide scatter of points in Figure 3 indicates that this function has considerable variance. However, plotting of the residual values in both the linear and log regressions indicated no further obvious relationships of the two variables.

The second regression analysis on the same data (Table 3B) indicates that the main cause of variation in the butterflies derives from the plants. However, there is also a contribution based on the plants fed upon by the different sexes in the different areas, i.e., the area × sex interaction is significant ($P < 0.019$). This is apparent by examining the mean values for the six areas in Table 1: plants for area 4 and area 6 have very low plant concentrations for both "sexes," but their male–female ratios are reversed. It is likely that this

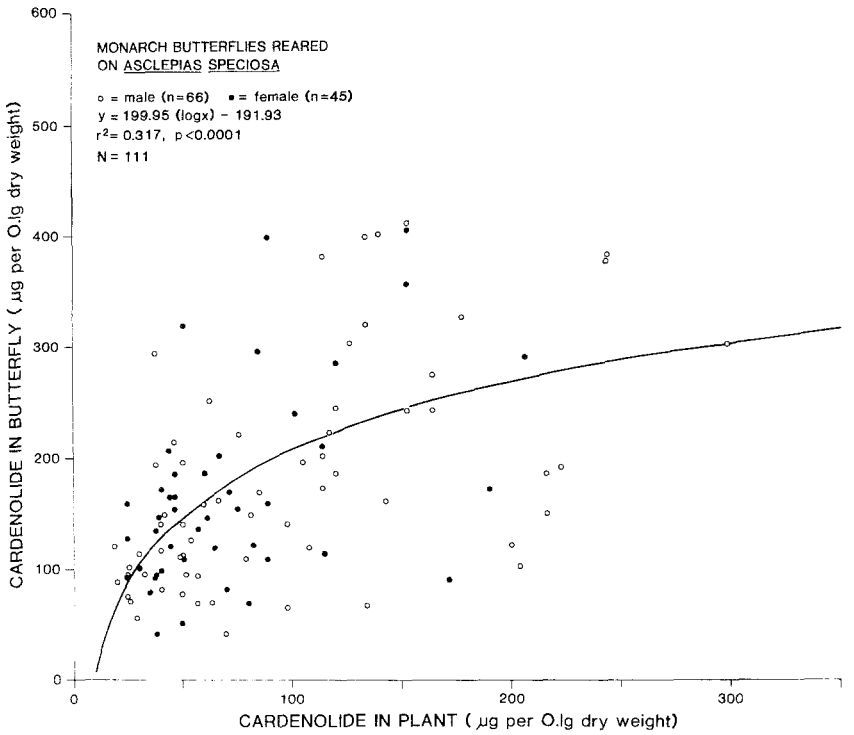

FIG. 3. Gross cardenolide concentrations of adult monarch butterflies (Y axis) as a function of the gross cardenolide concentrations of their larval foodplants (X axis). Each of the 111 data points represents one corresponding individual butterfly-plant rearing experiment. Open circles are males and solid circles are females. The line is derived from the regression equation $Y = b\ (\log_{10} X) + a$.

reversal was responsible for the female butterflies not having a significantly higher cardenolide content than the males, as we have found in several previous studies (see Discussion).

Dry Weights of Butterflies

The dry weights of the butterflies (Table 4) are normally distributed (grand mean = 0.169 g, median = 0.171 g, $D = 0.066$; $P > 0.15$) and the variances of the two sexes do not differ significantly ($F = 1.54$; $df = 65, 44$; $0.10 > P > 0.05$). A two-way ANOVA indicates a significant difference of the means both by sex and by geographic area, but no significant interaction (Table 5A). The average dry weight of the males is 0.175 g and the females 0.159 g (Table 4), i.e., the males on average weigh 1.10 times the females. Duncan's test indicates a significant difference between the geographic areas

TABLE 3. LINEAR REGRESSION ANALYSES OF CARDENOLIDE CONCENTRATIONS (μg/0.1 g DRY WT) IN BUTTERFLIES (DEPENDENT VARIABLE) VS. CARDENOLIDE CONCENTRATIONS ($\log_{10}$ μg/0.1 g DRY WT) IN THEIR RESPECTIVE PLANTS, ACCORDING TO FUNCTION $Y = b\ (\log_{10} X) + a$

A. Overall regression pooling sex and geographic areas ($r^2 = 0.317$; type IV sum of squares)

Source of variation	*df*	*SS*	*MS*	*F*	*P*
Plant concentration	1	377225	377225	50.59	< 0.0001
Error	109	812810	7457		
Corrected total	110	1190035			
Estimated value of parameters		SE	*T* for H = O		*P*
a = *Y* intercept = −191.93		52.85	−3.63		< 0.0004
b = slope = 199.95		28.11	7.11		< 0.0001

Equation for the line: $Y = 199.95\ (\log_{10} X) - 191.93$

B. Butterfly concentrations on plant concentrations by area, by sex, and for all interactions ($r^2 = 0.599$; type IV sum of squares)

Source of variation	*df*	*SS*	*MS*	*F*	*P*
Model	23	712941	30997	5.65	< 0.0001
Error	87	477093	5484		
Corrected total	110	1190035			
Log plant	1	35633		6.50	< 0.013
Area	5	39846		1.45	> 0.212
Sex	1	7287		1.33	> 0.252
Log plant × area	5	60616		2.21	> 0.059
Log plant × sex	1	8331		1.52	> 0.221
Sex × area	5	78978		2.88	< 0.019
Log plant × sex × area	5	95491		3.48	< 0.007

TABLE 4. SUMMARY OF DRY WEIGHTS AND TOTAL CARDENOLIDE CONTENT (EQUIVALENT TO DIGITOXIN) OF 111 ADULT MONARCH BUTTERFLIES REARED ON *A. speciosa* PLANTS POOLED FROM ALL SIX GEOGRAPHIC AREAS IN TABLE 1

		Dry weights (g)			Total cardenolide (μg)		
Sample size		Means	SD	Range	Means	SD	Range
Male	66	0.175	0.042	0.077–0.273	334	223	69– 991
Females	45	0.159	0.033	0.096–0.224	296	236	54–1279
Both	111	0.169	0.039	0.077–0.273	319[a]	234	54–1279

[a]The mean of the males + the mean of the females ÷ 2 = 315 μg.

TABLE 5. TWO-WAY ANOVAS OF (A) DRY WEIGHTS AND (B) TOTAL CARDENOLIDE CONTENT OF BUTTERFLIES BY SIX AREAS AND TWO SEXES

Source of variation	*df*	*SS*	*MS*	*F*	*P*
A. Dry Weights of Butterflies[a] ($r^2 = 0.52$)					
Model	11	0.08779	0.00798	9.82	< 0.0001
Error	99	0.08048	0.00081		
Corrected total	110	0.16827			
Area	5	0.07916		19.47	< 0.0001
Sex	1	0.00424		5.22	< 0.025
Sex × area	5	0.00216		0.53	> 0.75
B. Total cardenolide per butterfly[b] ($r^2 = 0.47$)					
Model	11	4.58545	0.41686	7.85	< 0.0001
Error	99	5.25799	0.05311		
Corrected total	110	9.84344			
Area	5	4.29419		16.17	< 0.0001
Sex	1	0.02076		0.39	> 0.53
Sex × area	5	0.21646		0.82	> 0.54

[a] Type II sum of squares (data are normally distributed).
[b] Type II sum of squares ($\log_{10}$ data are normally distributed).

grouped as follows: area 2 (0.203 g); areas 6 (0.179 g), 1 (0.177 g), and 3 (0.173 g); and areas 5 (0.143 g) and 4 (0.123 g). However, it is doubtful that these differences are ecologically relevant because of the varied conditions of their collection and/or rearing.

Dry Weights of Butterflies As a Function of Cardenolide Concentrations in Plants

The same linear regression models as in Table 3A and B were run to relate the dry weights of the butterflies (Y) to the cardenolide concentration of the plants (X). The overall model indicated a significant but weak positive correlation ($r^2 = 0.177$, $F = 23.51$; $P < 0.0001$), and the equation for the line was: dry weight = 0.000255 (plant concentration) + 0.146. (We did not use dry weight vs. $\log_{10}$ plant concentration because of a lower r^2: = 0.167, $F = 21.79$.) Two further regressions were carried out dropping the interactions shown in Table 3B because they were insignificant. The results were (overall model): $r^2 = 0.54$, $F = 17.52$, $P < 0.0001$; both plant concentration and area showed a significant effect ($P < 0.007$ and $P < 0.0001$, respectively), whereas sex did not ($P < 0.054$).

The data thus indicate a very slight positive correlation between cardenolide concentration in the plants and the weight of the butterflies with some effect of geographic area on the relationship.

Total Cardenolide in Butterflies

The $\log_{10}$ data for all six areas are normally distributed (mean = 2.401, median = 2.393; $D = 0.053$; $P > 0.15$). Total cardenolide per butterfly ranged from 54 to 1279 μg with a mean of 319 μg and a standard deviation of 234 μg (Table 4). The variances of the males and females (transformed data) do not differ significantly ($F = 1.07$; $0.50 > P > 0.25$). The males have an average of 334 μg and the females 296 μg. The two-way ANOVA on the transformed data indicates a highly significant difference among the areas but neither the sex nor the interaction is significant (Table 5B). Duncan's test of the differences among the areas indicates the following groupings: areas 2 (567 μg and 1 (407 μg); areas 1, 3 (321 μg), 6 (253 μg), and 5 (248 μg); and area 4 (129 μg).

The area results suggest that monarchs which fed on *Asclepias speciosa* in the northern populations contain larger amounts of cardenolide than those that fed on the more southern populations. Part of this difference is correlated with the body weight differences (areas 2, 4, and 5 have the same weight and total cardenolide ranking), and it is therefore doubtful that the differences are ecologically relevant. Area-derived differences in total cardenolide for the most part thus probably reflect the varied conditions of the collections and/or rearing as pointed out in the dry weight section.

Total Cardenolide in Butterflies as a Function of Cardenolide Concentrations in Plants

Regression analyses indicated that total μg of cardenolide per butterfly is highly dependent on the $\log_{10}$ plant concentrations ($Y = 466.46 \log X - 547.63$; $r^2 = 0.341$; $P < 0.0001$), and both the Y intercept and the slope are significantly different from 0 at the $P < 0.0001$ level (Table 6A). This regression equation predicts that butterflies which fed upon plants containing as little as 20 μg/0.1 g would contain approximately 59 μg of cardenolide, those on 50-μg plants would contain 245 μg, those on 100 μg plants would contain 385 μg, and those on 300 μg plants would contain 608 μg, etc. The second regression (Table 6B) indicated that the main cause of variation in the butterflies derives from the plant concentrations together with a significant contribution based on the concentrations in the plants fed upon in the different areas, i.e., the area effect is significant ($P < 0.013$), as is the plant $\times$ area interaction ($P < 0.001$). However, for the reasons given above, the effects of area appear of minor importance, and the sex of the butterflies appears not to effect the relationship ($P > 0.452$).

Blue Jay ED_{50} Bioassays

The results of the ED_{50} tests are shown in Table 7. The ED_{50}s per 100 g jay of the plants and butterflies are, respectively 0.165 and 0.086 g, i.e., the dried butterfly material is about 1.92 times more emetic than the dried plant

TABLE 6. LINEAR REGRESSION ANALYSES OF TOTAL CARDENOLIDE (μg) PER BUTTERFLY (Y AXIS, DEPENDENT VARIABLE) VS. CARDENOLIDE CONCENTRATIONS ($\log_{10}$ μg/0.1 g DRY WT) IN THEIR RESPECTIVE PLANTS, ACCORDING TO FUNCTION $Y = b\ (\log_{10} X) + a$

A. Overall regression pooling sex and geographic areas ($r^2 = 0.341$; type IV sum of squares)

Source of variation	*df*	*SS*	*MS*	*F*	*P*
Plant concentration	1	2052860	2052860	56.35	< 0.0001
Error	109	3971188	36433		
Corrected total	110	6024048			

Estimated value of parameters	SE	*T* for H = O	*P*
a = *Y* intercept = −547.63	116.8	−4.69	< 0.0001
b = slope = 466.46	62.1	7.51	< 0.0001

Equation for the line: $Y = 466.46\ (\log_{10} X) - 547.63$

B. Total cardenolide per butterfly on $\log_{10}$ plant concentrations by area, by sex, and for all interactions ($r^2 = 0.685$; type IV sum of squares)

Source of variation	*df*	*SS*	*MS*	*F*	*P*
Model	23	4124242	179315	8.21	< 0.0001
Error	87	1899806	21837		
Corrected total	110	6024048			
Log plant conc.	1	161777		7.41	< 0.008
Area	5	338173		3.10	< 0.013
Sex	1	12425		0.57	> 0.452
Log plant × area	5	518894		4.75	< 0.001
Log plant × sex	1	16441		0.75	> 0.387
Sex × area	5	239300		2.19	> 0.061
Log plant × sex × area	5	293468		2.69	< 0.027

material. The 95% confidence intervals do not overlap, so the difference is significant. Based on the spectroassay determinations of the cardenolide contents of the plant or butterfly material which was force fed to the blue jays, we calculated the emetic potencies of the gross mixture of cardenolides. For the plants and butterflies, these are, respectively, 5.62 and 5.25 ED_{50} units per mg of cardenolide per 84.25 g jay (Table 7), i.e., the butterfly cardenolides have about the same emetic potency (93%) as those of the plants. However, if we did in fact overestimate the plant cardenolide content (see analytical procedures), then the emetic potency of the plant material is greater than indicated above.

By combining the data on the mean and range in total cardenolide in the 111 *Asclepias speciosa*-reared butterflies (54–1279 μg, Table 4) with the calculated 190.6 μg of cardenolide per ED_{50} unit (Table 7), this sample of butterflies on emergence would contain an average of 1.67 and a range of 0.28–6.71 ED_{50} units per butterfly.

TABLE 7. CARDENOLIDE CONCENTRATIONS AND EMETIC POTENCIES TO BLUE JAYS OF POOLED DRY POWDERED *Asclepias speciosa*-REARED BUTTERFLIES AND PLANTS UPON WHICH THEY WERE REARED

Material tested	Absorbance[a]	Cardenolide concentration (μg/0.1 g dry wt)[b]	Emetic dose 50 data per 100 g blue jay			Blue jay emetic potencies[c]		Mean ED_{50} units per mg cardenolide
			Number of birds tested	ED_{50} (g)	95% confidence limits	Cardenolide (μg/ED_{50} unit)	Mean ED_{50} units per butterfly	
B'fly	0.423	263	10	0.086	0.078–0.094	190.6	1.67	5.25
Plant	0.211	128	8	0.165	0.149–0.184	177.9		5.62

[a]Standard spectroassay, see text.
[b]For the butterfly and plant material, the μg of cardenolide are equivalent to μg of digitoxin (see Brower et al., 1982).
[c]Adjusted for the mean of the mean male + the mean female cardenolide content (315 μg, from Table 4) and for the mean weight of blue jays (84.25 g, from Brower et al., 1982). The value of 84.25 g for the jays is based on a sample of 226 birds collected in the wild in Massachusetts (Brower and Moffitt, 1974).

TLC Cardenolide Profiles of Plants and Butterflies: Chloroform–Methanol–Formamide (CMF) System

Examples from two of the 10 TLC plates run in the CMF system are reproduced in Figure 4. Figure 4A shows three individual plants and the respective male butterflies reared thereon from area 3. Figure 4B shows three plants and the respective male butterflies from area 5. Table 8 presents the means and standard deviations for both the $R_{\text{digitoxin}}$ (R_d) and the spot intensity (SI) values for all the spots in the 58 plant and respective butterfly samples. Table 8 also gives each spot's probability of occurrence (PO), based on the proportion of plants or butterflies in which each spot occurred. Although several spots were frequently weak or absent in the individual plant or butterfly channels, the standard deviations of the R_d values of all spots in the plants are low, i.e., resolution of the cardenolides is good.

Figure 5 is based on the mean R_d and SI values from Table 8 and depicts the average cardenolide profile for the CMF system for the plants and butterflies representing most of the geographic range of *Asclepias speciosa* in California. The shapes and sizes of the spots are generalized in the figure. Twenty-four discernible cardenolides of varying intensities were resolved in this TLC system. Of these, 23 occurred in the plants and 21 occurred in the butterflies. Spot 7 did not occur in any of the plants and spots 17, 23, and 24 did not occur in any of the butterflies.

In Figure 5 the migration distance for the digitoxin standard (mm above the origin) is based on three spots each on nine of the 10 plates ($N = 27$, mean $R_f = 51.55$ mm, SD = 2.47 mm, range = 48.1–57.8 mm). Corresponding values for digitoxigenin (two spots on each of the 10 plates) are: $N = 20$; mean $R_f = 97.63$ mm, SD = 2.59 mm, range = 93.1–102.8 mm). The calculated R_d values for digitoxigenin are: mean = 1.89, standard deviation = 0.059, range = 1.78–2.02 ($N = 18$).

Plants. As pointed out in the methods section, the overall spot intensities in the plants frequently were less than anticipated from the spectroassay data. As a result, 15 of the 23 spots in the plants were frequently not detected on the TLC plates and had PO values of less than 0.40 (spots 1–6, 8, 9, 12, 14, 18, 20–22, and 24; Table 8). This leaves eight spots as particularly diagnostic: 10, 11, 13, 15–17, 19, and 23.

The 23 plant spots occur in three distinct R_f regions, those below digitoxin ($N = 15$, or 65%), those above digitoxin but below digitoxigenin ($N = 6$, or 26%), and those above digitoxigenin ($N = 2$, or 9%).

Butterflies. The SI values for the butterflies (Table 8) are generally greater than the corresponding plant SIs, which undoubtedly accounts for their higher PO values. Thus of the 21 spots, only 10 (numbers 1, 2, 5, 7, 12, 14, 18, and 20–22) have a PO of 0.40 or less. Of the remaining 11 spots (3, 4, 6, 8–11, 13, 15, 16, and 19), only spots 9 and 13 have SI values of less than 1.25.

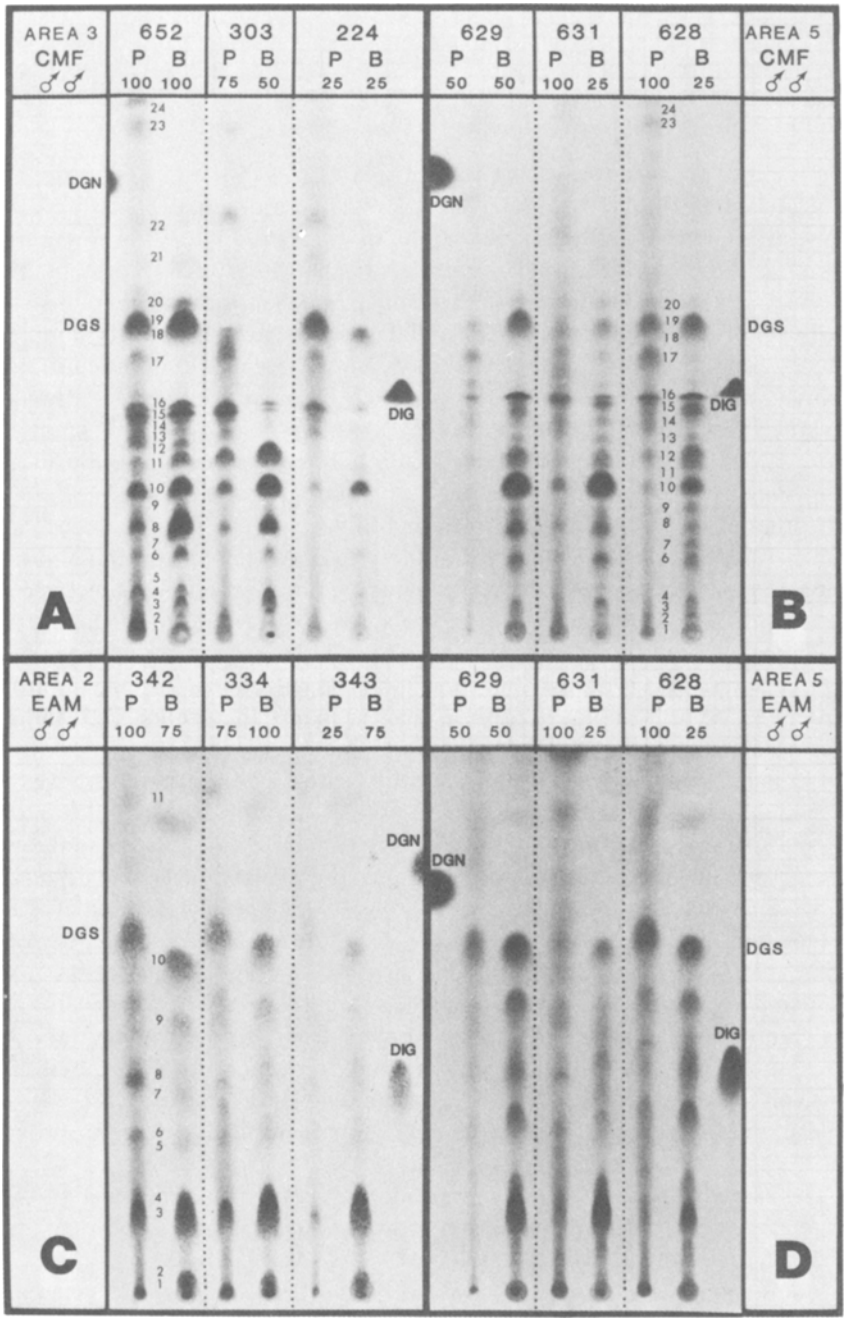

FIG. 4. Photographic reproduction of the thin-layer chromatographic profiles of the cardenolides stored by adult monarch butterflies from their *Asclepias speciosa* larval foodplants in California. Each of the four sections of the plate (A–D) shows the TLC profiles of three pairs of plants and corresponding individual monarch butterflies reared on these plants, as well as digitoxin (DIG), and digitoxigenin (DGN) as stan-

This leaves nine spots as particularly diagnostic of the butterfly cardenolide pattern: Nos. 3, 4, 6, 8, 10, 11, 15, 16, and 19. Of these, spot 19 occurs above and the remaining eight occur below the digitoxin standard.

As was true of *A. eriocarpa*, all the butterfly cardenolides occur in only two of the three TLC regions found in the plants: 16 (76%) of them have R_f values below digitoxin, five (24%) above the digitoxin, and none occurs above digitoxigenin. Overall, the butterflies are capable of storing cardenolides within approximately 76% of the R_d range of those found in the plants (= R_d spot 22/ R_d spot 24).

Plant–Butterfly Relationships: Effects of Sex, Geography, and Plate. As pointed out in our *A. eriocarpa* study, the effectiveness of cardenolide fingerprinting depends upon a high degree of correlation between R_d values of the respective cardenolides of the plants and butterflies. We utilized the SAS linear regression analysis in two steps to test simultaneously for the dependence of the R_d for all butterfly spots on the R_d of their respective plant spots, the effect of the six geographic areas, the effect of sex, the effect of plate-to-plate variation, and the interactions of plant R_d with plate, plant R_d with sex, and plant R_d with area as listed in Table 9. Because of the low PO values for many spots, this analysis could use only 259 of the 1160 possible spot pairs (58 plant–butterfly pairs × the 20 spots in common). The five most frequently occurring spot pairs (Nos. 10, 11, 13, 16, and 19) are evident in the footnote to Table 9. The same analyses were carried out for the SI values (Table 10).

$R_{digitoxin}$ *Values.* The results in Table 9A indicate a highly significant dependence of butterfly R_d on plant R_d ($P < 0.0001$, $r^2 = 0.945$) and that all other factors and their interactions are without significant effects. We therefore reran the test for the main effect alone (Table 9B) which gave virtually the same high degree of dependence of butterfly R_d on plant R_d ($r^2 = 0.943$). Thus the butterfly R_d values are very strongly dependent on those in the plants and are insignificantly affected by sex, area, or plate. The

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dards. Sections A and B were run in the chloroform–methanol–formamide system which separates a total of 24 spots. Twenty-three of these (all but spot 7) occur in the plants, and 21 (all but spots 17, 23, and 24) occur in the butterflies (see generalized drawing in Figure 5). Section A shows three male butterflies reared on three plants from area 3 in the northern Sierra Nevada. Section B shows three male butterflies reared on three plants from area 5 in the southern Sierra Nevada. Sections C and D were run in the ethylacetate–methanol TLC system which separates 11 cardenolides in both the plants and the butterflies. Section C shows three male butterflies reared on three plants from area 2 in the southern Cascades, and section D shows the same plant–butterfly pairs as in section B. Spot 19 in the CMF system and spot 10 in the EAM system correspond to desglucosyrioside. Evidence given in the discussion suggests that spot 22 (CMF) is uzarigenin, spot 16 (CMF) and spot 8 (EAM) is syriogenin, spot 23 and or 24 (CMF) is a mixture of labriformin and labriformidin, and spot 11 (EAM) is labriformidin.

TABLE 8. SUMMARY OF MEANS AND STANDARD DEVIATIONS FOR $R_{\text{DIGITOXIN}}$ VALUES AND SPOT INTENSITY VALUES FOR 24 CARDENOLIDES AND PROBABILITY OF THEIR OCCURRENCE IN 58 PAIRED PLANTS AND BUTTERFLIES[a]

Spot no.	Means				Standard deviations				Probability of spot		Subsample sizes	
	$R_{\text{digitoxin}}$ values		Spot intensity values		$R_{\text{digitoxin}}$ values		Spot intensity values					
	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly
24	2.24		1.33		0.102		0.577		0.06		4	
23	2.10		1.00		0.055		0.000		0.50		29	
22	1.70	1.70	1.00	1.00	0.055		0.000		0.33	0.02	19	1
21	1.59	1.56	1.00	1.00	0.028	0.000	0.000	0.000	0.03	0.02	2	1
20	1.35	1.35	1.00	1.29	0.012	0.039	0.000	0.611	0.05	0.24	3	14
19	1.19	1.24	1.79	1.97	0.102	0.054	0.820	1.090	0.57	0.50	33	29
18	1.18	1.18	1.24	1.14	0.033	0.031	0.452	0.378	0.21	0.12	12	7
17	1.08		1.38		0.057		0.554		0.55		32	
16	0.97	0.97	2.06	2.16	0.025	0.016	1.010	0.996	0.91	0.95	53	55
15	0.93	0.93	1.83	2.40	0.028	0.021	1.130	1.040	0.41	0.43	24	25
14	0.91	0.90	1.13	1.67	0.025	0.021	0.352	0.816	0.26	0.26	15	15
13	0.87	0.87	1.05	1.07	0.027	0.030	0.223	0.255	0.67	0.76	39	44
12	0.83	0.79	1.17	1.24	0.024	0.024	0.408	0.625	0.10	0.36	6	21
11	0.72	0.72	1.13	2.04	0.044	0.040	0.341	0.886	0.55	0.98	32	57
10	0.60	0.60	1.84	4.28	0.030	0.029	0.987	0.744	0.88	1.00	51	58
9	0.52	0.49	1.00	1.16	0.021	0.030	0.000	0.370	0.09	0.66	5	38
8	0.44	0.43	1.46	2.10	0.018	0.028	0.660	1.190	0.24	1.00	14	58
7		0.37		1.33		0.008		0.516		0.12		7
6	0.32	0.31	1.16	1.57	0.015	0.019	0.408	0.652	0.10	1.00	6	58
5	0.23	0.22	1.00	1.00	0.035	0.021	0.000	0.000	0.03	0.10	2	6
4	0.15	0.14	1.50	1.73	0.032	0.023	0.756	0.899	0.14	0.76	8	44
3	0.13	0.11	1.50	1.71	0.021	0.018	0.707	0.854	0.03	0.48	2	28
2	0.06	0.09	1.40	1.33	0.021	0.016	0.548	0.500	0.09	0.16	5	9
1	0.04	0.04	1.27	1.10	0.006	0.015	0.647	0.316	0.17	0.16	10	9

[a]Based on chromatograms of 58 plants and the corresponding butterflies reared thereon; chloroform–methanol–formamide TLC system.

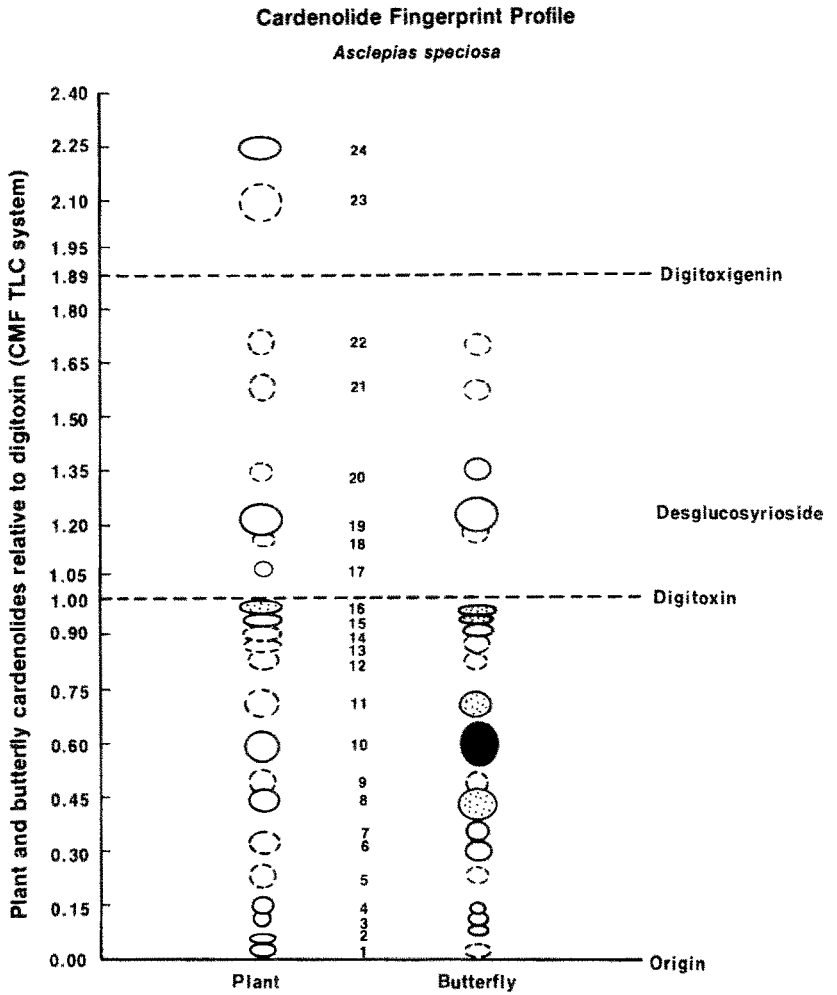


FIG. 5. The cardenolide fingerprint profile for freshly emerged monarch butterflies reared as larvae on *Asclepias speciosa* based on the CMF (chloroform–methanol–formamide) TLC system. The diagram shows mean $R_{digitoxin}$ and spot intensity values for each of the 23 plant and 21 butterfly cardenolides and is based on the data in Table 8 for 58 plants and respective butterflies (30 males, 28 females) reared thereon. The mean migration distance for digitoxin was 51.6 mm, for digitoxigenin 97.6 mm, and the mean digitoxigenin/digitoxin ratio was 1.89 (see text). Spot intensities are represented as follows: 1.00–1.25, dotted circle; >1.25–2.00, closed circle; >2.00–3.00, stippled circle; and >4.00–5.00, blackened circle. Nine spots (numbers 3, 4, 6, 8, 10, 11, 15, 16, and 19) have spot intensity values > 1.50, have probabilities of occurrence >0.40, and are particularly diagnostic of the cardenolide fingerprint profile of freshly emerged monarch butterflies which have fed on *Asclepias speciosa*. Spot 19 is desglucosyrioside and evidence given in the discussion suggests that spot 16 is syriogenin, spot 22 is uzarigenin, and spot 23 (or 24) is a mixture of labriformin and labriformidin.

TABLE 9. LINEAR REGRESSION ANALYSES OF $R_{DIGITOXIN}$ VALUES OF 20 BUTTERFLY CARDENOLIDES (Y) AS FUNCTION OF $R_{DIGITOXIN}$ VALUES OF RESPECTIVE PLANT CARDENOLIDES (X)[a]

A. Dependence of butterfly R_Ds on plant R_Ds as affected by the two sexes, by the six geographic areas, and by the 9 TLC plates ($r^2 = 0.945$)[b]

Source of variation	*df*	*SS*	*MS*	*F*	*P*
Model	21	19.2476	0.9165	196.7	< 0.0001
Error	237	1.1039	0.0047		
Corrected total	258	20.3515			
Plant R_D	1	19.1844		4118.6	< 0.0001
Area	5	0.0375		1.61	> 0.15
Sex	1	0.0023		0.49	> 0.48
Plate	4	0.0058		0.31	> 0.86
Plant $R_D \times$ area	5	0.0116		0.50	> 0.78
Plant $R_D \times$ sex	1	0.0013		0.28	> 0.59
Plant $R_D \times$ plate	4	0.0046		0.25	> 0.90

B. Dependence of butterfly R_Ds on plant R_Ds alone ($r^2 = 0.943$)[b]

Source of variation	*df*	*SS*	*MS*	*F*	*P*
Model	1	19.18	19.18	4224	< 0.0001
Error	257	1.17	0.005		
Corrected total	258	20.35			
Estimated value of parameters		SE	*T* for H = O		*P*
a = *Y* intercept = 0.0057		0.013	0.45		> 0.65
b = slope = 0.995		0.015	65.00		< 0.0001

Equation for the line: $y = 0.995x + 0.0057$

[a]Visualized in the chloroform–methanol–formamide TLC system in 58 samples of plants and respective butterflies reared thereon. The regressions are based on 259 plant–butterfly spot pairs as follows: spot 1 = 2 pairs; 2 = 2; 3 = 1; 4 = 8; 6 = 6; 8 = 14; 9 = 4; 10 = 51; 11 = 32; 12 = 3; 13 = 33; 14 = 7; 15 = 18; 16 = 52; 18 = 4; 19 = 21; 20 = 1.

[b]Type I sum of squares.

virtual one-to-one correspondence of the R_d values is indicated by the estimated regression equation $Y = 0.995X + 0.0057$ (Table 9B).

SI Values. The butterfly spot intensities showed considerably less correspondence with the plants than did the R_d values. The low r^2 (0.191) for the first test (Table 10A) indicates a weak (but statistically significant) correspondence of butterfly SIs on their respective plant SIs ($P < 0.001$) as well as significant effects of both area ($P < 0.028$) and sex ($P < 0.007$). The dependence of butterfly SIs solely on plant SIs (Table 10B) is also weak ($r^2 = 0.087$). At low SI values, the butterflies have SI values about twice those of their plants, and at maximal, SI values are about equal to them ($Y = 0.44X + 1.81$, Table 10B).

The A. speciosa *CMF Fingerprint Profile.* As summarized in Figure 5 and Table 8, the butterflies contain 21 of the 23 cardenolides present in the *A.*

TABLE 10. LINEAR REGRESSION ANALYSES OF SPOT INTENSITY VALUES OF 17 BUTTERFLY CARDENOLIDES (Y) AS FUNCTION OF SPOT INTENSITY VALUES OF RESPECTIVE PLANT CARDENOLIDES (X)[a]

A. Dependence of butterfly SIs on plant SIs as affected by the two sexes, by the six geographic areas, and by the 9 TLC plates ($r^2 = 0.147$)[b]

Source of variation	*df*	*SS*	*MS*	*F*	*P*
Model	21	85.42	4.068	2.67	< 0.0002
Error	237	361.21	1.524		
Corrected total	258	446.63			
Plant intensity	1	39.02		25.61	< 0.0001
Area	5	19.40		2.55	< 0.029
Sex	1	11.51		7.55	> 0.007
Plate	4	1.65		0.27	> 0.89
Plant intensity × area	5	8.76		1.15	> 0.33
Plant intensity × sex	1	0.24		0.16	< 0.68
Plant intensity × plate	4	4.81		0.79	> 0.53

B. Dependence of butterfly SIs on plant SIs alone ($r^2 = 0.087$)[b]

Source of variation	*df*	*SS*	*MS*	*F*	*P*
Model	1	39.03	39.03	24.61	< 0.0001
Error	257	407.61	1.59		
Corrected total	258	446.63			
Estimated value of parameters		SE	*T* for H = O		*P*
$a = y$ intercept = 1.809		0.164	11.06		< 0.0001
b = slope = 0.440		0.089	4.96		< 0.0001

Equation for the line: $y = 0.440x + 1.809$

[a]Visualized in the chloroform-methanol-formamide TLC sytem in 58 samples of plants and respective butterflies reared thereon. The regression is based on the same plant-butterfly spot pairs as in Table 9.
[b]Type I sum of squares.

speciosa plants as resolved in the CMF solvent system. In nine of these (numbers 3, 4, 6, 8, 10, 11, 15, 16, and 19) spot intensity values are >1.50, and probabilities of occurrence are >0.40). These nine spots therefore are particularly diagnostic of the cardenolide fingerprint profile of freshly emerged monarch butterflies which have fed on *A. speciosa*.

TLC Cardenolide Profiles of Plants and Butterflies: Ethyl Acetate–Methanol (EAM) System

As in the *A. eriocarpa* study, resolution of fewer cardenolides occurred in this TLC system, and we therefore did not quantitatively analyze the data as in Tables 8–10. Examples from two of the 10 plates are reproduced in Figure 4. Eleven spots in the plants and 10 in the butterflies are resolved in this system. Of these, spots 3, 4, 5, 9, and 10 were the ones most frequently present and spot

10 (desglucosyrioside, see discussion) occurred in all 58 samples. Spot 11 did not occur in the butterflies. As was found in the CMF system, several spots of lower R_f than desglucosyrioside, and especially those below digitoxin, were more concentrated in the butterflies than in the plants. This was most notable for spots 3 and 4 which probably correspond to spots 8 and/or 10 in the CMF system. No major differences occurred in the TLC profiles of the 58 plant–butterfly pairs either in the sexes of the butterflies or in the six geographic areas.

Conclusion from both TLC Systems

The TLC evidence suggests that the majority of cardenolides stored by the butterflies are chemically identical to those in the leaves of *A. speciosa* plants. In both systems, the butterflies showed a general enrichment of cardenolide spots which could simply reflect the quantitatively higher concentrations of the cardenolides in the butterflies, or it could result from metabolic transformations of some of the leaf cardenolides during larval feeding or during metamorphosis. For example, spots 23 and 24 of the CMF system occur in the plants but not in the butterflies and could have been transformed and stored as one or more of the other spots. Likewise, spot 7 of the CMF system occurs in the butterflies but not the plants, and spot 17 occurs in the plants, but not in the butterflies. However, it is doubtful that metabolism to storable forms is quantitatively as significant in monarchs reared on *A. speciosa* as in monarchs reared on *A. eriocarpa* (see Discussion).

As established in the *A. eriocarpa* study, the cardenolide profiles in both TLC systems are highly consistent in the plants, both sexes of butterflies, and throughout a wide geographic range in California. However, once again, we emphasize that this *Asclepias speciosa* cardenolide fingerprint profile is that of freshly eclosed monarchs and could change during their normal life-spans (as long as 9–10 months; Brower and Calvert, in preparation).

DISCUSSION

Quantitative Variation of Cardenolides in Plants and Butterflies

As was found in the *A. eriocarpa* study, large variation occurred in the gross cardenolide concentrations of the 111 *A. speciosa* plants (19–344 μg/0.1 g), in the butterflies reared on these plants (41–574 μg/0.1 g), and in the total cardenolide per butterfly (54–1279 μg). However, the leaves of *A. speciosa* have only about one fifth the mean cardenolide concentration of the leaves of *A. eriocarpa*. This difference is reflected in the butterflies which have a mean cardenolide concentration of only 56% and a mean total cardenolide content of only 52% of that found in the *A. eriocarpa*-reared butterflies. Another

important difference is that when reared on *A. speciosa*, the butterflies increase both the mean and the variance of their cardenolide concentrations compared to their plants, whereas they decrease both when reared on *A. eriocarpa*. In other words, the shapes of the plant and butterfly histograms are reversed in the two studies (compare Figure 2 in the present study with Figure 3 in the *A. eriocarpa* study).

Overall geographic variation in concentrations of the *A. speciosa* plants is relatively minor as was the case in the *A. eriocarpa* study. An overall trend of high concentrations in the butterflies to the north, intermediate concentrations in the Sierras, and low concentrations in the Owen's River Valley appears statistically significant, but, as in the *A. eriocarpa* study, of minor biological importance.

Higher mean cardenolide concentrations in females compared to males occurred in monarchs reared on *Asclepias eriocarpa* (Brower et al., 1982), in monarchs reared on several other *Asclepias* species (Brower and Glazier, 1975; Brower, et al., 1975), and in monarchs collected from several wild United States populations (Brower et al., 1972; Brower and Moffitt, 1974). The lack of a sexual difference in the present study may be explained by the fact that the average concentrations of the plants on which the females were reared were lower in five of the six areas than were the average concentrations of the plants on which the males were reared (Table 1). Curiously, in each of the six areas the number of males exceeds the number of females such that for all six areas there is a significant excess of males (59.5%; X_1^2, $df = 3.97$, $P < 0.05$). We have not previously found a significant difference of this nature.

Relationship of Cardenolide Concentrations, Total Cardenolide, and Dry Weights of Butterflies to Cardenolide Concentrations in Plants

The butterflies are able to regulate their uptake of cardenolides from *A. speciosa*. Thus both cardenolide concentrations and total cardenolide in the butterflies are proportional to the logarithm of the cardenolide concentrations in the plants, i.e., the butterflies can significantly increase their cardenolide contents when feeding on low concentration plants and reach an upper capacity when reared on plants containing high concentrations. We did not find this logarithm relationship in our *A. eriocarpa* study because even those individual plants with the lowest concentrations were so high in content that the resulting data plot represented only the flattened out portion of the logarithmic curve and therefore appeared as a straight line with a slope that did not differ significantly from zero [compare Figure 3 in this study with Figure 4 in Brower et al. (1982)].

Regression analyses also determined that the major contribution to variation in concentration and total cardenolide in the butterflies is dependent

on the concentrations in the plants with only minor differences resulting either from differences in plant concentrations in the different geographic area or from the sex of the butterflies.

The average dry weight of the males in this study was 1.10 times that of the females. Lighter females also characterized the *A. eriocarpa* study (the male-female ratio was 1.14), as well as wild populations of overwintering butterflies along the California Coast (Brower et al., 1982; Brower and Moffitt, 1974; Tuskes and Brower, 1978). Dry weight showed a weak positive correlation to plant cardenolide concentration without significant effects of sex or area. It seems possible that plants which have higher concentration of cardenolides may also contain more nutrients or be generally healthier, thereby producing heavier butterflies. Alternatively, higher concentrations of cardenolides in the plants might stimulate growth in the butterflies. In any case, our *A. speciosa* data provide no evidence for a metabolic cost of cardenolide incorporation.

Differences in Emetic Potencies of Cardenolides of A. speciosa *and* A. eriocarpa.

The emetic potency of monarchs reared on *A. speciosa* was 190.6 μg of cardenolide per ED_{50} unit. The corresponding value for *A. eriocarpa*-reared butterflies was 37.5 μg. Expressing emetic potency as the number of ED_{50} units per mg of cardenolide, values for the cardenolides in the *A. speciosa* plants and butterflies are, respectively, 5.62 and 5.25. (Table 7). Corresponding values for *A. eriocarpa* are 17.2 and 26.7 (see Table 5 in Brower et al., 1982). In other words, the gross mixture of cardenolides in *A. eriocarpa* plants is 3.1 times more emetic than in *A. speciosa* plants, and the cardenolide mixture in the *A. eriocarpa* butterflies is 5.1 times more emetic than in the *A. speciosa* butterflies. However, if the spectroassay has resulted in a 30% overestimation of *A. speciosa* cardenolide concentration, then the emetic potency of the plants is greater than 5.62 ED_{50} units per mg of cardenolide and the differences in emetic potency of *A. eriocarpa* and *A. speciosa* would be reduced.

The *A. speciosa* butterfly material is 0.93 as emetic as the corresponding plant material (5.25/5.62). This near equality undoubtedly reflects the very similar cardenolide profiles of the *A. speciosa* plants and butterflies. In contrast, the cardenolides in the *A. eriocarpa*-reared butterflies were 1.55 times more emetic than those in their plants. This difference undoubtedly results from the different plant and butterfly TLC profiles characteristic of *A. eriocarpa*-reared monarchs. Furthermore, these differences in profile are amplified by metabolic conversion of high to low R_f cardenolides in butterflies which feed on *A. eriocarpa* (see below). Thus monarchs which have fed on *A. eriocarpa* differentially store and/or metabolically manipulate the mixture of

cardenolides in the plant to produce greater emetic potency, whereas monarchs reared on *A. speciosa* do not.

This sample of *Asclepias speciosa*-reared butterflies on emergence contained an average of 1.67 ED_{50} units and a range of 0.28–6.7 units per butterfly. In contrast, the *Asclepias eriocarpa*-reared butterflies contained an average of 16.5 units and a range of 5.9–26.6 units per butterfly. The overall mean number of ED_{50} units per *A. speciosa* butterflies is therefore only 10% (range = 5–25%) that of the *A. eriocarpa* butterflies. About 80% of this 10-fold difference is due to the fact that the cardenolides in the *A. speciosa* butterflies had only one fifth the emetic potency of the *A. eriocarpa* butterflies (i.e., 5.25 vs. 26.7 ED_{50} units/mg cardenolide). The remaining 20% of the difference is attributable to the lower cardenolide concentrations in and lighter weights of the *A. speciosa*-reared butterflies.

Thus monarchs which feed upon *A. speciosa* will be substantially less well protected from vertebrate predators than if they feed upon *A. eriocarpa*. The implications of feeding on milkweeks containing cardenolides of low emetic potency in the natural environment may be very great (see Ecological Predictions).

Emetic Potency and Cardenolide Polarity

As reviewed in Roeske et al. (1976), high polarity cardenolides (i.e., those of low R_f) appear to be absorbed poorly by the intestinal mucosa of vertebrates and consequently have low emetic potencies. This was supported by their experiments which demonstrated substantially higher emetic potencies of monarchs reared on *A. curassavica* compared to those reared on *Gomphocarpus sp.* (=*G. physocarpus* Mey; Brower et al., 1982). Although these two have similar TLC profiles, *G. physocarpus* has quantitatively higher amounts of more polar as well as structurally different cardenolides from those in *A. curassavica* (Roeske et al., 1976; Seiber et al., 1983). Brower et al. (1982) also adduced evidence that in *A. eriocarpa* cardenolides of intermediate polarity were more emetic than those of lowest polarity. Specifically, their evidence strongly suggested that the relatively high concentration of desglucosyrioside in the butterflies accounted for their greater emeticity compared to the plants which contained a higher proportion of the less polar labriformin and labriformidin.

The lower emetic potency of the *A. speciosa* material is consistent with this explanation in that this plant is characterized by an array of more polar cardenolides in higher relative concentrations compared to those in *A. eriocarpa*. An alternative explanation is that emeticity is somehow masked by noncardenolide material in the plants, owing in part to the relatively low concentrations of cardenolides in *A. speciosa*. However, it would be quite coincidental if this masking material were also sequestered by the butterflies;

in fact, the butterflies have virtually the same low emetic potency as the plants. A more complete understanding of the cardenolide-caused differences in the emeticity requires the isolation and purification of individual cardenolides of differing R_d values which are then force fed to the blue jays as done in our *A. eriocarpa* study.

Chemistry of the Cardenolides in Asclepias speciosa

Previous research, including the cospotting of desglucosyrioside as a standard under several TLC solvent conditions, has established for *A. speciosa* that spot 19 of the CMF system and spot 10 of the EAM system is desglucosyrioside (Seiber et al., 1982; Seiber and Nelson, unpublished data). Coincidence of TLC R_f value is the only direct evidence we presently have to support the chemical identity of the cardenolides below the R_f of digitoxin. In fact, spots 1–6 and 8–16 of the CMF system and spots 1–10 of the EAM system are all present in both the leaves and the butterflies. Storage per se, rather than metabolic conversion and storgae, thus appears to predominate in monarchs reared on this milkweed.

As pointed out elsewhere (Seiber et al., 1978; Seiber et al., 1982; Seiber et al., 1984), desglucosyrioside has been identified in at least four other milkweeds including *A. eriocarpa, A. syriaca, A. erosa* Torr., and *A. labriformis* Jones. Consideration of the known cardenolides in *A. eriocarpa* and *A. syriaca* therefore bears importantly on the cardenolide chemistry of *A. speciosa*, even though their common cardenolide chemistry cuts across Woodson's (1954) taxonomic subdivisions within the subgenus *Asclepias* (*A. speciosa* is in series 6: Purpurascentes; *A. syriaca* is in series 5: Syriacae; and the other three are in series 8: Roseae).

Analogy with Asclepias eriocarpa. Considerable progress has been made in cardenolide characterization of *A. eriocarpa* in which desglucosyrioside occurs along with two less polar chemical relatives, labriformin and labriformidin (Brower et al., 1982; Nelson et al., 1981; Seiber et al., 1983). All three are unusual 2, 3-dihydroxy cardenolides with a cyclic glycoside bridge and epoxide functionality at carbons 7 and 8 (Brown et al., 1979; Cheung et al., 1980; Cheung and Watson, 1980). We have established that monarchs are not able to store labriformin and labriformidin directly, but rather metabolize labriformin to labriformidin and labriformidin to desglucosyrioside which they then store (Seiber et al., 1980; Nelson et al., 1981; Brower, et al., 1982).

Whereas desglucosyrioside is the principal cardenolide monarchs store when fed *A. eriocarpa* (spot 13, CMF, Figure 6 in the *A. eriocarpa* study), they store it to a substantially lesser degree from *A. speciosa* (spot 19 CMF, Figure 5, present study). There are at least three reasons for this difference: first, the comparative TLC evidence indicates that the amount of desglucosyrioside in

A. speciosa leaves is much less than in *A. eriocarpa.* Secondly, labriformin and labriformidin occur in large quantities in the leaves of *A. eriocarpa*, so that a surfeit of precursor cardenolides is available for metabolic conversion to desglucosyrioside. Thirdly, *A. eriocarpa* has an additional reservoir of these three cardenolides in its latex (Nelson et al., 1981), whereas the latex of *A. speciosa* lacks or contains immeasurable amounts of cardenolide (Seiber et al., 1982).

Because desglucosyrioside does occur in small quantities in the leaves of *A. speciosa* and because it is derivable from labriformin and labriformidin, it seems possible that spot 23 and/or 24 in the CMF system may represent a mixture of small amounts of these compounds. By similar reasoning, spot 11 in the EAM system may be labriformidin (compare Figures 4C and 4D in the present study with Figures 5C and 5D in the *A. eriocarpa* study). However, the low intensities and infrequent occurrence of spots 23 and 24 and 11 in the respective TLC systems preclude a definitive statement for the existence of labriformin and/or labriformidin in our *A. speciosa* leaf samples. Moreover, it is clear that if these two cardenolides do occur in *A. speciosa* plant tissue, they are very minor components, and they definitely are not present in *A. speciosa*-reared butterflies.

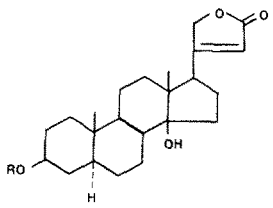
We have separately determined (unpublished data) that spot 22 of *A. speciosa* (CMF system) coincided with the R_f of a cospotted uzarigenin standard, and Seiber and Lee (unpublished data) have recently isolated small amounts of uzarigenin from aerial portions (mostly leaves and some stem material) of *A. speciosa* collected in the summer of 1982 at the Sierraville locality (see area 3, Methods and Materials). Nelson et al. (1981) found that uzarigenin occurs in the stems but not leaves of *A. eriocarpa.* Thus the fact that the plant samples in this study consisted mostly of leaves may explain the low spot intensity and only moderate probability of occurrence of presumed uzarigenin in *A. speciosa.*

From their similar R_f values in the CMF TLC system, it is likely that spots 3 and 4 of both *A. eriocarpa* and *A. speciosa* are the same cardenolides, although chemical confirmation of this is needed.

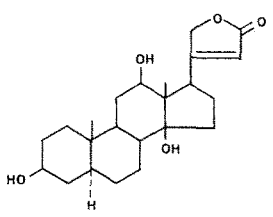
Analogy with A. syriaca. Because of its early introduction from North American to the Old World, *Asclepias syriaca* has been the subject of many investigations which have shown it to vary in cardenolide content from immeasurable to large amounts (review in Roeske et al., 1976). Recent studies also indicate variable numbers of cardenolides in different North American populations. Thus Duffey and Scudder (1972) found 15 cardenolides in the seeds and five of intermediate to high polarity in leaves from Ontario; Rothschild et al. (1975) reported only one of high polarity in a leaf sample from New York; while Brower (1984) and Brower, Seiber, and Nelson (in preparation) separated 12 across a moderate polarity range in monarch adults reared on leaves in western Massachusetts.

Considerable progress has been made on chemical characterizations of the cardenolides in *A. syriaca*. Bauer et al. (1961), Masler et al. (1962a,b), and Petricic (1967) isolated low yields of five cardenolides from aerial parts of the plant: uzarigenin, desglucouzarin, syriogenin, syriobioside, and syrioside. Mitsuhashi et al. (1970) also found xysmalogenin (5,6-dehydrouzarigenin) after acid hydrolysis of material of Japanese origin, while Brown et al. (1979) isolated syrioside, syriobioside, a glucosyl derivative of syriobioside named dihydrosyrioside, and most importantly, small amounts of desglucosyrioside from roots grown in Basle. The structures of these compounds are indicated in Figure 6.

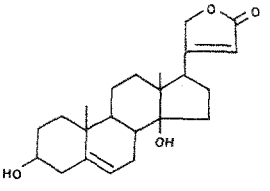
Reasoning from the common chemical link represented by desglucosyrioside, we speculate that *A. speciosa* contains other cardenolides in common with *A. syriaca*. The R_f values of spot 16 (CMF system) and spot 8



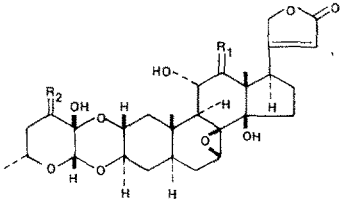
	R
Desglucouzarin	glu
Uzarigenin	H



Syriogenin



Xysmalogenin



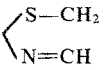
	R_1	R_2
Desglucosyrioside	O	α-R, β-OH
Dihydrosyrioside (hypothetical)	α-OH, β-H	α-H, β-O-glu
Labriformidin	O	O
Labriformin	O	
Syrioside	O	α-H, β-O-glu
Syriobioside	α-OH, β-H	α-H, β-OH

FIG. 6. Structures of cardenolides present or inferred in *Asclepias syriaca* (desglucosyrioside, desglucouzarin, dihydrosyrioside, syriobioside, syriogenin, syrioside, uzarigenin, xysmalogenin) and in *A. eriocarpa* and *A. speciosa* (desglucosyrioside, labriformidin, labriformin, and uzarigenin). For further details, see discussion.

(EAM system) coincided with a syriogenin standard cospotted with *A. speciosa* plant extracts. However, this cardenolide has not been isolated from *A. speciosa* for confirmation. More polar spots in the R_f region (CMF system) characteristic of syrioside and uzarin (spots 2–4) and syriobioside (spots 10,11) were present in both our *A. speciosa* plant and butterfly samples, but more definitive evidence (coinciding R_f values in the EAM system and chemical isolations for proof of identities) for these specific compounds is lacking.

Conclusions on Chemistry of Cardenolides in Asclepias speciosa. Our *A. speciosa* plant samples definitely contained desglucosyrioside, probably contain small amounts of labriformin and labriformidin, as well as uzarigenin, syriogenin, and possibly other polar cardenolides with R_f values lower than digitoxin. We speculate that these very polar cardenolides are the same derivatives of desglucosyrioside and uzarigenin as reported in *A. syriaca* by other workers (Brown et al., 1979; Bauer et al., 1961; Masler et al., 1962a,b; Petricic, 1967).

Ecological Predictions Based on A. speciosa *and* A. syriaca

Asclepias speciosa occurs widely in the western United States and the eastward limit of its distribution overlaps the western edge of the distribution of *Asclepias syriaca* L. (Woodson, 1954). *A. syriaca* has a nearly identical latitudinal distribution, ranging to the Atlantic Coast from North Carolina to New Brunswick. Although the two species are placed in separate taxonomic series by Woodson (1954), they hybridize in their zone of geographical overlap (Woodson, 1954) and are ecologically similar in many respects. Both have the northernmost distribution of all the North American *Asclepias*, both occur commonly as weeds, and both have extended their ranges and abundances due to agricultural and other human activities (Woodson, 1954) as have many native North American plants (e.g., see Shapiro, 1974). As a consequence, *Asclepias syriaca* and *A. speciosa* may have become the major foodplants of the monarch butterfly in North America (Urquhart, 1960; Urquhart and Urquhart, 1979, 1980; Fink and Brower, 1981).

Extensive bird predation occurs upon monarch butterflies in their overwintering colonies in Mexico (Calvert et al., 1979; Fink et al., 1983; Brower (1984) in contrast to the overwintering colonies in California (Brower, personal observations). Fink and Brower (1981) proposed that this may be a consequence of the historical changes in the eastern North American milkweed flora producing a decline in the emetic potencies in the overwintering monarch populations. Evidence supporting this important ecological possibility has emerged from TLC fingerprinting analyses (CMF system) of individual monarch butterflies collected in Michoacan, Mexico, at their overwintering sites. It strongly appears that CMF spots 8, 10, and 11, as

well as several of the other more polar cardenolides of *A. speciosa* (and/or *A. syriaca*), occur in a majority of these butterflies (Brower, Seiber, and Nelson, in preparation). Further research into the emetic potencies of cardenolides in the precolonial milkweed flora of the United States and Canada thus appears an exciting area for future study.

Comparison of Fingerprint Profiles of A. speciosa *and* A. eriocarpa

Twenty cardenolides were resolved in the *A. eriocarpa* plants and/or butterflies in the CMF system, whereas in *A. speciosa* 24 are resolved. Butterflies reared on *A. eriocarpa* store 16 cardenolides, and butterflies reared on *A. speciosa* store 21. In both TLC systems, the butterflies stored no spots above digitoxigenin, and in the CMF system the ratio of the R_d range of the spots in the butterflies divided by that of the plants is similar, i.e., 62% in *A. eriocarpa* and 76% in *A. speciosa*.

The overall similarity in cardenolide TLC profiles in the leaf–butterfly pairs in the EAM system is more striking for *A. speciosa* than for *A. eriocarpa*. This is undoubedly due to the low quantities or lack of labriformin and labriformidin in *A. speciosa* leaves and latex as indicated above.

Comparison of the TLC fingerprint profiles (CMF system) of butterflies reared on the two plants reveals strikingly different patterns [(compare Figure 5 of this paper with Figure 6 in Brower et al. (1982)]. Major differences are the greater number of polar cardenolides below digitoxin (16 in *A. speciosa* vs. 11 in *A. eriocarpa*) and the very prominent spot 10 at $R_d = 0.60$ in the *A. speciosa* butterflies. In contrast, the *A. eriocarpa*-reared butterflies have two prominant spots (spots 13 and 14) above the digitoxin line and three prominent spots (spots 4, 8, and 9) below it. These latter three spots of the *A. eriocarpa* butterflies conspicuously straddle spot 10 of the *A. speciosa* butterflies.

Distinction of wild captured butterflies which fed as larvae on these two plants should be simple and clear-cut in the CMF TLC system if no major changes in cardenolide composition occur during the aging of adult monarchs. This bodes auspicously for the utilization of cardenolide fingerprints of wild-captured monarch butterflies to determine the milkweed foodplants that they, as larvae, consumed in the natural environments. This is especially so in California where both *A. speciosa* and *A. eriocarpa* are abundant and widely distributed milkweeds. Our results thus indicate that the profiles of insect-stored allelochemics can predictably mirror those present in their foodplants, thereby refuting a recent conclusion by Blum (1981, p. 451) "that the cardenolide sequestration pattern exhibited by the adult may in no way mirror the cardenolide content of its host plant" (see also Blum, 1983).

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(*Z, E*)-α-FARNESENE: MAJOR COMPONENT OF SECRETION FROM METATHORACIC SCENT GLAND OF COTTON SEED BUG, *Oxycarenus hyalinipennis* (COSTA) (HETEROPTERA; Lygaeidae)

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Abstract—The isolation and identification of (*Z,E*)-α-farnesene [(3*Z*, 6*E*)-3,7,11-trimethyldodeca-1,3,6,10-tetraene] (III) as the major component of the secretion from the metathoracic scent gland of the cotton seed bug, *Oxycarenus hyalinipennis*, is reported. The compound was identified by a combination of [^{1}H] NMR, [^{13}C] NMR, UV, and mass spectral data and by comparison with a synthetic sample, prepared by dehydration of (*E*)-nerolidol. (*Z,E*)-α-Farnesene (III) has been recorded previously in insects (ants and termites), but this is the first report of its occurrence in a member of the Heteroptera. (*E*)-2-Octenyl acetate (XIII) and 2-octenal (XIV) were identified as minor components of the secretion. In addition, three minor monoterpenoid and two other sesquiterpenoid components were detected and tentatively identified.

Key Words—*Oxycarenus hyalinipennis*, cotton seed bug, metathoracic scent gland, (*Z,E*)-α-farnesene, (*E*)-2-octenyl acetate, 2-octenal, isoprenoids.

INTRODUCTION

The occurrence of an unidentified sesquiterpenoid as the major component of the secretion from the metathoracic scent gland in the cotton seed bug, *Oxycarenus hyalinipennis* (Costa), has recently been reported (Olagbemiro

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and Staddon, 1983). The isolation and identification of this component of the secretion as (*Z,E*)-α-farnesene (III) is reported in this paper. (*Z,E*)-α-Farnesene has been recorded previously in insects (ants and termites: Morgan and Wadhams, 1972; Morgan et al., 1979; Vander Meer et al., 1981, Cammaerts et al., 1981; Attygalle et al., 1982, 1983; Naya et al., 1982), but not in any members of the Heteroptera (Staddon, 1979; Blum, 1981; Pasteels et al., 1983). Clues to the origin and biological significance of the farnesene in *O. hyalinipennis* adults are also reported.

METHODS AND MATERIALS

O. hyalinipennis was maintained in laboratory culture at 26° C, under a 14 light–10 dark photophase on dry cotton seeds and water. Glandular samples were isolated by dissection under 200mM NaCl. Steam distillation of bulk samples of adult material was carried out in all-glass apparatus. The oily distillate was extracted with redistilled diethyl ether, the organic solution dried with magnesium sulfate, and stored under nitrogen at −20° C. Column chromatography was performed using Kieselguhr G and fractions were analyzed by TLC using Polygram Sil G/UV_{254} silica gel plates, visualized by potassium permaganate spray. Hydrogenations were performed using 10% palladium on carbon catalyst in ethanol and under 1 atmosphere of hydrogen.

Gas chromatography (GC) was performed with a Varian 1440 gas chromatograph equipped with a flame ionization detector. Separations were effected on a 2m × 2 mm ID glass column packed with 3% OV-225 on 60–80 mesh Gas Chrom Q with the injector at 150° C, the detector at 230° C, and a flow rate of 20 or 30 ml of nitrogen per minute. Isothermal runs were carried out at 110° C while programed runs were carried out between 70° C and 200° C with a temperature increase of 6° C/min. Pentadecane was used as external standard for the standardization of peak area responses. Separations for combined gas chromatography–mass spectrometry (GC-MS) were similarly achieved using a 2 m × 2 mm ID glass column packed with 3% OV-225 on 100–120 mesh Gas Chrom Q. The mass spectra were recorded on a 7070 VG Micromass mass spectrometer operating at 70 eV with the ion source at 105° C, the separator at 180° C, and a 200-μA ionizing current. Glandular samples were injected by an open column procedure (Staddon et al., 1979).

The ultraviolet (UV) absorption spectra were recorded on ethanol solutions with a Pye Unicam SP 8 400 UV/VIS spectrophotometer, and the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra on samples in $CDCl_3$ using a Bruker WM 250 instrument operating at 250 MHz.

RESULTS

Steam distillation of ca. 9 g of *Oxycarenus* adult material (4000–5000 individuals) followed by column chromatography using 2% diethyl ether in *n*-pentane as eluant yielded four fractions: 15 mg consisting of the major sesquiterpene and some very minor sesquiterpenes (R_f-0.75); 1 mg of 2-octenyl acetate (XIII) (R_f-0.2); 0.7 mg of 2-octenal (XIV) (R_f-0.15), and 1 mg of a mixture of monoterpenes (R_f-0.8). The R_f values were determined by TLC using 2% diethyl ether in *n*-pentane. These data are in good agreement with previous findings on the composition of the oil from the metathoracic scent gland in *O. hyalinipennis* (Olagbemiro and Staddon, 1983).

Sesquiterpene. The molecular formula is $C_{15}H_{24}$ (Olagbemiro and Staddon, 1983). Hydrogenation added four molecules of hydrogen ($M^+204 \rightarrow M^+212$), thus indicating the presence of four double bonds. The hydrogenated product was found to be identical with authentic farnesane (2,6,10-trimethyldodecane, Aldrich), thus indicating that the natural material was a farnesene.

The farnesenes (I–VI) (Figure 1) can be grouped into pairs using their UV spectral data (Anet, 1970), as follows: λ_{max} 224 nm, β-farnesenes (I) and

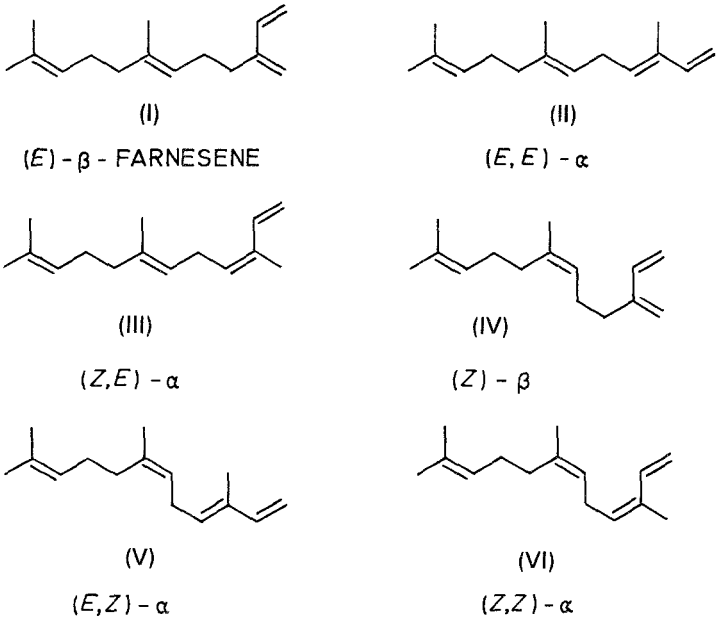


FIG. 1.

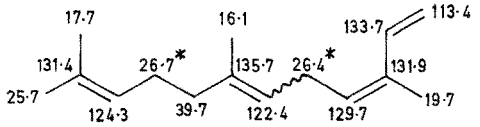
(IV); λ_{max} 232–233 nm, (*E*,-)-α-farnesenes (II) and (V); λ_{max} 237–238 nm, (*Z*,-)-α-farnesenes (III) and (VI). The UV spectrum of the *Oxycarenus* farnesene showed λ_{max} 237 nm, (ε19,000) indicating the presence of a (*Z*)-1,3-butadiene unit as in (III) or (VI). The extinction coefficient is lower than that quoted for (VI) (ε22,500) and unfortunately cannot be compared with (III), because the literature value (ε11,300) is apparently in error (Anet, 1970; footnote to Table 1).

A [^{1}H] NMR spectrum of the unknown from *Oxycarenus* showed the following resonances: 1.60 (CH_3·C:), 1.63 (CH_3·C:), 1.68 (d, *J*-0.9 Hz, CH_3·C:), 1.82 [d, *J*-1.2 Hz, CH_3·C:(CH:CH_2)], 1.94–2.14 (m, 2× CH_2), 2.87 (bt, *J-ca.* 7.4 Hz, :CH·CH_2·CH:), 5.05–5.15 (m, 3×:CH), 5.20 (d, *J* 17.3 Hz, CH:C$H_t$$H_c$), 5.36 (bt, *J-ca.* 7.5 Hz, :CH·CH_2·C*H*:), and 6.81 (ddd, *J*-17.3, 10.8, and 0.7 Hz, C*H*:CH_2). These and other data derived from appropriate decoupling experiments clearly indicated that the natural material was an α-farnesene. The signal at δ6.81 for H-2 is closely similar to the values quoted for (III) and (VI) (δ6.74 and 6.77, respectively) and contrasts with the corresponding resonances in (II) and (V) (δ6.30 and 6.35) (Anet, 1970).

A [^{13}C] NMR spectrum of the natural material showed all the 15 resonances expected; the assignments shown in Figure 2 were deduced using off-resonance [^{13}C] NMR data and by comparison with data for the (*E*)- and (*Z*)-farnesols (VII) and (VIII) (Crombie et al., 1975). The assignments in the unknown farnesene are also in excellent agreement with those made for a series of related furanosesquiterpenes (Bowden et al., 1983), and in particular confirm the presence of a (*Z*)-1,3-butadiene unit. [See data associated with partial structures (IX) and (X).] (*Z*)-Farnesol (VIII) is distinguished by a methyl resonance at 23 ppm and an olefinic resonance at 110 ppm. Hence the geometry of the central double bond in the natural material is probably *E*. This was confirmed by an experiment in which (*Z*)-nerolidol (XI) was dehydrated as described by Anet (1970); GC analysis revealed that *none* of the products corresponded to the natural material (Figure 3).

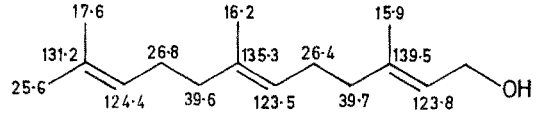
Therefore, we reasoned that the natural material from *Oxycarenus* was the (*Z,E*)-α-farnesene (III). A sample was prepared as described by Anet (1970) by dehydration of (*E*)-nerolidol (XII) [obtained from (*E*)-geranylacetone (Fluka) and vinylmagnesium bromide] (Figure 3). Isolation of (III) was achieved by addition of the crude mixture consisting of (I), (II), and (III) and some cyclic products to a solution of maleic anhydride in dry chloroform. The Diels-Alder adducts of only (I) and (II) formed rapidly ($t_{½}$ ca. 0.5 hr at 20°C) (cf. Oroshnik, 1956) and were easily separated from the much less polar (III) by column chromatography using *n*-pentane as eluant. As expected, the sample of synthetic (*Z,E*)-α-farnesene (III) was identical by GC, GC-MS, [^{1}H]-and [^{13}C] NMR with the natural material.

Finally, the identity of the steam distilled (*Z,E*)-α-farnesene with that

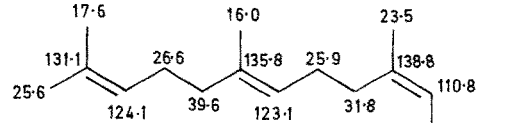


Natural farnesene

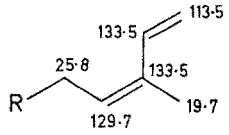
* assignments may be reversed



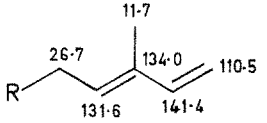
(VII)



(VIII)



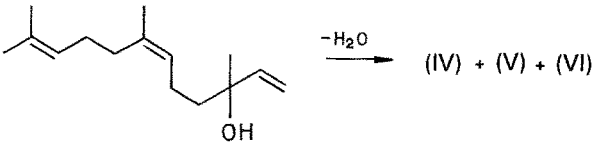
(IX)



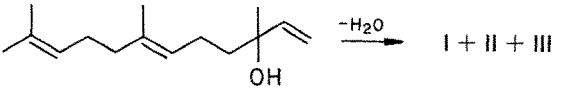
(X)

^{13}C n.m.r. data in p.p.m.

FIG. 2.

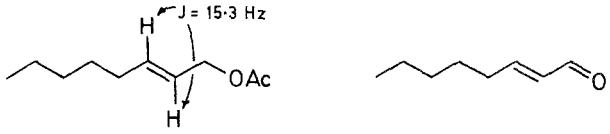


(XI)



(XII)

FIG. 3.



(XIII) (XIV)

FIG. 4.

present in the insect was established by GC and GC-MS, and by comparison of the UV data for the steam-distilled material and unheated material isolated directly from the lateral reservoir of the metathoracic scent gland [lateral reservoir sample: λ_{max} 236 mn (ϵ19,810)].

Other Scent Volatiles. 2-Octenyl acetate (XIII) was identified by comparative GC-MS data. The [^{1}H] NMR spectrum of the natural sample showed the following resonances: 0.88 (t, *J*-7.2 Hz, 8-CH_3), 1.18–1.45 (m, 3 × CH_2), 2.04 (m, 4-CH_2), 2.07 (OAc), 4.51 (dd, *J*-6.4, and 0.9 Hz, CH_2 · OAc), 5.56 (dtt, *J*-15.3, 6.4, and *ca.* 1.3 Hz, 2-H), and 5.78 (dtt, *J*-15.3, 6.7, and 0.9 Hz, 3-H). The coupling constant of 15.3 Hz between the olefinic protons clearly shows that the compound possesses the *E* configuration (XIII) (Figure 4). No evidence of the corresponding *Z* isomer was detected either by GC or [^{1}H] NMR spectroscopy on steam distilled or freshly isolated scent material.

The 2-octenal (XIV) present in the sample was also identified by comparative GC-MS data and, by analogy with (XIII), we assume that this also has an *E* configuration. It was isomerically homogeneous by GC, and its mass spectrum was superimposable on that of authentic (*E*)-2-octenal (XIV) (PPF International).

The monoterpenoid fraction contained α-pinene (identified by comparative GC-MS of the natural and hydrogenated material with authentics) and two previously unrecorded monoterpenes, which were tentatively identified from their mass spectra as α- and γ-terpinene.

Two other minor components (M^+ 204) were tentatively identified as monocyclic sesquiterpenes (M^+ 210 in hydrogenated samples). There can be no assurance that these materials were not artifacts formed by heating of the sample in the injector port during GC-MS. However, the major product arising when *Oxycarenus* (*Z,E*)-α-farnesene is strongly heated is a less polar acyclic isomer (M^+ 204), presumably the thermodynamically more stable allofarnesene, 3, 7, 11-trimethyl-2, 4, 6, 10-dodecatetraene, which would be produced by a thermally-allowed (1.5) hydride shift.

DISCUSSION

With the identification of the major component of the secretion as (*Z,E*)-α-farnesene (III), interest in the metathoracic scent gland of *O. hyalinipennis* now shifts to questions of biological function.

The reflex emission which occurs when the bugs are handled is an indication that the secretion from the gland is capable of exerting a repellent action against at least some kinds of predators. It is interesting to note that the emission of scent oil is accompanied by an audible stridulation which, as far as we are aware, has not been recorded previously within the genus *Oxycarenus* (Ashlock and Lattin, 1963; Schaefer and Pupedis, 1981). In their survey of the biotic relations of *Oxycarenus* spp. in Ghana, Adu-Mensah and Kumar (1977) found no evidence to implicate the metathoracic scent gland in defense, but Formicidae were not among the predators studied by these workers. As noted previously, the only occurrences of (*Z,E*)-α-farnesene reported in insects have been in ants (Morgan and Wadhams, 1972; Morgan et al., 1979; Vander Meer et al., 1981; Commaerts et al., 1981; Attygalle et al., 1982, 1983) and in termites (Naya et al., 1982). The latter apparently compete with *Oxycarenus* for seeds of Malvaceae (Adu-Mensah and Kumar, 1977); thus, an adaptive explanation for the production of (*Z,E*)-α-farnesene by *Oxycarenus* adults is perhaps to be found in predation or competition involving ants or termites.

In a search for volatiles in the different body regions of *Oxycarenus,* using GC and GC-MS, we were surprised to discover (*Z,E*)-α-farnesene in samples consisting of the body wall from the pregenital segments of the abdomen of both males and females. It is perhaps unlikely that an independent site of production of the farnesene exists in the abdomen, and therefore the compound probably reaches these locations following a redistribution of the secretion from the metathoracic scent gland. Although this could have occurred accidentally while the bugs were handled prior to sampling, it is quite possible that the cause is a voluntary release of secretion from the scent gland followed by grooming movements of the hind limbs (cf. Maschwitz, 1971). Self-marking with the farnesene could be adaptive and is perhaps an indication of sexual maturity (Olagbemiro and Staddon, 1983). Alternatively, the compound could form part of an aggregation mechanism, or simply act as an external, renewable, defensive film against ants or some other, as yet unspecified, predators.

On the question of biosynthetic origin, our failure to detect (*Z,E*)-α-farnesene is steam distillates of the dry cotton seed on which our culture of *Oxycarenus* was maintained indicates that cotton seed bugs are able to form the compound themselves. Presumably, the secretory cells of the gland tubules are equipped with the biogenetic machienry required to produce the farnesene from acetate, *via* the conventional isoprenoid pathway.

The farnesenes (*E,E*)-α-(II) and (*E*)-β-(I) have also been found in exocrine secretions from insects. (*E,E*)-α-Farnesene has been isolated from ants (Dufour's gland; Cavill et al., 1967, according to Anet, 1970; Vander Meer et al., 1981), andrenid bees (Dufour's gland; Fernandes et al., 1981), and *Papilio* larvae (osmeterial gland; Honda, 1981). (*E*)-β-Farnesene occurs

widely in aphids as an alarm pheromone (Bowers et al., 1972, 1977; Edwards et al., 1973; Wietjens et al., 1973; Pickett and Griffiths, 1980) and, so far as we are aware, has been found elsewhere in insects only in *Papilio* larvae (Honda, 1981). Interestingly, farnesenes produced by some flowering plants have been found to elicit both repellent and attractant responses in various phytophagous insects (Sutherland and Hutchins, 1973; Wearing and Hutchins, 1973; Gibson and Pickett, 1983).

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VOLATILE COMPOUNDS FROM PONERINE ANTS IN THE GENUS *Mesoponera*

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Abstract—Volatile constituents have been characterized from two species of ponerine ants in the genus *Mesoponera*. 2,5-Dimethyl-3-isopentylpyrazine has been identified from cephalic extracts of *M. castanea* and *M. castaneicolor*, by gas chromatography–mass spectrometry. Combined gasters and thoraces of both species are also characterized by the presence of nonanal, nonanoic acid, isovaleric acid, phenylacetic acid, and undecanal, as well as a series of aliphatic amines and amides. *N*-Isoamylnonylamine was a major constituent that was accompanied by *N*-isoamylnonenylamine, *N,N*-diisoamylnonylamine, *N*-acetyl nonylamine, *N*-formyl isoamylnonylamine, *N*-isovaleroyl nonylamine, and several other secondary and tertiary amines. The possible significance of the amines and amides as idiosyncratic natural products of *Mesoponera* species is discussed.

Key Words—Hymenoptera, Formicidae, *Mesoponera*, ants, ponerine ants, secondary and tertiary aliphatic amines, formamides, acetamides, isovaleramides, 2,6-dimethyl-3-isopentylpyrazine, aliphatic aldehydes and acids, phenylacetic acid.

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INTRODUCTION

Chemical eclecticism characterizes the exocrine chemistry of ants and a large number of pheromones and allomones have been identified in the secretions of these insects. Species in the subfamily Ponerinae are particularly distinctive in producing a variety of compounds belonging to several chemical classes. For example, alkyl sulfides (Casnati et al., 1967, Longhurst et al., 1979) ethyl ketones (Duffield and Blum, 1973), pyrazines (Wheeler and Blum, 1973, Longhurst et al., 1978), and a salicylate ester (Duffield and Blum, 1975) have been identified as mandibular gland products of ponerines in a variety of genera. These results demonstrate that ponerine species produce a diversity of compounds and suggest that analysis of other ponerine genera may yield additional distinctive exocrine products.

In the present report, the volatile constituents produced by two ponerine species in the genus *Mesoponera* are described. The identification of alkylamines and amides as products of these species further documents the ability of ponerines to synthesize novel natural products.

METHODS AND MATERIALS

Ants

Mesoponera, previously considered a subgenus within the genus *Euponera*, is given provisional generic status by Brown (1958) and by Wilson (1958). From further studies toward a reclassification of the Ponerinae, Brown (1973) proposes inclusion of *Mesoponera*, along with several other genera, in *Pachycondyla*. However, pending a formal designation, its provisional generic status is retained in this paper. The genus *Mesoponera*, apparently unremarkable phylogenetically, is mainly tropicopolitan but is also found in temperate regions such as New Zealand. Those species inhabiting the Australian (including New Zealand), Melanesian and Indo-Malayan regions appear to be closely related (group of *M. melanaria*). New Zealand is represented by two species, *M. castanea* (Mayr) and *M. castaneicolor* (Dalla Torre). Brown (1958) recognizes only one species in New Zealand (*M. castanea*) but Taylor (in Wilson and Taylor, 1967), after preliminary studies on New Zealand *Mesoponera* material, concludes that *castanea* applies to a species with ergatoid (wingless) queens, apparently limited to the North Island, while *castaneicolor* applies to a sibling relative of *castanea* which has alate queens and which is found in both major islands, North and South. *M. castaneicolor* is confined in the South Island to the northern sector.

Mesoponera workers in New Zealand vary in color from yellowish (teneral) to dark red or dark brown. Most fall within a size range (total length)

of 5.5–7 mm, with *M. castaneicolor* workers larger on average than *M. castanea* workers. They possess a respectable sting and rapidly disappear into their nest galleries when disturbed. Colonies are modest in size. One of us (A.W.D.) has made ecological observations which suggest that *M. castanea* prefers to nest in shady forest, in soil under stones or in rotting logs, where *M. castaneicolor* shows a preference for similar nest sites outside forests in more open situations, such as road verges and pastures. The biology of these species has yet to be studied in depth but the lower intensities of the unknown GC peaks in *M. castaneicolor* (see below) lends some support to their taxonomic distinction.

Workers of *M. castaneicolor* (Dalle Torre) were collected in New Zealand at Aorere Valley, northwest Nelson province, 18 miles from Collingwood. *M. castanea* workers were collected at Ohinetonga Scenic Reserve, Owhanga, near Taumarunui. Workers were decapitated, and heads and combined gasters and thoraces were placed in methylene chloride and mailed to the United States for analysis.

Chemical Analysis

Gas chromatography–mass spectrometry was performed on an LKB 2091 EI-CI system operating in the electron impact mode at 250° source temperature using 20 μA ionizing current and 70 eV when scanning. A 15 m $\times$ 0.26 mm ID glass capillary column with SE-30 liquid phase (J and W Scientific, Inc., Rancho Cordova, California) operating in the splitless mode (J and W Scientific injector system) was used for all mass spectral analyses. Packed columns (2 m $\times$ 2.5 mm ID) containing 1% SP-1000 were used for gas chromatographic analysis (see Synthesis).

Synthesis of Amines and Amides

Secondary amines. These were synthesized by combining one drop of amine with one drop of aldehyde in 1 ml of methanol and, after 10 min, adding sodium borohydride in excess. After an additional 10 min, three drops of glacial acetic acid, followed by three drops of concentrated hydrochloric acid were added and the mixture extracted with cyclohexane to remove any excess aldehyde as the corresponding alcohol. Addition of excess 50% sodium hydroxide and extraction with cyclohexane provided the amine which in all cases showed only 5–10% of the corresponding dialkylated tertiary amine by GLC. All retention temperatures (°C) were obtained on a 1% SP-1000 column.

Amylnonylamine. This was prepared from amylamine and nonanal, retention temperature 122°, MS: see Figure 4, spectrum B.

1-Methylbutylnonylamine. This was prepared from 1-methylbutylamine and nonanal, retention temperature 118°, MS: see Figure 4, spectrum C.

Isoamylnonylamine. This was prepared from isoamylamine and nonanal, retention temperature 115°, MS: Figure 4, spectrum A. Diisoamylnonylamine was present as a by-product (10%), retention temperature 128°, MS: *m/z* 283 ($M^{+}\cdot$, 7), 268 (1), 226 (100), 170 (96), 156 (4), 114 (27), 100 (4), 58 (18), 44 (8), 43 (7), m* 127.9 (226 → 170), 76.4 (170 → 114).

Isoamylnonylamine. This was also prepared in larger quantity for testing purposes. Isoamylamine (4.3 g, 0.05 M) and nonanal (8.0 g, 0.05 M) were combined with 40 ml of ethanol and after the initial reaction had subsided, 1.5 g sodium borohydride was added. The mixture evolved heat and was allowed to stand 2 hr after which 5 ml of glacial acetic acid followed by 5 ml of concentrated hydrochloric acid were added. Hexane (20 ml) was added and the solutions filtered from 1.9 g insoluble residue. The hexane layer was discarded, and the methanolic solution basified with concentrated sodium hydroxide and again extracted with hexane. Evaporation of the residue gave 6.70 g (48%) of an oil showing one peak on GC-MS. A sample was converted to the hydrochloride and recrystallized from methanol–water, mp 210–220° C (subl.)

Diisoamylamine. This was prepared from isoamylamine and isoamyl aldehyde for use in synthesis of diisoamylnonylamine (see Synthesis), retention temperature 59°, MS: *m/z* 157 ($M^{+}\cdot$, 10), 142 (1), 140 (1), 100 (49), 57 (4), 56 (5), 55 (4), 44 (100), 43 (27), 41 (11), m* 63.7 (157 → 100), 19.4 (100 → 44). Triisoamylamine was present as a by-product (~5%), retention temperature 83°, MS: *m/z* 227 ($M^{+}\cdot$, 5), 210 (8), 170 (100), 114 (48), 100 (9), 98 (6), 58 (25), 44 (32), 43 (3), m* 76.4 (170 → 114).

Heptylnonylamine. This was prepared from heptylamine and nonanal, retention temperature 140°, MS: *m/z* 241 ($M^{+}\cdot$, 5), 182 (7), 156 (85), 128 (100), 98 (7), 84 (9), 70 (14), 57 (20), 56 (18), 55 (18), 44 (61), 43 (30), 42 (6), 41 (7). Diheptylnonylamine was a by-product (~5%), retention temperature 171° MS: *m/z* 339 ($M^{+}\cdot$, 2) 338 (3), 337 (2), 336 (1), 335 (1), 306 (2), 290 (17), 254 (100), 226 (57), 170 (3), 168 (3), 156 (3), 154 (3), 142 (5), 140 (3), 128 (3), 126 (3), 112 (3), 98 (3), 84 (3), 70 (3), 69 (3), 58 (5), 57 (10), 56 (3), 55 (7), 44 (9), 43 (16), 41 (12). Nonylamine–heptaldehyde Schiff's base was identified as a third peak in the above preparation, retention temperature 135°, MS: *m/z* 239 ($M^{+}\cdot$, 2), 224 (1), 210 (4), 196 (6), 182 (24), 169 (5), 168 (5), 154 (100), 140 (5), 126 (17), 112 (8), 98 (14), 84 (14), 71 (7), 70 (14), 57 (15), 56 (17), 55 (12), 43 (17), 42 (8), 41 (15).

Heptylisoamylamine. This was prepared from isoamylamine and heptanal (for use in synthesis of diisoamylheptylamine) retention temperature 106°, MS: *m/z* 185 ($M^{+}\cdot$, 9), 128 (76), 100 (51), 57 (13), 44 (100), 30 (20), m* 19.4 (100 → 44).

Tertiary Amines. These were prepared from secondary amines by combining the corresponding amine and aldehyde in methanol with 10% Pd on C catalyst and bubbling hydrogen through the solution for 15 min.

Filtration from the catalyst and evaporation of solvents gave the crude tertiary amine in good yield (GLC) in all cases.

Heptylisoamylnonylamine. This was prepared from isoamylnonylamine and heptanal, retention temperature 153°, MS: see Figure 4, spectrum D, m* 127.8 (226 → 170), 113.8 (254 → 170), 101.8 (198 → 142).

Hexylisoamylnonylamine. This was prepared from isioamylnonylamine and hexanal, retention temperature 142°, MS: 297 ($M^{+}\cdot$, 6) 282 (2), 240 (75), 226 (91), 184 (100), 170 (22), 156 (23), 128 (26), 114 (10), 100 (22), 58 (12), 44 (36), 43 (19), m* 127.8 (226 → 170), 89.1 (184 → 128), 120.4 (240 → 170),

Dinonylisoamylamine. This was prepared from isoamylnonylamine and nonanal, retention temperature 180°, MS: 339 ($M^{+}\cdot$, 4), 338 (3) 282 (39), 252 (1), 226 (100), 170 (15), 156 (2), 114 (2), 112 (2), 100 (2), 98 (1), 71 (1), 69 (1), 58 (4), 57 (2), 44 (4), 43 (4), m* 127.8 (226 → 170),

Amylethylnonylamine. This was prepared from amylnonylamine and acetaldehyde, retention temperature 112° MS: see Figure 5, spectrum B, m* 19.4 (100 → 44)

N-*Methyl Isoamylnonylamine.* This was prepared from isoamylnonylamine and aqueous formaldehyde, retention temperature 108°, MS: *m/z* 227 ($M^{+}\cdot$, 5), 212 (1), 210 (1), 170 (87), 140 (3), 114 (100), 98 (5), 84 (5), 71 (5), 70 (5), 58 (81), 57 (10), 55 (9), 44 (26), 43 (27), 41 (9), 40 (13), m* 29.5 (114 → 58),

N-*Acetyl Derivatives.* These were prepared by combining the secondary amines and acetic anhydride with pyridine and allowing the mixture to stand for 1 hr. Evaporation of reactants afforded the *N*-acetyl derivatives. Complete conversion (GC) was realized in all cases.

N-*Acetyl Isoamylnonylamine.* This was prepared as above, retention temperature 182°, MS: see Figure 5, spectrum C, m* 225.8 (255 → 240), 122.9 (198 → 156), 136.3 (212 → 170), 52.1 (142 → 86).

N-*Acetyl Nonylamine.* This was prepared as above, retention temperature 171°, MS: *m/z* 185 ($M^{+}\cdot$, 27), 170 (8), 150 (9), 142 (13), 128 (12), 114 (23), 100 (31), 87 (15), 86 (34), 73 (70), 72 (69), 60 (27), 58 (7), 57 (5), 56 (7), 55 (14), 44 (22), 43 (52), 42 (7), 41 (23), 39 (4), 30 (100), 29 (11), 27 (6).

N-*Acetyl Heptylisoamylamine.* This was prepared as above, retention temperature 158°, MS: *m/z* 227 ($M^{+}\cdot$, 11) 212 (19), 184 (13), 170 (32), 156 (53), 142 (36), 128 (100), 114 (11), 100 (48), 87 (29), 86 (56), 73 (12), 72 (9), 57 (10), 55 (10), 44 (71), 43 (50), 41 (17), 30 (12), 29 (9), m* 197.9 (227 → 212), 109.5 (184 → 142), 96.3 (170 → 128).

N-*Isovaleroyl Derivatives.* These were prepared from the primary and secondary amines by combining them with isovaleroyl chloride and pyridine in benzene. After one hour, samples were withdrawn for direct GC analysis.

N-*Isovaleroyl Isoamylamine.* This was prepared as above, retention temperature on 3% SE-30: 152°, MS: *m/z* 171 ($M^{+}\cdot$, 25), 156 (41), 129 (94), 115 (40), 114 (43), 102 (32), 100 (13), 87 (12), 86 (18), 85 (90), 73 (100), 72 (22), 71 (23), 70 (11), 69 (11), 60 (15), 57 (100), 56 (7), 55 (18), 44 (80), 43 (79), 42 (16),

41 (56), 30 (77), 29 (31), 27 (20), m* 77.3 (171 → 115). *N,N*-Diisovaleroyl isoamylamine was fortuitously present as a by-product, retention temperature on 3% SE-30 186°, MS: *m/z* ($M^{+\cdot}$, 1) 240 (6), 198 (52), 172 (38), 170 (31), 171 (27), 156 (69), 129 (67), 115 (44), 114 (57), 112 (16), 85 (95), 78 (55), 73 (51), 60 (23), 57 (100), 44 (25), 43 (65), 41 (60). On 1% SP-1000 the above mono- and divaleroyl isoamylamines eluted together at 138°.

N-*Isovaleroyl Nonylamine.* This was prepared as above, retention temperature 181°, MS: see Figure 5, spectrum D, m* 150.7 (227 → 185).

Formyl Derivatives. These were prepared by combining the secondary amine with an excess of ethyl formate in a sealed tube and heating overnight on a steam bath. Evaporation of excess ethyl formate provided the amide in >70% yield (GC) in all cases.

N-*Formylisoamylnonylamine.* This was prepared as above, retention temperature 176°, MS: see Figure 5, spectrum A.

N-*Formyl Amylnonylamine.* This was prepared as above, retention temperature 184°, MS: *m/z* 241 ($M^{+\cdot}$, 4), 240 (2), 226 (1), 224 (1), 212 (7), 198 (6), 184 (90), 170 (4), 156 (6), 142 (6), 128 (65), 115 (5), 100 (9), 72 (100), 69 (6), 60 (4), 59 (2), 58 (6), 57 (4), 56 (3), 55 (10), 44 (15), 43 (26), 42 (5), 41 (16), 30 (7), 29 (10), m* 140.5 (184 → 241).

N-*Formyl Diisoamylamine.* This was prepared as above, retention temperature 122°, MS: *m/z* 185 ($M^{+\cdot}$, 2) 170 (3), 142 (4), 128 (91), 116 (4), 114 (3), 100 (2), 96 (4), 73 (38), 72 (100), 60 (12), 58 (5), 55 (6), 44 (16), 43 (22), 42 (6), 41 (12), 30 (6), 79 (8), m* 88.6 (185 → 128).

N-*Nonyl*-N-*isoamyl*-Δ′-*isopentenylamine.* This compound was obtained by heating a solution of isoamylnonylamine with an excess of isovaleraldehyde at 100° in a sealed tube for 2 hr. Gas chromatography revealed about 70% conversion to the enamine, retention temperature 210°, MS: *m/z* 281 (M+·, 13) 266 (100), 238 (7), 224 (49), 210 (5), 168 (37), 154 (9), 112 (39), 98 (11), 82 (4), 69 (5), 57 (6), 55 (8), 43 (18), 41 (12).

RESULTS

Gas Chromatographic—Mass Spectrometric Analysis of M. castaneicolor and M. castanea Extracts.

Figure 1 shows the gas chromatogram obtained from worker heads of *M. castaneicolor.* An important peak eluting at 125° shows a mass spectrum identical with that of 2,5-dimethyl-3-isopentylpyrazine, MS: *m/z* 178 ($M^{+\cdot}$, 0.2), 177 (1), 163 (8), 136 (3), 135 (13), 123 (9), 122 (100), 121 (8), 109 (0.3), 108 (1), 107 (3), 88 (8), 80 (5), 66 (3), 53 (9), 43 (5), 42 (15), 41 (11), a compound found in the mandibular glands of ponerine ants by Wheeler and Blum (1973). Following this peak, from 140–200°, a series of compounds appeared whose structures have not yet been elucidated. The intensities of these unknown

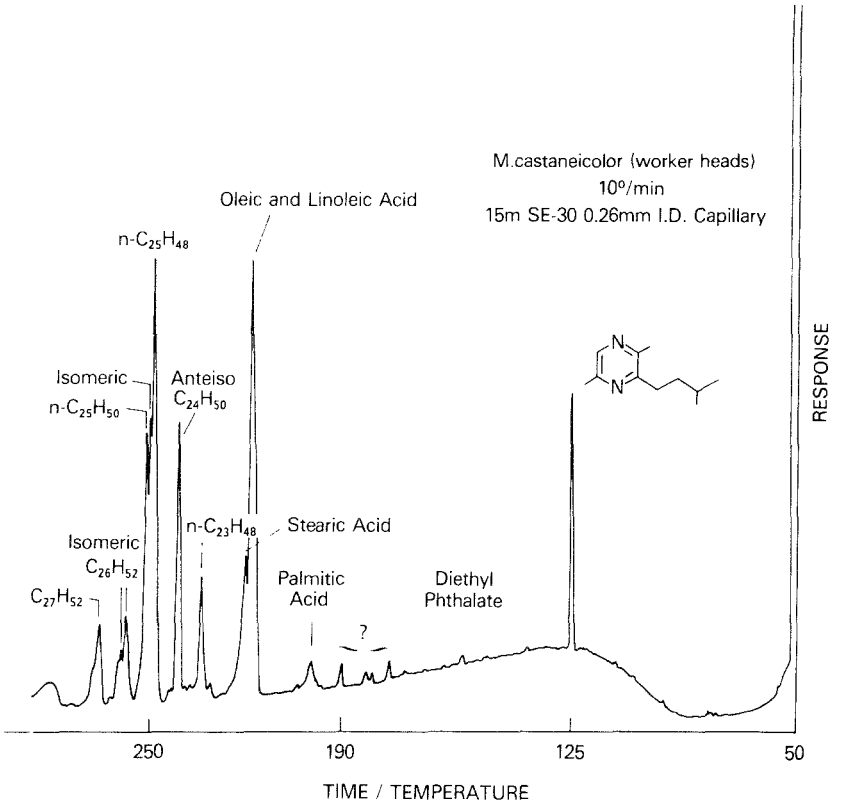

FIG. 1. Gas chromatogram of extracts of *M. castaneicolor* worker heads. Conditions on figure.

peaks were much higher in *M. castanea* worker heads than in *M. castaneicolor* but otherwise the chromatograms were very similar. Eluting last was a typical series of C_{16}- and C_{18}-saturated and unsaturated fatty acids (identified by reference to mass spectral compilations) (Heller and Milne, 1980) followed by a series of C_{23}–C_{27} normal and branched saturated hydrocarbons.

Extracts of the decapitated bodies of both *M. castaneicolor* and *M. castanea* workers showed nearly identical chromatograms (Figure 2) and also contained a similar series of fatty acids and hydrocarbons as well as isovaleric acid, nonanal, nonanoic acid, phenylacetic acid, and undecanal. All were identified by reference to mass spectral compilations (Heller and Milne, 1980) and spectra of authentic samples. The acids themselves were best observed after treating a sample of the extract with diazomethane to convert them to their methyl esters (Figure 3). An important peak eluting at 155° (Figures 2 and 3) showed a mass spectrum (Figure 4, spectrum A) characterized by an

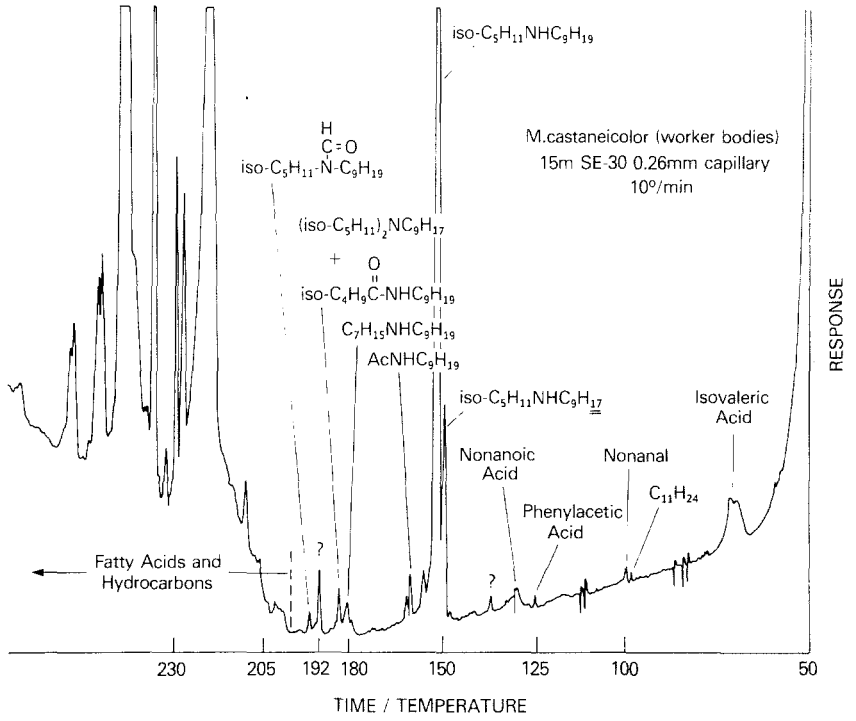

FIG. 2. Gas chromatogram of extracts of *M. castaneicolor* worker bodies. Conditions on figure.

odd mass molecular ion at m/z 213 and a few intense fragment ions, strongly suggesting an aliphatic amine. From the mass differences (57 and 113 amu) between these cleavage ions and the molecular ion, the simplest interpretation is that α-cleavage has resulted in the loss of $C_4H_9\cdot$ and $C_8H_{17}\cdot$, respectively, from amyl and nonyl groups attached to the nitrogen. The ion at m/z 44 $(CH_2{=}NHCH_3)^+$ is typical of aliphatic secondary amines and results from cleavage and rearrangement with hydrogen transfer. The obvious structure deduced for this compound is therefore *N*-amylnonylamine. However, the mass spectrum of a sample of this compound (Figure 4, spectrum B), prepared readily from amylamine and nonanal (see above) in methanol with sodium borohydride is not completely in agreement with that of the natural product. The latter shows greater loss of $C_4H_9\cdot$ and slightly greater loss of $CH_3\cdot$, suggesting the presence of branching in the amyl side chain. Possible alternative structures are 1-methylbutylnonylamine and isoamylnonylamine. The first, synthesized in the same fashion from 1-methylbutylamine and nonanal, is shown in Figure 4, spectrum C. Three modes of α-cleavage are available from this molecule [M-CH_3 (m/z 198), M-C_3H_7 (m/z 170), and

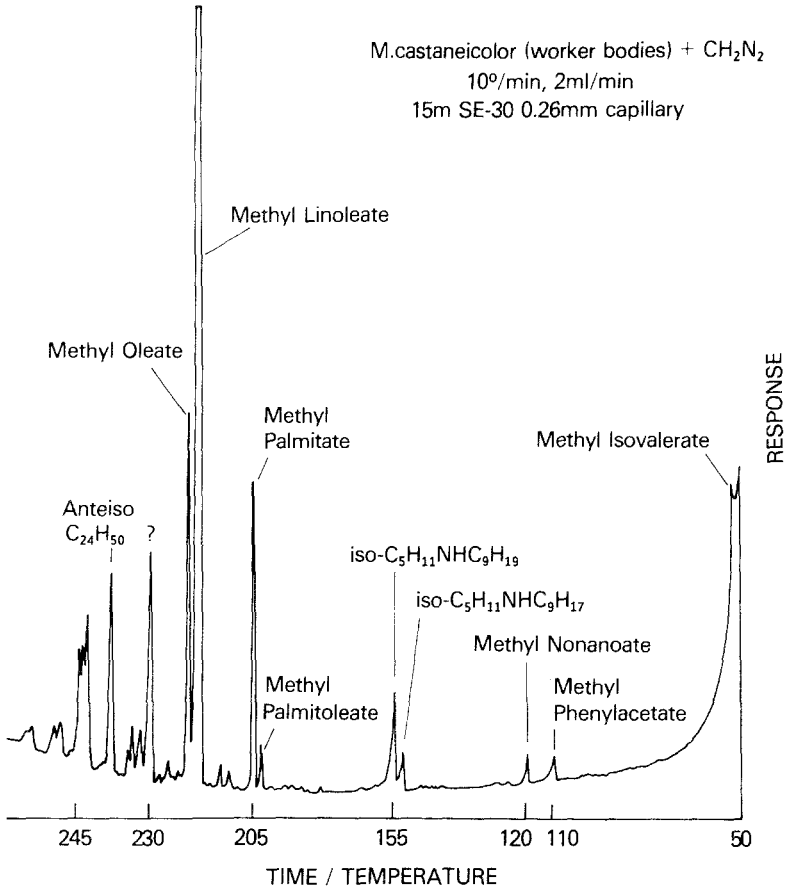

FIG. 3. Gas chromatogram of extracts of *M. castaneicolor* worker bodies after treatment with ethereal diazomethane. Gas chromatographic conditions on figure.

$M-C_8H_{17}$ (m/z 100)], differing markedly from the natural product. Another isomer, *N*-isoamylnonylamine synthesized from isoamylamine and nonanal showed a spectrum and retention time identical with that of the natural product (Figure 4, spectrum A).

A peak eluting slightly earlier than *N*-isoamylnonylamine (Figures 2 and 3) shows the following mass spectrum: m/z 211 (0.1, $M^{+}\cdot$), 210 (0.2), 209 (0.2), 196 (0.4), 194 (0.3), 154 (4), 152 (1), 126 (2), 114 (1), 105 (1), 100 (75), 91 (5), 83 (1), 81 (1), 71 (1), 70 (1), 69 (2), 67 (1), 57 (1), 56 (1), 55 (3), 45 (2), 44 (100), 43 (12), 42 (2). A suggested structure is *N*-isoamylnonenylamine since its molecular ion ($M^{+}\cdot$ 211) shows one unit of unsaturation and α-cleavage produces an ion at m/z 100 ($M^{+}\cdot - C_8H_{15}$). The location of the double bond is not positively known but the intensity of the ion at m/z 100 suggests it may be

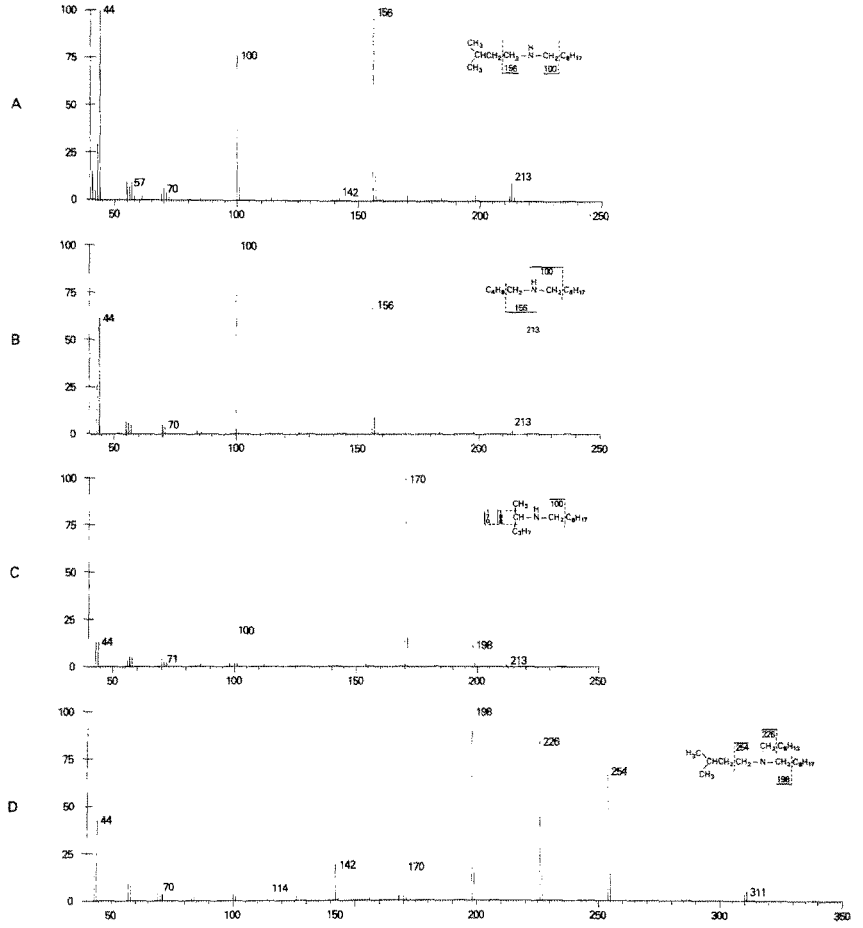

FIG. 4. Mass spectra of (A) isoamylnonylamine, (B) *n*-amylnonylamine, (C) 2-methylbutylnonylamine, and (D) heptylisoamylnonylamine, all run at 70 eV.

Δ^3 to the nitrogen, facilitating cleavage by the allylic nature of the departing radical. On the other hand, by analogy with piperidines and pyrrolidines in other ant species (Jones et al., 1981), it may be at the terminal end of the side chain.

Eluting slightly earlier still and visible in other chromatograms is a small peak identified as isoamylmethylnonylamine by its intense α-cleavage ions at m/z 170 ($M^{+\cdot} - C_4H_9$) and m/z 114 ($M^{+\cdot} - C_8H_{17}$) as well as its molecular ion at m/z 227. Its spectrum was identical with that of a synthetic sample.

A compound related to *N*-isoamylnonylamine, eluting later (196°, Figure 2), showed a molecular ion 28 mass units higher (Figure 5, spectrum A)

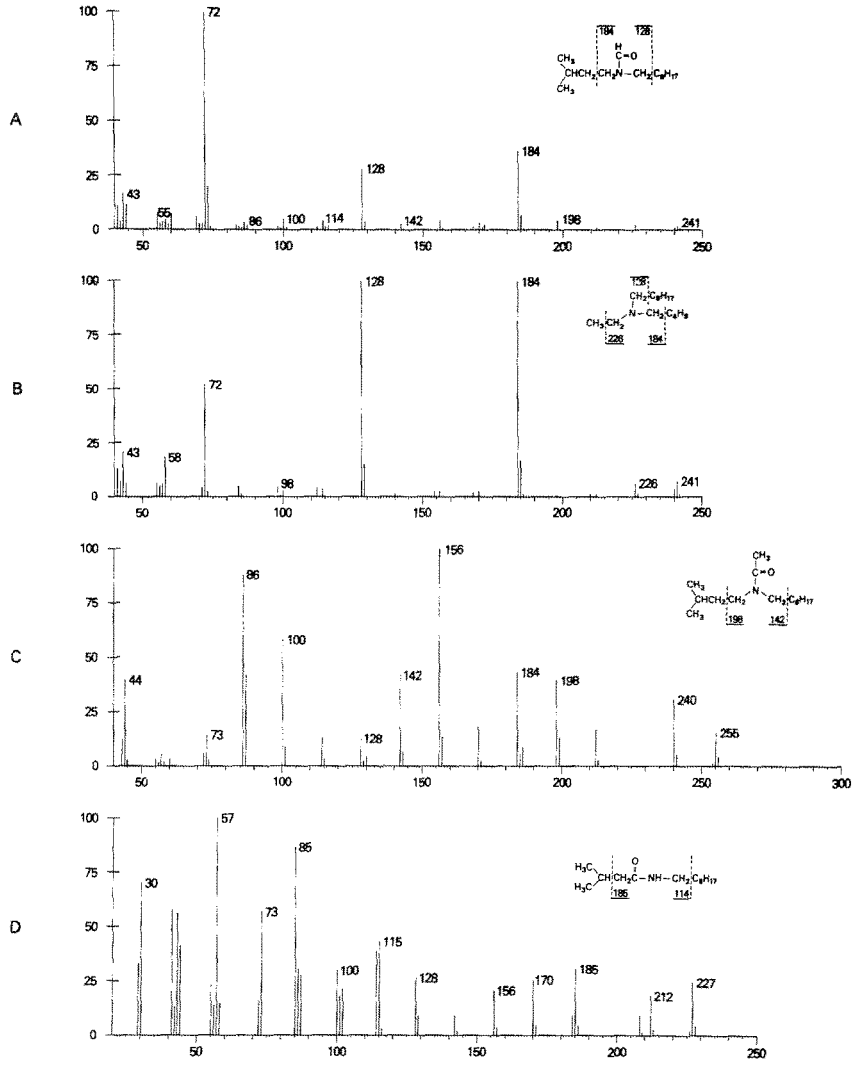

FIG. 5. Mass spectra of (A) *N*-formylisoamylnonylamine, (B) amylethylnonylamine, (C) *N*-acetyl isoamylnonylamine, and (D) *N*-isovaleroyl nonylamine, all run at 70 eV.

than that of isoamylnonylamine. The intensity of this peak relative to that of isoamylnonylamine inexplicably varied over a wide range. However, ions at m/z 184 and m/z 128 characteristic of C_5 and C_9 side chains are still present, suggesting the presence of a third substituent on nitrogen. That this group is formyl rather than ethyl is suggested both by the late retention time of the compound and the relatively low intensity of the α-cleavage ions. Synthesis of

the *N*-ethyl derivative of amylnonylamine from acetaldehyde and amylnonylamine provided a sample eluting much earlier (see under Synthesis), although its mass spectrum (Figure 5, spectrum B) was surprisingly similar to that of the natural product. Thus, loss of methyl from the *N*-ethyl derivative by α-cleavage leads to an ion (m/z 226) that is no more intense relative to the molecular ion than in the case of methyl loss from the side chains of the *N*-formyl analog. Enhanced loss of larger alkyl groups in such cleavages is, of course, well documented (Budzikiewicz et al., 1967). In a second similarity, the ion at m/z 72 (44 + 28), due to double rearrangement, is intense in both derivatives. *N*-Formylisoamylnonylamine, prepared from ethyl formate and the same amine, showed both spectrum and retention time identical with those of the natural product. *N*-Formylamylnonylamine was also synthesized and, as expected, showed a spectrum (see Synthesis) differing slightly but distinctly from the natural product.

A compound of the same mass (241) eluting much earlier (180°, Figure 2) is clearly the secondary amine heptylnonylamine, identified by intense α-cleavage ions at m/z 128 (loss of C_8H_{17}) and m/z 156 (loss of C_6H_{13}) and through comparison with its synthetic counterpart. Accompanying this peak in several chromatograms was a substance tentatively identified as diisoamylnonenylamine by a molecular ion at m/z 281 intense ions at m/z 170 (M-C_8H_{15}) and m/z 224 (M-57). From its mass and certain features of its spectrum, this was originally considered to be the enamine derived from isoamylnonylamine and valeraldehyde. Synthesis of this substance and comparison of its mass spectrum (see under Synthesis) proved that this was not the case.

Eluting just prior to *N*-formylisoamylnonylamine in heavily loaded chromatograms (not visible in Figure 2) was a compound showing a molecular ion at m/z 339 with intense ions at m/z 282 (M-C_4H_9) and 226 (M-C_8H_{17}). Its structure is therefore dinonylisoamylamine, and this was confirmed by comparison with a synthetic sample (see under Synthesis).

N-Acetylisoamylnonylamine, eluting at 195° (Figure 2), was similarly identified by its retention time on the polar SP-1000 and by loss of 42 mass units (ketene) from all of its even-electron α-cleavage ions (Figure 5C). As in the case of the *N*-formyl derivative, a substituted double rearrangement ion is present at m/z 86(44 + C_2H_2O). A second possibility for this mass is *N*,*N*-diisovaleroylisoamylamine. Formation of this substance as a by-product during synthesis of *N*-isovaleroylisoamylamine (see under Synthesis) provided a sample showing a similar but distinctly different spectrum: m/z 57 ($C_4H_9^+$) from the isovaleroyl groups is the base peak rather than m/z 156 (Figure 5, spectrum C) and there are no ketene losses. The first structure, *N*-acetylisoamylnonylamine, was confirmed by comparison with a synthetic sample (see under Synthesis).

The *N*-acetyl derivative of nonylamine eluting at 160° (Figure 2) was similarly identified by its molecular ion at m/z 185 and double rearrangement

with loss of ketene to give an intense ion at m/z 44. Comparison of its spectrum with a synthetic sample (see under Synthesis) confirmed the identification.

The *N*-isovaleroyl derivative of nonylamine eluting at 185° (Figure 2) was also identified by its molecular ion at m/z 227 accompanied by many fragment ions (Figure 5, spectrum D). Comparison with the mass spectrum of a synthetic sample confirmed the assignment. This peak also showed the presence of a second substance identified, after subtraction of ions due to *N*-isovaleroylnonylamine, as diisoamylnonylamine via its molecular ion at m/z 283, loss of C_4H_9 (m/z 226), and loss of C_8H_{17} (m/z 170). Comparison with a synthetic sample, isolated as an impurity in the synthesis of isoamylnonylamine confirmed the identification. Eluting slightly later, but not visible in Figure 2, was a compound whose mass spectrum (Figure 4, spectrum D) showed a molecular ion at m/z 311 and three intense α-cleavage ions at m/z 198 (M-C_8H_{17}), m/z 226 (M-C_6H_{13}), and m/z 254 (M-C_4H_9), clearly divulging its nature as the tertiary amine, heptylisoamylnonylamine, again confirmed by comparison with a synthetic sample (see under Synthesis).

In all of the above mass spectra, the presence of the isoamyl group was indicated by intense metastable ions for the loss of butylene from the ions formed by α-cleavage of the other substituents. For example, in heptylisoamylnonylamine (Figure 4, spectrum D) intense metastable ions are present for fragmentations of m/z 226 → m/z 170 and m/z 198 → m/z 142. Table 1 summarizes all compounds found in these species.

TABLE 1.

Compound	Location in workers	Identification
2,5-Dimethyl-3-isopentylpyrazine	heads	MS (literature data)
C_{16}–C_{18} saturated and unsaturated fatty acids	heads, bodies	MS (literature data)
C_{23}–C_{27} normal and branched hydrocarbons	heads, bodies	MS (literature data)
Isovaleric acid	bodies	MS (authentic samples)
Nonanal	bodies	MS (authentic samples)
Nonanoic acid	bodies	MS (authentic samples)
Phenylacetic acid	bodies	MS (authentic samples)
Undecanal	bodies	MS (authentic samples)
N-Isoamylnonylamine	bodies	MS (authentic samples)
N-Isoamylnon-3-enylamine	bodies	MS (proposed structure)
Isoamylmethylnonylamine	bodies	MS (authentic sample)
N-Formylisoamylnonylamine	bodies	MS (authentic sample)
Heptylnonylamine	bodies	MS (authentic sample)
Diisoamylnonenylamine	bodies	MS (proposed structure)
Dinonylisoamylamine	bodies	MS (authentic sample)
N-Acetylisoamylnonylamine	bodies	MS (authentic sample)
N-Acetylnonylamine	bodies	MS (authentic sample)
N-Isovaleroylnonylamine	bodies	MS (authentic sample)
Diisoamylnonylamine	bodies	MS (authentic sample)
Heptylisoamylnonylamine	bodies	MS (authentic sample)

DISCUSSION

2,5-Dimethyl-3-isopentylpyrazine is the major exocrine compound identified as a cephalic product of workers of *Mesoponera* species. This pyrazine has also been identified as a mandibular gland product of ponerines in several genera (Wheeler and Blum, 1973; Longhurst et al., 1979) and appears to be a rather typical natural product of workers in this subfamily. On the other hand, most of the compounds identified in extracts of the gaster–thorax of the *Mesoponera* species constitute new natural products of insects, especially the alkylamines and their amides.

Although the anatomical source of the amines and amides has not been established, it will not prove surprising if they constitute poison gland products. The poison glands of ants have proven to be a rich source of both high- and low-molecular-weight nitrogenous compounds, indicating that this gland is dedicated to the synthesis of nitrogen-containing natural products. For example, 2-methyl-6-alkyl- and alkylidenepiperidines (MacConnell et al., 1971) and 2,5-dialkyl- and alkylidenepyrrolidines (Ritter and Persoons, 1975; Jones et al., 1979) have been identified as poison gland products of a variety of *Solenopsis* and *Monomorium* species in addition to a pyrrolizidine (Jones et al., 1980) and an indolizidine (Ritter et al., 1973). These results document the ability of the poison gland to produce small nitrogenous compounds and suggest that the poison gland of the *Mesoponera* species may be responsible for the biosynthesis of the amines and amides that have been identified in extracts of the gaster–thorax. Indeed, the venoms of a variety of arthropods are enriched with low-molecular-weight amines such as norepinephrine (Ishay et al., 1974) and spermine (Gilbo and Coles, 1964).

Identification of nonanal, undecanal, and isovaleric acid (implicating valeraldehyde) in extracts of the gaster–thorax is especially interesting since their presence suggests the secondary and tertiary amines are formed by reductive alkylation processes involving the aldehydes. In addition to these secondary and tertiary amines, their amides with formic and isovaleric acids constitute very unusual natural products. Isovaleric acid and presumably formic acid are present in these ants, and it is possible that their amides are artifacts. However, mixtures of isoamylnonylamine and either 10% formic or 10% isovaleric acid did not produce detectable amounts of these amides when analyzed on the same gas chromatography columns.

The amines and amides produced by these *Mesoponera* species constitute idiosyncratic natural products that distinguish the species in this genus from those in other ponerine genera that have been subjected to similar analytical scrutiny. These compounds are perhaps utilized as defensive compounds against selected predators. Although the chemistry of relatively few ponerine genera has been examined, the presence of these distinctive nitrogenous

compounds in *Mesoponera* species should act as a spur to analyze species in additional genera in this primitive subfamily.

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MALE WING-GLAND PHEROMONE OF *Ephestia elutella*[1]

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Abstract—Sex pheromone extracted from glands on the forewings of male *Ephestia elutella* (Hübner) elicits a stereotyped courtship response from conspecific females. A bioassay for this sex pheromone was developed based on this behavior. Maximum production and responsiveness for males and females, respectively, occurred in insects more than 24 hr old. *E. elutella* females were not responsive to extracts made from *E. figulilella* Gregson, *E. kuehniella* Zeller, *E. cautella* (Walker), or *Plodia interpunctella* (Hübner) males.

Key Words—*Ephestia elutella*, Lepidoptera, Pyralidae, male pheromone, pheromone bioassay.

INTRODUCTION

Male-specific organs thought to produce and release courtship pheromones are found in many lepidopteran species (Birch, 1974, and references therein). However, behavioral activity has been attributed to characterized male secretions in only a few cases (Nishida et al., 1982; Conner et al., 1981), primarily because the female response to these chemicals is often inconspicuous and, therefore, difficult to observe and quantify.

Male courtship pheromones have been implicated in the mating behavior of three stored-products-infesting phycitine pyralid moths: *Plodia interpunctella* (Hübner) (Grant and Brady, 1975; McLaughlin, 1982), *Ephestia cautella* (Walker) (Grant and Brady, 1975; Barrer and Hill, 1978), and *Vitula edmandsae* (Packard) (Grant, 1976a). We examined the courtship behavior of

[1]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

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another of the stored-product Phycitinae,[3] *Ephestia elutella* (Hübner), in order to determine whether a male pheromone also might play a role in courtship in this species.

We describe here a bioassay for male-produced pheromone based on a female courtship response to volatiles emanating from specialized male wing-gland scales, and report results on the effects of dose, age, and species specificity upon female response.

METHODS AND MATERIALS

General. Ephestia elutella larvae were reared in our laboratory on the diet described by Silhacek and Miller (1972). Cultures were maintained on a 14:10 light–dark photoperiod at 26 ± 1°C and ca. 50% relative humidity. Pupae were segregated by sex and placed in separate incubators with 14:10 light–dark photocycles offset 12 hr from that of the larval rearing chamber. Newly eclosed adults (0–12 hr old) were collected in the last hour of their first photophase and were placed in an experimental room maintained at 26 ± 2°C. Illumination of the 14:10 light–dark photoperiod (identical to pupal photoperiod) was ca. 140 lux (fluorescent light) and 3 lux (Westinghouse® 25 A-R red bulb, 25 W, 120 V) for the photophase and scotophase, respectively, both measured 1 m from the light source with a light meter adjusted for human spectral sensitivity.

Mating Observations. Twenty-five newly emerged females were placed in 25 × 25 × 25-cm Plexiglas sleeve-cages and kept in an experimental room until they were 2–4 days old. (Light and temperature regimes were the same as in the eclosion chambers.) Twenty unmated 2- to 4-day-old males were released singly into each cage during the period from 3 to 5 hr after the onset of darkness, and the ensuing courtships were observed. Fifty matings were analyzed.

Bioassay. Solvent rinses of whole bodies of *E. elutella* males were prepared by soaking live unmated males of known age (±12 hr) in pentane (10 ♂♂/ml) for 3–5 hr. Rinses of excised male scent organs were prepared by soaking the parts in pentane (20 male equivalents/ml) for 2–3 hr. All rinses were filtered and adjusted in volume by evaporation under dry nitrogen so that the required number of male equivalents (ME) could be delivered in a 10-μl aliquot. Controls were prepared by concentrating an aliquot of solvent under dry nitrogen. Unless otherwise noted, rinses were used for 2 days and then discarded.

Equal numbers of unmated *E. elutella* females (2–4 days old unless

[3]The stored-produce Phycitinae is an artificial species complex comprised of *Plodia interpunctella*, *Ephestia cautella*, *Ephestia elutella*, *Ephestia figulilella*, and *Ephestia kuehniella*.

otherwise noted) were assigned at random to each of several 25 × 25 × 25-cm Plexiglas sleeve-cages that were placed in the experimental room 1–2 hr before the onset of the scotophase on the day of testing. Bioassays were conducted during the period from 4.5 to 5.0 hr after the onset of the scotophase, the period of maximum female calling activity for *E. elutella* females (Krasnoff et al., 1983).

An aliquot (1 ME unless otherwise specified) of the sample was pipetted onto the end of a glass rod (12 cm long × 3 mm diam). The solvent was allowed to evaporate (10–15 sec), and the rod was then introduced into a sleeve-cage and presented successively to individual calling females by holding the tip of the rod ca. 2 mm in front of their heads. The number of females that responded within 1 sec by lowering the abdomen and flexing it ventrally was recorded. A surplus of females was always placed in the sleeve cage to ensure an adequate number of calling females.

Except where noted, females were discarded after they were exposed to one treatment. The order in which the treatments were tested was randomized daily. Except in the dose–response study, treatments were tested blind. Unless otherwise noted, response percentages were transformed to arcsin $\sqrt{x}$ and submitted to analyses of variance.

Determination of Origin of E. elutella *Male Pheromone.* Modified scale clusters displayed during courtship are found in two sites: (1) costal folds on the forewings (Barth, 1937), and (2) the 8th abdominal tergite (Dickens, 1936). Both were considered as possible sources of male pheromone. Solvent rinses of (1) male wing glands, (2) male abdominal tips, and (3) wing glands and abdominal tips combined were assayed along with a control. An aliquot of each treatment was tested on five females (out of 15 in a cage) on each of six days.

Dose–Response Study. Female response to male pheromone (whole body rinse) was measured at 0.0, 0.1, 0.32, 1.0, and 3.2 ME. Each of five cages contained 25 females, 12 of which were tested with each dose. The experiment was replicated once each day for 15 days. Mean percentage responses were plotted against $\log_{10}$ of the dose.

Effect of Age on Female Response to Male Pheromone. Whole-body solvent rinses of males from three age classes (0–1, 3–4, and 6–7 days old) were tested separately in all possible combinations on 20 females (50 ♀♀/sleeve-cage) of each of the corresponding age classes for four days. On each day the same pool of females was used to test each male rinse. The pools of 0- to 1- and 3- to 4-day-old females on the first and second days of the experiment were reused as 3- to 4- and 6- to 7-day-old females on the third and fourth days of the experiment, respectively. The same male rinses were used throughout the test.

Species Specificity of E. elutella *Male Pheromone.* Treatments consisted

of whole-body rinses prepared from male moths (0–4 days old) of the following species: *P. interpunctella, E. elutella, E. cautella, E. kuehniella* Zeller, and *E. figulilella* Gregson. An aliquot of each treatment was tested on 10 *E. elutella* females (out of 15 in a cage) on each of the three days of the experiment. The same male rinses were used throughout the test.

RESULTS

Courtship. In courtship a wing-fanning male *E. elutella* walks toward a calling female and maneuvers into a head-to-head orientation with her. When this head-to-head position is established, the female's antennae come into close apposition to the male's inflated costal wing folds (Figure 1a). At this time the female drops her abdomen out of the calling position and withdraws her ovipositor and pheromone gland. She simultaneously lowers and flexes her abdomen ventrally. She may touch the tip of the abdomen against the substrate and dab it up and down several times (Figure 1b–d). This behavior, which we refer to as the ventral flexion response, occurs just prior to the male's attempt to engage the female's terminalia. We observed this behavior in 49 of 50 courtships, all of which culminated in copulation.

Bioassay. Preliminary experiments showed that the ventral flexion response could be induced in a calling *E. elutella* female by holding an excised male wing with the costal fold forward 1–2 mm in front of the female's head. One ME of a pentane extract of whole *E. elutella* males delivered to the tip of a glass rod and simliarly presented also produced the ventral flexion response (Figure 2). No female ever responded to an untreated glass rod, to one that had been treated with solvent alone, or to one treated with a rinse made from conspecific females.

Pheromonal Source. Out attempts to determine the origin of the pheromone producing the ventral flexion response demonstrated that at least the primary component issues from the costal wing gland of the *E. elutella* male (Table 1). Rinses of male abdominal tips alone had no significant activity, indicating that any odorant produced by the 8th tergal scale clusters is not involved in eliciting the response.

Dose Response. The response of *E. elutella* females to male pheromone increases with dose in the range between 0.1 and 1.0 ME and drops off thereafter with 3.2 ME producing a slightly lower response than 1.0 ME (Figure 3).

We observed that some *E. elutella* females responded to higher doses of pheromone (3.2–10 ME) with quick evasive movements (e.g., turning, walking, jumping, or flying away), suggesting a possible repellent effect. This may account for the drop-off in response at the highest dose tested.

Age Effects. Newly emerged females (0–1 days old) were less responsive to male pheromone than were older females. However, no further increase in

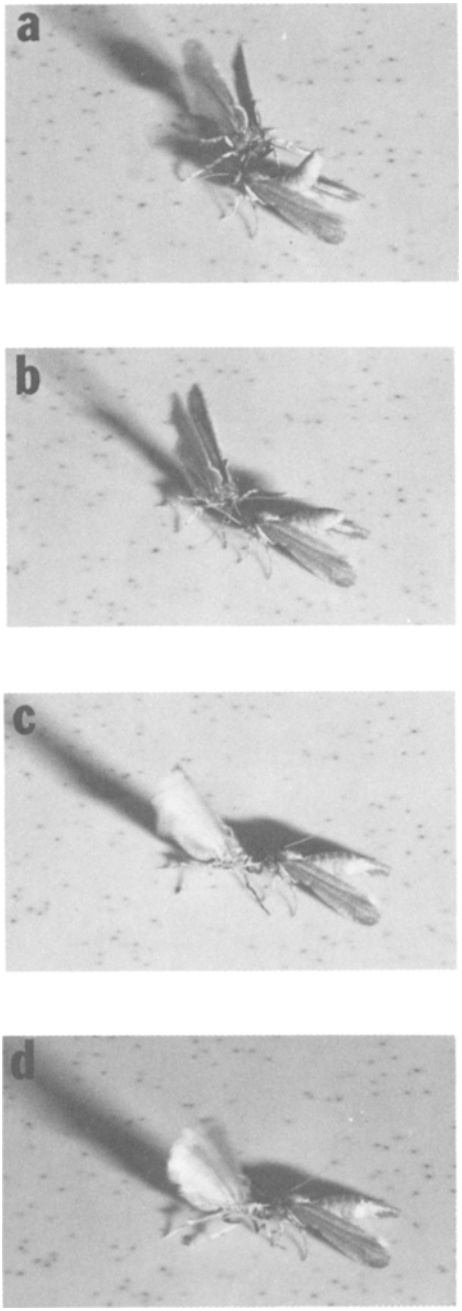

FIG. 1. Courtship of *Ephestia elutella:* female ventral flexion response. Sequence was photographed at 0.25-sec intervals and proceeds from top to bottom (a–d). Male (left) approaches calling female (a). Note expanded costal fold and exposed scent scales. Females lowers abdomen and flexes it ventrally (ventral flexion response) (b–d).

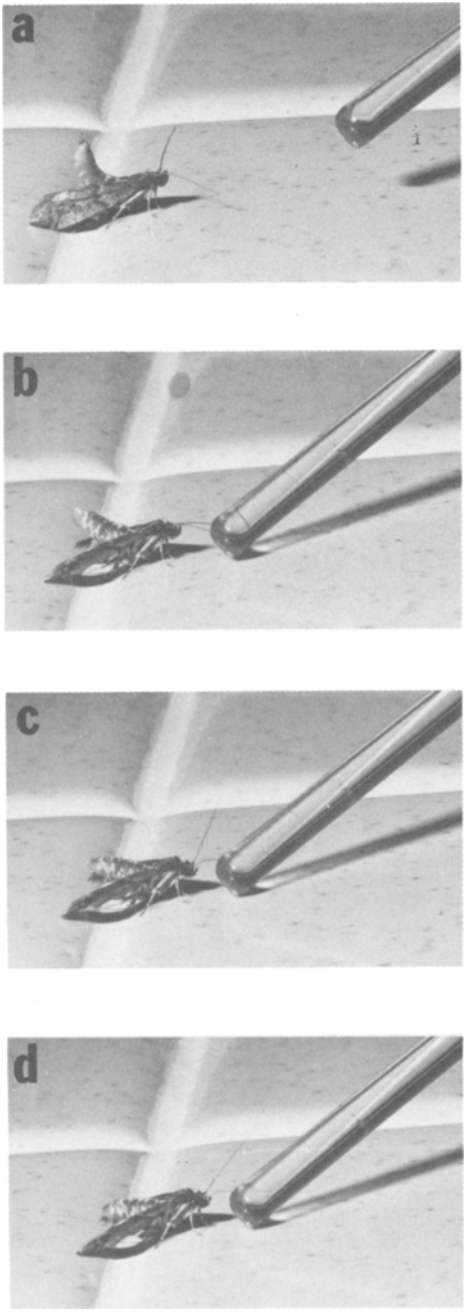

FIG. 2. Response of female *Ephestia elutella* to a glass rod treated with one male equivalent of a whole-body solvent rinse from *E. elutella* males. Sequence was photographed at 0.25-sec intervals and proceeds from top to bottom. Female exhibits ventral flexion response (b–d).

TABLE 1. RESPONSE OF *Ephestia elutella* FEMALES TO SOLVENT RINSES OF MALE WING GLANDS AND ABDOMINAL TIPS TESTED SEPARATELY AND IN COMBINATION

Source of rinse	Mean % response[a]
Wing glands	23.4 a
Wing glands and abdominal tips	37.0 a
Abdominal tips	3.4 b
Solvent blank	0.0 b

[a]Means not followed by the same letter are significantly different at the 5% level according to Duncan's multiple-range test.

response with increasing age could be detected (Figure 4A). Concomitantly, rinses of newly emerged males (0–1 days old) were less active than rinses of older males (Figure 4B).

Pheromone Specificity. The failure of *E. elutella* females to respond to any of the heterospecific male rinses that were presented to them (Table 2) demonstrates that the *E. elutella* male scent is unique among the stored-products Phycitinae.

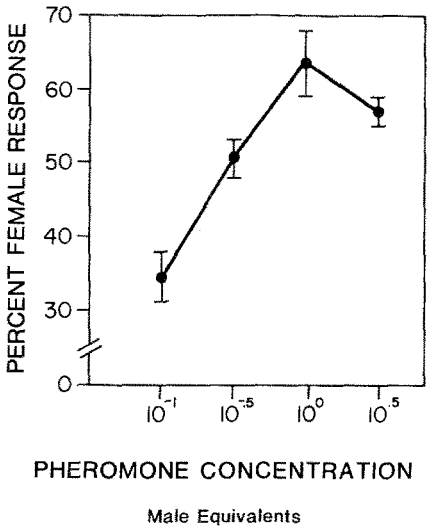

FIG. 3. Percentage of *Ephestia elutella* females responding to four concentrations of male pheromone. Each point represents the mean of 15 replications with 12 insects used in each replicate. Vertical lines represent standard errors of the indicated mean percentages.

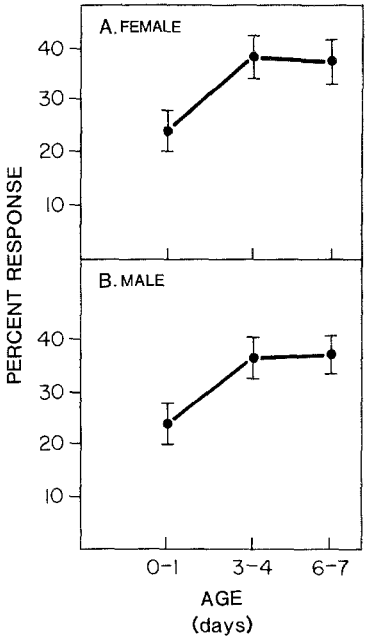

FIG. 4. Percentage of *Ephestia elutella* females responding to male pheromone as a function of (A) female age, and (B) age of males from which pheromone (whole-body solvent rinse) was derived. Each point represents the average of four replicates. Twenty females were tested in each replicate. Vertical lines represent standard errors of the indicated mean percentages.

DISCUSSION

The responses of female Lepidoptera to male pheromones (possibly in combination with other stimuli such as acoustic, visual, or tactile signals) may be categorized as follows: (1) long-range attraction (Zagatti, 1981), (2) short-range attraction (Baker and Cardé, 1979), (3) arrestment of flight (Brower et al., 1965), (4) inhibition of the female's tendency to flee (Birch, 1970), and (5) stationary precopulatory posturing such as the wing raising of *Utetheisa ornatrix* L. (Conner et al., 1981) or the abdominal extension of *Eurema lisa* (Boisduval and LeConte) (Rutowski, 1977). The ventral flexion response of *E. elutella* females described in this paper is of this latter type.

Although the courtship of *E. elutella* is similar in many details to that already described for other phycitine species (Grant and Brady, 1975; Barrer and Hill, 1977; Grant, 1976a), the ventral flexion response has not been previously defined. Both *P. interpunctella* (Grant and Brady, 1975) and *E. cautella* females (Grant and Brady, 1975; Barrer and Hill, 1977) lower the

TABLE 2. RESPONSE OF *Ephestia elutella* FEMALES TO WHOLE-BODY SOLVENT RINSES OF HETEROSPECIFIC MALES[a]

Species	Mean % response[b]
E. elutella	70.0 a
E. cautella	0.0 b
E. figulilella	0.0 b
E. kuehniella	0.0 b
Plodia interpunctella	0.0 b

[a] Means are based on three replications with 10 ♀ ♀ used in each replicate.
[b] Means not followed by the same letter are significantly different at the 5% level according to Duncan's multiple- range test.

abdomen during the head-to-head confrontation with the male. However, in these species the females do not bend the abdomen toward the substrate (Krasnoff and Vick, personal observation). They do subsequently raise the abdomen in what has been called the "acceptance response."

Grant and Brady (1975) linked this acceptance response in *P. interpunctella* to a wing gland pheromone by inference from studies of courtships involving antennectomized females and normal males as well as courtships between normal females and wingless (i.e., wing-glandless) males. Barrer and Hill (1978) were able to elicit this response artificially in *E. cautella* females calling in groups by directing jets of air toward their heads. Air passed over freshly excised male wings was more active than blank jets of air, but the blanks nonetheless did induce the acceptance response in 20% of the observations.

The ventral flexion response of *E. elutella* females is, we believe, not homologous to the "acceptance response" of *P. interpunctella* and *E. cautella* but rather to the abdominal lowering seen in those species. Because it is so distinctive and easily observed in the courtship of *E. elutella*, and can be readily induced in the absence of males or male parts, it was unnecessary to resort to ablative procedures to establish the existence of a male pheromone. This response lent itself especially well to quantification in that it did not require any additional mechanical stimulation from moving air or direct contact and was never observed in response to sham stimuli (e.g., solvent only).

This is not to say that stimuli through other sensory modalities may not contribute to eliciting the response in the context of actual courtship. Antennectomized *E. elutella* females courted by conspecific males and normal *E. elutella* females courted by males of the other species in the stored-products phycitine group may occasionally exhibit this behavior (Krasnoff and Vick,

personal observation). These are both situations in which the female presumably cannot perceive the pheromonal cue. This suggests to us that a wing-fanning male may produce either mechanical stimuli from moving air or direct contact, or an accoustic stimulus akin to that used by male galleriine pyralids to attract females (Spangler, Greenfield, and Takessian, unpublished data). Nonolfactory cues may then also elicit a behavior that can be produced by an odor cue alone.

The involvement of two different scent glands that must be brought into contact for the biosynthesis of a male courtship pheromone has been documented for the butterfly, *Danaus chrysippus* L. (Boppre et al., 1978). Although no such contact between the abdominal and alar glands of *E. elutella* has ever been observed, it was considered that a synergistic relationship might exist between two pheromone components with different anatomical points of origin. Our analysis, however, did not support this hypothesis. The few responses of *E. elutella* females to male abdominal tip extracts that were recorded in our assays may have been the result of contamination by wind-gland volatiles. In a preliminary trial, six of 30 *E. elutella* females gave a positive response to 1 ME of a solvent rinse of the distal portions of male forewings that were left over after the pheromone glands had been excised. Rutowski (1977) found that wind sections other than those containing the source of male pheromone in *E. lisa* were behaviorally active. He demonstrated that this contamination was due to direct contact between nonsecretory portions of the wings and the secretory androconia. Palaniswamy et al. (1979) reported electroantennogram (EAG) responses from female *Choristoneura fumiferana* (Clemens) antennae to extracts of a region of the male abdomen that contained no scent structures. They also pointed to the possibility that this activity was due to contamination by volatiles from active secretory organs. In the case of *E. elutella*, wing-gland volatiles may contaminate the surface of the male's body. This might explain why rinses of wing glands by themselves (Table I) appear to be much less active than rinses of whole males bodies (Figure 3).

It is not surprising that newly eclosed *E. elutella* females are not as responsive to male pheromone as their older counterparts in light of the evidence that has been compiled to show that female moths in general, and phycitines in particular, are not sexually mature at eclosion (Calvert and Corbet, 1973, and references therein). The finding that newly eclosed *E. elutella* males are apparently not as well endowed with courtship pheromone as their older counterparts also has parallels in the literature. Clearwater (1972) found that benzaldehyde, the putative male pheromone of *Pseudaletia separata* (Walker), does not appear in the male hairpencils until the first night after emergence and does not reach its peak concentration until the second night after eclosion. There may be a similar secretory cycle in *E. elutella* males.

The presence of (*Z,E*)-9,12-tetradecadien-1-ol acetate as the primary

component in the female sex pheromone of all five species in the stored-products Phycitinae may result in cross-attraction (e.g., Ganyard, 1971). Interspecific courtship encounters are likely to occur where species are sympatric (e.g., Hoppe and Levinson, 1979) and sexually active at the same time of day (Krasnoff et al., 1983). In the absence of long-range isolating mechanisms, a species-specific male scent would be a likely means by which a female could discriminate against heterospecific males. Our results would seem to leave open the possibility that male pheromones serve a de facto species isolating function in the stored-products Phycitinae. However, as noted above, nonspecific stimuli may, in some cases, also elicit the response that is linked to the wing-gland pheromone in *E. elutella*.

There are several European species of the genus *Ephestia* that are considered to be more closely related to *E. elutella* than any of the stored-products species (Roesler, 1973). Further insights into the possible reproductive isolating function of the male pheromone causing the response monitored in our bioassay might be gained by testing interspecific effects among these species.

Several recent studies have downplayed the reproductive isolating function of male pheromones in favor of an emphasis on the role of sexual selection in the evolution of male lepidopteran scent systems (Baker and Cardé, 1979; Conner, 1981; Willis and Birch, 1982). A female moth exhibiting the outward signs of sexual receptivity may often reject a courting male, perhaps on the basis of a quantitative assessment of the male pheromone. This "coyness" (Richards, 1927; Grant, 1976b; Baker and Cardé, 1979), the putative result of sexual selection for female choosiness in courtship, may make female moths more likely targets than males in attempts to control infestations by pheromone-based mating disruption techniques, i.e., females may be more susceptible than males to perturbation by a supernormal stimulus. Isolation, identification, and synthesis of the *E. elutella* male pheromone should allow us to test the effects of superphysiological doses of pheromone on mating success in this species.

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BIOSYNTHESIS OF SEX PHEROMONE COMPONENTS AND GLYCEROLIPID PRECURSORS FROM SODIUM [1-^{14}C]ACETATE IN REDBANDED LEAFROLLER MOTH

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Abstract—Sodium [1-^{14}C]acetate in water–dimethyl sulfoxide (1:1) was applied topically to sex pheromone glands of *Argyrotaenia velutinana*. Radiolabel was incorporated into the pheromone components (*Z*)-11-tetradecenyl acetate and (*E*)-11-tetradecenyl acetate, and also into triacylglycerols, diacylglycerols, ethanolamine phosphatides, and choline phosphatides. In the triacylglycerols, radiolabel appeared in (*Z*)-11-tetradecenoate, (*E*)-11-tetradecenoate, tetradecanoate, hexadecanoate, and octadecanoate. In the choline phosphatides, the same acyl moieties incorporated radiolabel but at lower levels. In the diacylglycerols and ethanolamine phosphatides, only the radiolabel in hexadecanoate and octadecanoate was above the limit of detection. At different times following application of sodium [1-^{14}C]acetate, the relative proportions of labeled (*Z*)-11-tetradecenyl acetate and (*E*)-11-tetradecenyl acetate changed very little, but the relative proportions of labeled fatty acyl moieties in the triacylglycerols and choline phosphatides changed markedly. After 8 min, triacylglycerols had incorporated about equal amounts of radiolabel into (*Z*)-11-tetradecenoate, (*E*)-11-tetradecenoate, and tetradecanoate. As the incubation time was increased, triacylglycerols accumulated proportionately more radiolabeled (*E*)-11-tetradecenoate than (*Z*)-11-tetradecenoate, and accumulated proportionately less radiolabeled tetradecanoate. In the choline phosphatides, at all times of incubation the amount of radiolabel incorporated into (*Z*)-11-tetradecenoate was small but above the limit of detection, and the amounts of radiolabel in (*E*)-11-tetradecenoate and tetradecanoate were smaller and often below the limit of detection. In both the triacylglycerols and the choline phosphatides, the relative proportion of radiolabeled hexadecanoate decreased with time, and that of octadecanoate increased.

Key Words—Sex pheromone biosynthesis, *Argyrotaenia velutinana*, redbanded leafroller moth, Lepidoptera, Tortricidae, radiolabel.

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INTRODUCTION

In addition to the volatile compounds (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, tetradecyl acetate, and dodecyl acetate (Roelofs et al., 1975), the sex pheromone gland of the female redbanded leafroller moth (*Argyrotaenia velutinana*) contains the corresponding fatty acyl moieties (*Z*)-11-tetradecenoate, (*E*)-11-tetradecenoate, tetradecanoate, and dodecanoate as components of several glycerolipid classes. These moieties occur in abundance in the triacylglycerols, less abundantly in the choline phosphatides, in small amounts in the ethanolamine phosphatides, and in very small amounts in the 1,2-diacylglycerols. The relative proportions of these fatty acyl moieties differ considerably among the glycerolipid classes, and differ in each case from the relative proportions of the corresponding volatile compounds (Bjostad et al., 1981). Because the fatty acyl moieties are evidently precursors for the volatile sex pheromone components, these differences pose interesting questions regarding the regulation of the sex pheromone blend.

In a previous study we demonstrated that the gland is able to incorporate radiolabel from sodium [1-^{14}C]acetate into the volatile components (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, tetradecyl acetate, and dodecyl acetate, and also into (*Z*)-11-tetradecenoate, (*E*)-11-tetradecenoate, tetradecanoate, dodecanoate, hexadecanoate, and octadecanoate (Bjostad and Roelofs, 1981). In the present study we analyzed each glycerolipid class separately for incorporation of radiolabel from sodium [1-^{14}C]acetate into the fatty acyl moieties, extracting glands at several times after application of radiolabel in order to examine the time course of incorporation.

METHODS AND MATERIALS

Sodium [1-^{14}C]acetate (1.5–2 GBq/mmol) was obtained from Amersham Corp. (Arlington Heights, Illinois 60005). A solution of 37 MBq in 0.5 ml water was made, and 50 μl of the solution was mixed with 50 μl dimethyl sulfoxide to prepare a stock solution (37 kBq/μl) for topical application to pheromone glands.

Insects were reared on a semisynthetic medium (Shorey and Hale, 1965), and sexes were separated as pupae and maintained in a 16:8 light–dark cycle. Females (48–72 hr posteclosion) were anesthetized with CO_2 gas for 15 sec, their glands were everted by squeezing the tip of the abdomen with a small clip, and a droplet (0.2 μl) of the sodium [1-^{14}C]acetate stock solution was applied to each gland with a 10-μl syringe mounted on a micromanipulator (Bjostad and Roelofs, 1981). After a defined time interval, glands were excised from the ovipositors with fine forceps and extracted with 2:1 chloroform–methanol. The extract was washed with water, the water layer was extracted once with chloroform, and the organic portions were combined and filtered (Folch et al., 1957).

Lipid classes were separated by thin-layer chromatography. Precoated 20 × 20-cm Whatman K5 thin-layer chromatographic plates (Whatman Inc., Clifton, New Jersey) were broken into small plates (2 × 9 cm) and cleaned before use (Bjostad et al., 1981). Lipid standards were used to evaluate TLC separations. Choline phosphatides, ethanolamine phosphatides, monoacylglycerols, cholesterol, 1,2-diacylglycerols, 1,3-diacylglycerols, and triacylglycerols were obtained from Sigma Chemical Co. (St. Louis, Missouri). Methyl (*Z*)-9-octadecenoate and (*Z*)-11-hexadecenyl acetate were synthesized in our laboratory. Solvent systems used were (I) 62:34:4 (by volume) chloroform–methanol–water, (II) 40:50:2:0.2 (by volume) ether–benzene–ethanol–acetic acid, (III) 80:20:2 (by volume) hexane–ether–acetic acid, and (IV) 95:5 (by volume) hexane–ether.

Lipids on TLC plates were visualized by brief exposure to I_2 vapor and marked. When color was no longer visible, fatty acyl moieties were converted to methyl esters by acid methanolysis. Fractions were scraped into vials with Teflon-lined caps, 1 ml 40:20:1 (by volume) methanol–benzene–sulfuric acid was added to each vial, and the tightly capped vials were heated at 100° for 1 hr. Hexane (2 ml) was added to the cooled vials, and each organic layer was washed three times with water and filtered. Methanolysis generated long-chain alcohols from the pheromone components, and these were converted to acetates by evaporating the solvent with N_2, adding 100 μl acetyl chloride, and evaporating the residual acetyl chloride with N_2 after 30 min. Evaluation with synthetic compounds indicated that these reactions were essentially quantitative.

Gas–liquid chromatographic (GLC) separation of methyl esters and long-chain acetates was done with a column (glass, 3 m × 2 mm id) packed with 10% (w/w) XF-1150 (50% cyanoethyl methyl silicone) on 100–120 mesh Chromosorb W-AW-DMCS at 140° C. GLC fractions were collected in 30-cm glass capillary tubes and rinsed into scintillation vials with scintillation fluid (Bjostad and Roelofs, 1981). Samples were counted for 10 min.

The solution of sodium [1-^{14}C]acetate in dimethyl sulfoxide was applied topically to the glands of nine females. The droplet applied to each female contained 10 kBq (about 250,000 counts per minute). The glands were extracted after 4 hr, the extract was applied to a TLC plate with a syringe, and adjacent to it a mixture of lipid standards was applied (20 μg each). The plate was developed for 2 cm (measured from the origin) with solvent system I to separate ethanolamine phosphatides and choline phosphatides (neutral lipids migrated near the solvent front). The plate was dried for 10 min at 40° and developed for 4 cm with system II to separate cholesterol from 1,2-diacylglycerols (but other lipid classes overlapped). The plate was again dried and developed for 7 cm with system III to separate 1,2-diacylglycerols, 1,3-diacylglycerols, fatty acids, triacylglycerols, acetates, and methyl esters. The plate was dried a third time and developed for 7 cm with system IV to improve the separation of triacylglycerols, acetates, and methyl esters. Each

development required about 5 min. The plate was dried and exposed briefly to I_2 to verify R_fs of the lipid standards and of lipids in the gland extract. When color was no longer apparent on the plate, acid methanolysis and treatment with acetyl chloride was performed on each TLC fraction as described above. An aliquot of each treated fraction was analyzed by scintillation counting. The acetates or methyl esters from each lipid fraction were then separated by GLC and analyzed by scintillation counting.

A control experiment was done to verify that the sodium [1-^{14}C]acetate stock solution was not contaminated with other radioactive compounds that might appear in the fractions of the GLC collections. A sample of 100 kBq of the sodium [1-^{14}C]acetate solution was extracted with 2:1 chloroform–methanol and washed with water (Folch et al., 1957). The organic portion was filtered and evaporated to apparent dryness with a stream of nitrogen. Acid methanolysis and acetylation were performed as described above, and fractions were collected from the GLC and analyzed by scintillation counting. Less than 25 cpm were observed in any of the fractions.

Changes in the pattern of incorporation with respect to time were examined with additional experiments conducted for 40 min (14 females), 15 min (54 females), and 8 min (100 females). It was anticipated that as incubations of shorter durations were performed, the number of females required to obtain significant incorporation of radiolabel would increase, and the experiments were designed accordingly. Because the pheromone glands were tiny (about 0.5 mm diameter) and hydrophobic, considerable patience was required to apply the sodium [1-^{14}C]acetate solution uniformly to each gland. Experiments involving more than 100 females were considered impractical, and incubations shorter than 8 min were not attempted. The applied droplets were completely absorbed by the glands only in the 4-hr and 40-min experiments. In the 15-min and 8-min experiments, excess sodium [1-^{14}C]acetate solution was removed from the glands with a syringe prior to their dissection from the ovipositors. Only the acetate, triacylglycerol, and choline phosphatide fractions were analyzed in this and subsequent experiments because chains with 14 carbon atoms appeared to incorporate detectable amounts of label only in these classes in the 4-hr experiment, and a simpler TLC protocol was used to separate the lipid classes. The plate was developed for 2 cm with system I, dried, and developed for 7 cm with system III. The changes in the pattern of incorporation were further examined in experiments conducted for 15 min (54 females) and for 40 min (14 females).

RESULTS

Sequential development of TLC plates with the four solvent systems described above allowed one-dimensional separation of all the lipid standards tested. Pheromone gland extracts were separated by this technique, and

exposure to iodine vapor revealed spots with the R_f values of choline phosphatides, ethanolamine phosphatides, cholesterol (very faint), 1,2-diacylglycerols, triacylglycerols, and acetates (Figure 1).

In the 4-hr incubation experiment, scintillation counting showed that triacylglycerols incorporated the most label, acetates and choline phos-

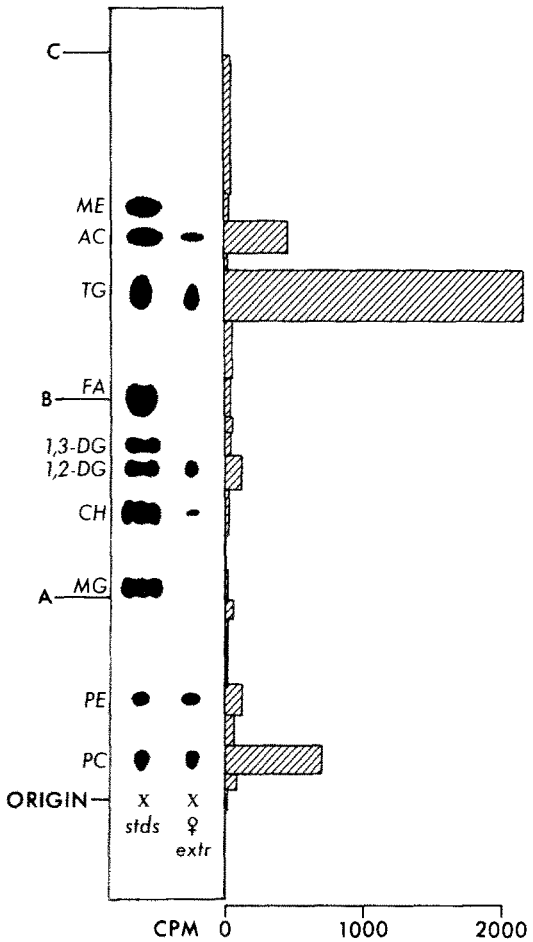

FIG. 1. Incorporation of [1-^{14}C]acetate into lipid classes in sex pheromone glands of *A. velutinana*. The plate was developed to A with solvent system I, to B with II, to C with III, and again to C with IV. Phosphatidylcholines = PC, phosphatidylethanolamines = PE, monoacylglycerols = MG, cholesterol = CH, diacylglycerols = DG, free fatty acids = FA, triacylglycerols = TG, acetates = AC, methyl esters = ME. Lipid standards were chromatographed in the left land and a female extract was chromatographed in the right lane.

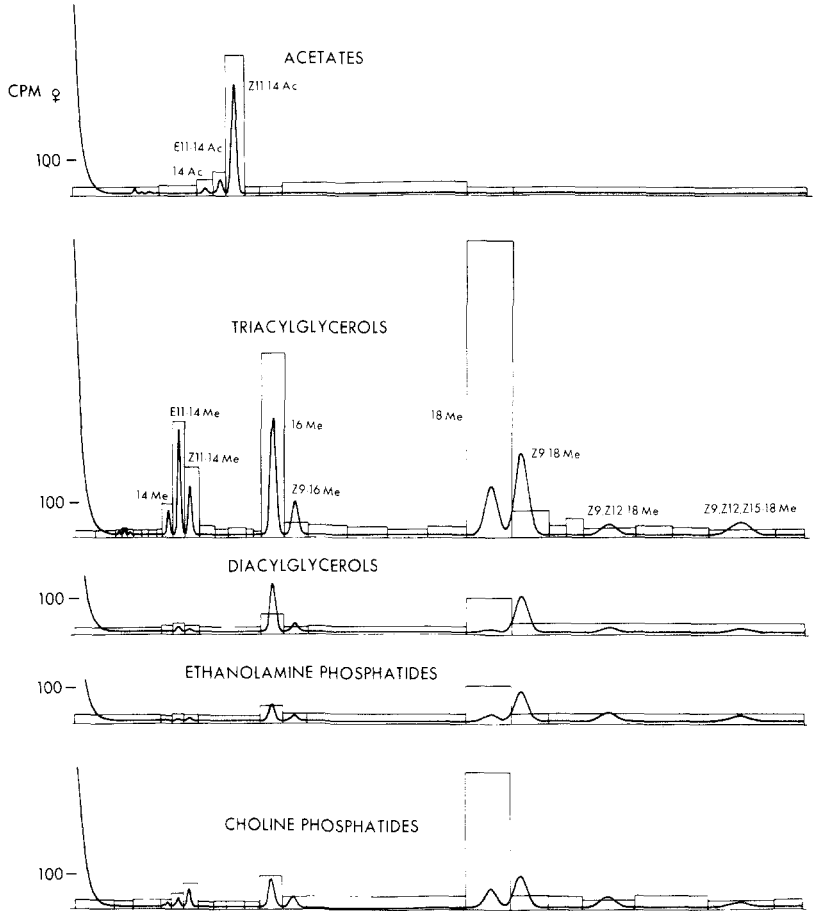

FIG. 2. Incorporation of [1-^{14}C]acetate after a 4-hr incubation. Histogram of counts per minute in each GLC fraction is superimposed on GLC peaks detected by flame ionization. (*Z*)-11-tetradecenyl acetate = Z11-14:Ac, methyl (*Z*)-11-tetradecenoate = Z11-14:Me, etc.

phatides incorporated less label, and 1,2-diacylglycerols and ethanolamine phosphatides incorporated only small amounts of label (Figure 1). In the acetate fraction, label was incorporated into (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, and tetradecyl acetate (Figure 2), essentially in proportion to their relative abundances in the gland. Dodecyl acetate also occurs in small amounts in the sex pheromone gland (Bjostad et al., 1981), but incorporation of label was below the limit of detection in this experiment. In the triacylglycerols, label was incorporated into (*Z*)-11-tetradecenoate, (*E*)-11-tetradecenoate, tetradecanoate, hexadecanoate, and octadecanoate. Ra-

diolabel was incorporated in smaller amounts into the same fatty acyl moieties in the choline phosphatides (except that radiolabeled tetradecenoate was below the limit of detection), but the relative proportions of radiolabel differed from the triacylglycerols. In the diacylglycerols and ethanolamine phosphatides, label was detected only in hexadecanoate and octadecanoate. Very little radiolabel was incorporated into (*Z*)-9-hexadecenoate, (*Z*)-9-octadecenoate, (*Z*,*Z*)-9,12-octadecadienoate, or (*Z*,*Z*,*Z*)-9,12,15-octadecatrienoate, despite the fact that these fatty acyl moieties were relatively abundant in all the glycerolipid classes in the gland. Dodecanoate also occurs in small amounts in these glycerolipids (Bjostad et al., 1981), but incorporation of label was below the limit of detection.

As experiments of shorter duration were conducted, the overall incorporation of radiolabel into pheromone components and fatty acyl moieties decreased, as expected. In the 4-hr experiment with nine females, the overall incorporation was 0.27%. In the 40-min experiment with 14 females, it was 0.028%. In the 15-min experiment with 54 females, it was 0.006%. In the 8-min experiment with 100 females, it was 0.005%. Despite the lower rates of incorporation for shorter incubation intervals, the greater number of females used in successively shorter incubations was sufficient in each case to allow observation of significant incorporation of radiolabel above background into the compounds of interest. The control experiment with the stock solution of sodium [1-^{14}C]acetate verified that a background of less than 25 counts per minute was present in any of the GLC fractions, the same value found for the scintillation fluid alone.

In the incubation experiment of shortest duration (8 min), (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, and tetradecyl acetate incorporated radiolabel (Figure 3) in about the same relative proportions as in the longest incubation tested (4 hr). The pattern of incorporation among the fatty acyl moieties in the 8-min experiment was very different than in the 4-hr experiment, however. After 8 min incubation, approximately equal amounts of label had been incorporated into (*Z*)-11-tetradecenoate, (*E*)-tetradecenoate, and tetradecanoate in the triacylglycerols. In contrast, in the 4-hr experiment, (*E*)-11-tetradecenoate contained more radiolabel than did (*Z*)-11-tetradecenoate in the triacylglycerols, and both contained more radiolabel than tetradecanoate. This change in the relative proportions of radiolabel in the saturated, (*E*)-11, and (*Z*)-11 isomers was a consistent function of time of incubation (Figure 4). Contingency chi-square analysis (Steele and Torrie, 1960) indicated that the observed change in proportions was statistically significant ($P < 0.05$).

The relative proportions of radiolabel in hexadecanoate and octadecanoate in the triacylglycerols also changed greatly with respect to time of incubation (Figures 2 and 3). Radiolabeled hexadecanoate decreased in relative abundance with increasing time of incubation, and radiolabeled

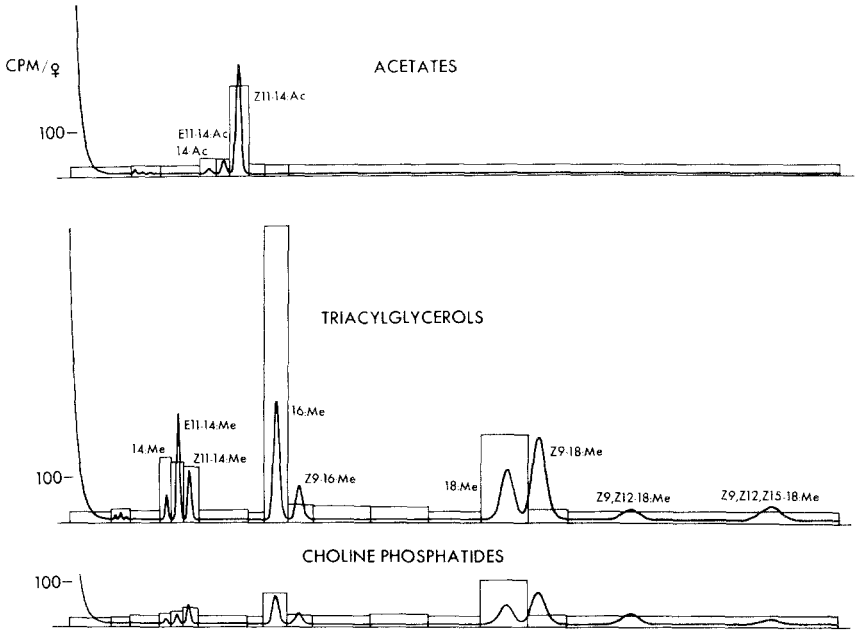

FIG. 3. Incorporation of [1-^{14}C]acetate after an 8-min incubation. Symbols as in Figure 2.

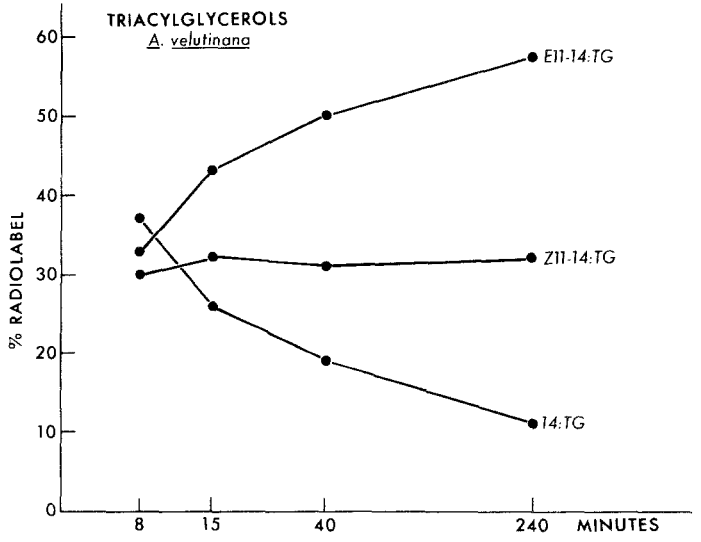

FIG. 4. Distribution of radiolabel among biosynthetic precursor fatty acyl moieties in the triacylglycerols. Z11-14:TG = (Z)-11-tetradecenoate moieties in the triacylglycerols, etc.

octadecanoate increased in relative abundance. In the choline phosphatides, radiolabeled octadecanoate was more more abundant than radiolabeled hexadecanoate at all times of incubation, but the proportion of radiolabel in octadecanoate relative to that in hexadecanoate increased as a function of incubation time as well. These changes were consistent with respect to time of incubation, and contingency chi-square analysis showed that the observed changes in proportions were statistically significant ($P < 0.05$).

DISCUSSION

The relative proportions of radiolabel found in (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, and tetradecyl acetate were essentially the same as the relative amounts of these compounds in the pheromone gland (91:9:3, respectively), and changed very little with respect to time of incubation with sodium [1-^{14}C]acetate. This is consistent with the results of Miller and Roelofs (1980), who analyzed individual pheromone glands by GLC and found little variation in the ratio of *E* to *Z* (9.1% ± SD 1.8%).

The relative proportions of the precursor fatty acyl moieties (*Z*)-11-tetradecenoate, (*E*)-11-tetradecenoate, and tetradecanoate were considerably different in the gland (Figure 2). The bulk of these fatty acyl moieties occurred in the triacylglycerols, in the proportions 2:3:1, respectively (Bjostad et al., 1981). In the longest incubation with sodium [1-^{14}C]acetate performed (4 hr), the proportions of radiolabel found in these fatty acyl moieties were essentially the same as their relative abundances in the gland. In contrast, after shorter incubations, the proportions of radiolabel incorporated into these fatty acyl moieties were significantly different (Figure 4). The relative proportion of tetradecanoate decreased as a function of time of incubation, and the relative proportion of (*E*)-11-tetradecenoate increased. Tetradecanoate is a precursor for (*Z*)-11-tetradecenoate and (*E*)-11-tetradecenoate (Bjostad and Roelofs, 1981), and this likely accounts for its relative decrease with time of incubation. Because the pheromone component (*Z*)-11-tetradecenyl acetate was more than ten times as abundant in the gland as the component (*E*)-11-tetradecenyl acetate, it appears that from the pool of precursor (*Z*)-11-tetradecenoate and (*E*)-11-tetradecenoate moieties in the gland, the *Z* isomer is preferentially selected for conversion to the corresponding acetate. The (*E*)-11-tetradecenoate therefore accumulates, and this likely accounts for its relative increase with time of incubation. It is also possible, however, that the results observed in Figure 4 are due to changes that occur after sex pheromone biosynthesis has taken place, perhaps involving selective removal and degradation of tetradecanoate and (*Z*)-11-tetradecenoate from the triacylglycerols.

These findings have some implications regarding regulation of the pheromone blend. If any particular carrier protein or enzyme (such as an

acyltransferase or a reductase) acted on both geometric isomers, but preferentially selected the *Z* isomer, then the relative proportion of the remaining *E* isomer would increase with time, and the *E*/*Z* ratio of the pheromone components would also be expected to increase with time. The gland might circumvent this problem by continually providing such an enzyme with a newly synthesized pool of *Z* and *E* fatty acyl moieties in a certain ratio and by making unavailable to the enzyme the pool of fatty acyl moieties from which selection had already been made. One candidate for such a pool may be the surfaces of the lipid spheres observed in the gland by Feng and Roelofs (1977). The lipid spheres were found to increase greatly in size following the emergence of the adult female. Triacylglycerols would be expected to occur in these lipid spheres. Because the triacylglycerols biosynthesized by the gland consist principally of hexadecanoate and octadecanoate, moieties that would be expected to increase the melting point of the triacylglycerols greatly, it is possible that triacylglycerols elaborated on the surface of a lipid sphere undergo very little mixing with the interior of the sphere. If this is true, newer triacylglycerols may bury older triacylglycerols, effectively presenting a newly synthesized pool of (*Z*)- and (*E*)-11-tetradecenoyl moieties to enzymes at the surface of the sphere. We cannot yet rule out other possible mechanisms that might, for example, involve a pair of enzymes, one specific for *Z* fatty acyl moieties and the other specific for *E* fatty acyl moieties. If the gland contained *Z*-specific and *E*-specific enzymes in an appropriate ratio, the correct blend of pheromone components would be produced despite changes in the blend of *Z* and *E* fatty acyl precursors, as long as sufficient quantities of both precursors were available to make the rates of reaction of the two enzymes independent of fatty acyl substrate concentrations.

In a previous paper (Bjostad and Roelofs, 1981) we concluded that hexadecanoate in the sex pheromone gland of *A. velutinana* is chain-shortened to tetradecanoate, which is then desaturated to form (*Z*)-11- and (*E*)-11-tetradecenoyl moieties that are reduced and acetylated to form the principal sex pheromone components (*Z*)-11- and (*E*)-11-tetradecenyl acetates. In the present study, the relative proportion of labeled hexadecanoate in the glycerolipids was much greater in short-term incubations than in long-term incubations. This is consistent with our proposal that tetradecanoate in the gland arises by chain-shortening of hexadecanoate (Bjostad and Roelofs, 1981). The chain-shortening reaction has recently been demonstrated in the related species *Argyrotaenia citrana* as well (Wolf and Roelofs, 1983). The great increase in radiolabeled octadecanoate with time is probably due to chain-lengthening of hexadecanoate (Volpe and Vagelos, 1976).

It was expected that after radiolabel began to appear in (*E*)- and (*Z*)-11-tetradecenoate moieties in the glycerolipids, there would be a time lag before radiolabel would begin to appear in the pheromone components. Instead,

appreciable amounts of radiolabel appeared in the pheromone components even at the earliest times tested. This implies that once hexadecanoate becomes available, the remaining steps in the biosynthesis of the sex pheromone components proceed rapidly. This may be because hexadecanoate is produced by fatty acid synthetase, which is soluble in the cytosol (Volpe and Vagelos, 1976), whereas the subsequent steps (chain-shortening, desaturation, reduction, and acetylation) are all likely to involve membrane-bound enzymes (Bell and Coleman, 1980; Holub and Kuksis, 1978) that may be closely associated.

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Journal of Chemical Ecology, Vol. 10, No. 4, 1984

Errata

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There was a typographical error in the title of this article; namely, *Glomeris* was spelled with a terminal *c* rather than with an *s*.

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CHEMICAL STIMULI IN HOST-HABITAT LOCATION BY *Leptopilina heterotoma* (Thomson) (Hymenoptera: Eucoilidae), A PARASITE OF *Drosophila*

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Abstract—Chemical stimuli play an important role in the process of searching for a host habitat by parasitic wasps. Volatile compounds originating from host habitats and/or hosts are the cues that enable such a location. *Leptopilina heterotoma,* a larval parasite of *Drosophila,* is attracted to the food of its host, baker's yeast. Analysis of the fermentation products of baker's yeast, using a mass spectrometer, and olfactometer studies indicate that three fermentation products of this yeast, the main component of the host habitat in our laboratory, attract *L. heterotoma:* ethanol (5%), ethyl acetate (10^{-2}, 10^{-3}%), and acetaldehyde (1%). A combination of these three compounds, however, cannot compete with baker's yeast in attracting the parasites. Thus other factors, such as different compounds, concentrations, and/or combinations, also play a role and remain to be tested. *Leptopilina heterotoma* does not use host-related olfactory cues in long-distance habitat location as it cannot distinguish between host habitat and host habitat with hosts.

Key Words—*Leptopilina heterotoma,* Hymenoptera, Eucoilidae, *Saccharomyces cerevisiae,* host-habitat searching, chemoreception, fermentation products, ethanol, ethyl acetate, acetaldehyde.

INTRODUCTION

The way in which a parasite locates and selects host habitats and the way in which it allocates searching time to patches of different host quality (host

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density, host species, and host age) are topics much discussed by students of optimal foraging and biological pest control (e.g., Waage, 1979; Nordlund et al., 1981; Van Alphen, 1982). The models that have recently been developed in the field of optimal foraging assume that host searching and parasitization have been optimized through the process of natural selection (e.g., Pyke et al., 1977; Waage and Hassell, 1982; McArthur and Pianka, 1966). This would mean that two decisions should be made by the foraging organism: (1) which sites to go to, and (2) how long to stay in one particular site.

In this paper the role played by chemical stimuli in the location of the host habitat (fermenting fruit) and of the host (larvae of *Drosophila*) by the parasite *Leptopilina heterotoma* (=*Pseudeucoila bochei*) is discussed. In another paper (Dicke et al., 1983) the influence of *Drosophila* kairomones on patch-time allocation as well as the purification of the kairomone will be discussed. We previously published studies on other factors that influence allocation of patch-time, i.e., size of the patch (Van Lenteren and Bakker, 1978) host density (Van Lenteren and Bakker, 1976, 1978), and host suitability (Van Lenteren, 1976, 1981; Carton et al., 1983). Doutt (1964) divided the process of locating and parasitizing hosts by parasites in three steps: (1) location of the host habitat, (2) location of the hosts, and (3) acceptance of the host.

The first two of these steps, which may result in contact with the host, correspond to the two decisions mentioned above that parasites are assumed to make, whereas the third step also influences the second decision. The importance of host-habitat location was shown by Picard and Rabaud (1914), who observed that many parasitic Hymenoptera attack larvae of insect species belonging to different families and even different orders if these species occur on the same plant (i.e., the same host habitat). An attraction to a specific host habitat has been reported for several species of parasites [e.g., *Cardiochiles nigriceps* (Vinson, 1975), *Apantheles glomeratus* (Salt, 1958), *Itoplectus conquistor* (Arthur, 1962), and *Leptopilina boulardi* (=*Cothonaspis* sp.) (Carton, 1976)]. This attraction is often mediated by volatile chemical stimuli (e.g., Carton, 1976). Vinson (1981) states that there has been relatively little work towards elucidating the factors important in habitat location, and he expects the potential sources of habitat-location cues to be present in the host's food or shelter, the host, non-host-associated organisms, or the interaction between these factors. He further says that chemicals appear to play the major role in the orientation of many insects.

METHODS AND MATERIALS

Host Habitat, Hosts, and Parasites. The most important part of the host habitat is yeast. We took baker's yeast (*Saccharomyces cerevisiae* Hansen) to

simulate the host habitat because it is routinely used for rearing *Drosophila* in the laboratory, although under natural conditions this yeast species is not found on breeding sites (Carson et al., 1956). Yet *Drosophila* is abundantly attracted to baker's yeast in the field. Because of the fact that extensive research has been done on baker's yeast, we decided to use this yeast species in our experiments. An additional reason is that baker's yeast is available in a form that favors standardization of use of this yeast in experiments. The yeast is available in living dry form (Engedura, Gist-Brocades, Delft, Netherlands). It is used as a suspension of 25 g/100 ml water. When used as medium for *Drosophila* larvae, the superfluous water was evaporated by placing the yeast suspension in the air stream of a ventilator.

Larvae of the WW strain of *Drosophila melanogaster,* that has been reared in our laboratory for the last 20 years, were used as hosts. For rearing methods see Bakker (1961). In all experiments larvae were kept at 25° C and used when 24 hr old.

The parasites used belonged to the Storrs strain of *Leptopilina heterotoma* that has been reared in our laboratory for the last 12 years. In some experiments parasites of the first generation from field-collected material reared in the laboratory were used. For rearing methods see Bakker et al. (1976). In all experiments the parasites used were 7–14 days old. During this period the parasites were kept at a temperature of 13° C. Parasites that parasitize hosts without previous experience have difficulties in stabbing the host and laying an egg. The parasites have to learn how to parasitize (Samson-Boshuizen et al., 1974). Oviposition experience also increases motivation to search for hosts (Van Lenteren, 1976). Therefore, experienced females were used in all experiments except for the climate-room experiment. All experiments were done at 25 ± 1° C and a relative humidity of 60 ± 10%.

Climate-Room Experiment. A preliminary experiment was conducted to ascertain the long-range orientation of this parasite. Six jars with agar, two of which contained host larvae, two yeast, and two yeast + host larvae, were placed in an illuminated climate room. The jars stood 1.5 m away from a central release point and the distance between the jars was equal. Five hundred inexperienced females were released and after 1 and 5 hr the wasps in each jar were counted.

The Olfactometer. An airflow olfactometer of the model of Pettersson (1970) was used to study stimuli that may give the parasite information on the quality and the location of the host habitat. For a detailed description of the set-up and use of this olfactometer, see Vet et al. (1983). The model of the olfactometer is shown in Figure 1 because we used it in a slightly different way. The parasites were tested individually; they were introduced through the central opening in the olfactometer. After introduction, the airflow tube was reconnected, the parasite was then exposed to a mixture of the odors offered until it reached the surface of the exposition chamber where it could

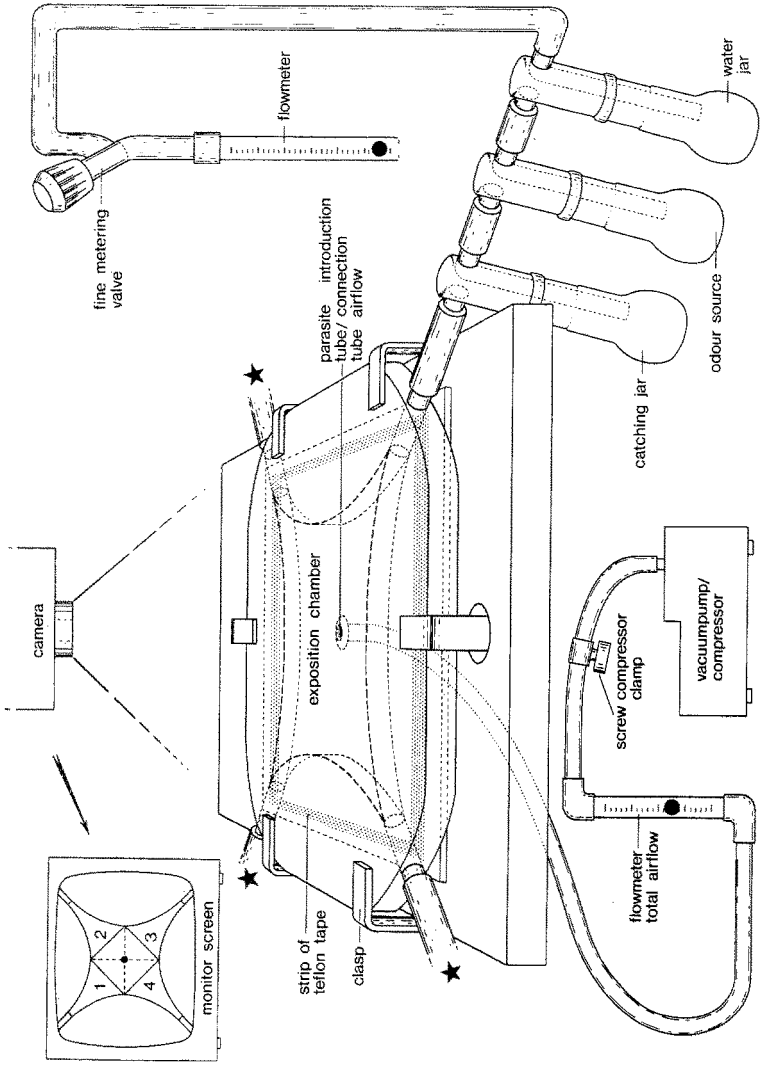

FIG. 1. Perspective view of the airflow olfactometer. Air is drawn through the exposure chamber equally from four odor sources and flows towards the center of the chamber and out through the central hole (parasite introduction tube) (after Vet et al., 1983).

choose and enter one of the odor fields. We have defined this entering of a first odor field as *first choice.* After having recorded the first choice, the parasite was left another 10 min in the olfactometer. For some of the experiments, data about a second choice are given; this *second choice* is defined as crossing of one of the lines of the arbitrary square (see Figure 1) by the parasite. [Vet et al. (1983) describe this as the first choice.] By distinguishing these two choices, the influence of odors that do not direct the parasite towards the source of odor production, but only arrest the parasite, can be taken into account. During the 10-min period, the times when the parasite left and entered different odor fields were recorded. The parasites might walk up one of the airflows out of the exposure chamber to end up in one of the collection jars. This is defined as a *final choice.*

Kennedy (1977) described different forms of orientation behavior of parasites. He distinguishes orthokinesis (upon perceiving the stimulus the parasite slows down its walking speed or stops), klinokinesis (the parasite increases its rate of turning influenced by external stimulation, the turning is random in orientation), and klinotaxis (the parasite increases its rate of turning, oriented with respect to the source of the stimulus, i.e., directed movement in our case). In our experiments we can speak of kinesis (either ortho- or klino-) when the odor source evokes a reaction in which the parasites make a first choice but seldom a second or final choice. On the other hand a high percentage of final choices means that the reaction is one of taxis. Another difference with Vet et al.'s (1983) description of the use of the olfactometer lies in the evaluation of the time spent in the different odor areas. For part of the experiments we give the number of parasites that spent the longest time period in the different odor fields. These numbers are evaluated using the chi square test. Vet et al. (1983) evaluate the relative times allocated to the different fields with the Friedman two-way analysis of variance by ranks.

Test Substances for Olfactometer. When yeast was used in the olfactometer, it was either as a thin 25 g/100 ml suspension or as a thicker suspension after evaporation of the superfluous water (called host-habitat). The host habitats with and without hosts were prepared as follows: In the first series of olfactometer experiments, yeast spots were made shortly before the start of the experiments; 2 ml of the yeast suspension was put on top of an agar substrate (4%) and the superfluous water was evaporated. To some of these yeast spots 200 host larvae were added shortly before the experiment started.

In the second series of olfactometer experiments, the preparation of spots with and without hosts was slightly different: 5 ml yeast solution from which the superfluous water was evaporated was incubated at 25° C for 20 hr with 200 host larvae or without larvae. Then these host habitats were transferred to the olfactometer vials in which some agar (4%) was present and

supplemented with 2.5 ml host-free host habitat (yeast) that had undergone the same procedure.

The solutions of chemicals that were tested had concentrations based on data from the literature [acetic acid (Barrows, 1907; Fluegel 1981)], our mass-spectrometric analysis (methanol, acetaldehyde, and formaldehyde) in which the ethanol concentration is assumed to be 5% (Hwang et al., 1978), or arbitrarily chosen concentrations (diacetyl, acetaldehyde). During the experiment these solutions were renewed every half hour. When several odor substances were offered in one arm of the olfactometer, they were present in separate vials, unless mentioned otherwise.

Analysis of Fermentation Products. To examine the fermentation products of *S. cerevisiae* (under conditions without an additional food source), these products were concentrated using a cold trap and were analyzed by means of a mass spectrometer. A LKB-2091 (EI/CI) GC-MS computer system was used (ion source temperature 150–210°C, box current 250 μA, box electrovoltage 250 eV). Chemical ionization spectra were obtained with isobutane as a reactant gas. The mass spectra were recorded continuously and stored in a Digital PDP-11 computer.

RESULTS

Climate-Room Experiment. One hour after release of the 500 females, the two yeast and the two yeast + host jars contained equal numbers of parasites (28–40 parasites per jar, see Table 1), whereas the jars with only hosts contained no parasites. Five hours after release, the jars with yeast and yeast + hosts again contained about equal numbers of parasites (58–85 per jar), and no parasites were found in the jars with host larvae only. From this we may conclude that long-range attraction occurs through a factor produced by the fermenting yeast. Further, we may conclude that at these distances the parasites cannot determine whether hosts are present in a potential habitat,

TABLE 1. HOST-HABITAT LOCATION IN *Leptopilina heterotoma*, CLIMATE-ROOM EXPERIMENT[a]

Number of females	2 jars with hosts (100 24-hr-old *D. melanogaster*)	2 jars with yeast (2 ml)	2 jars with yeast + hosts (2 ml + 100 24-hr-old *D. melanogaster*)
After 1 hour	0	73(40 + 33)	65(28 + 37)
After 5 hours	0	127(58 + 69)	146(61 + 85)

[a]500 ♀♀ released in center of room, jars with test material at 1.5 m from release point.

which means that we can exclude influence of sound and chemicals produced by hosts in long-range orientation. Similar data were obtained when the lights were switched off, so vision does not play a role in long-range orientation.

Olfactometer Experiments. The preliminary findings of the climate-room experiment were checked in the airflow olfactometer. In this set-up the influence of stimuli other than volatile chemical sources (e.g., sound and light) is excluded. To determine whether the olfactometer is set up properly, we first tested whether the parasites made a random choice when four fields were created containing the same odor (Table 2A). The data show no significant bias exists: about the same number of parasites chose each odor field and the amounts of time spent per odor field are also equal.

TABLE 2. HOST-HABITAT LOCATION IN *Leptopilina heterotoma*, OLFACTOMETER EXPERIMENTS[a]

A. Test for bias in the system. Four equal odor fields produced by living yeast (2 ml), 50 ♀♀ tested.

	No choice	Field 1 yeast	Field 2 yeast	Field 3 yeast	Field 4 yeast	*P*
First choice (No.)	0	12	9	15	14	>0.05
Final choice (No.)	25	6	8	6	5	>0.05
% time per odor field		22.4	24.8	24.0	28.8	

B. Test with host and habitat material prepared shortly before experiment, 200 ♀♀ tested.

	No choice	Field 1 control	Field 2 hosts (100 larvae)	Field 3 yeast (2 ml)	Field 4 yeast + hosts (2 ml + 100 larvae)	*P*
First choice (No.)	4	37	34	68	57	<0.005
Final choice (No.)	31	32	25	55	57	<0.005
% time per odor field		18.2	16.3	28.1	30.2	

C. Test with host and habitat material prepared 20 hr before the experiment, 82 ♀♀ tested.

	No choice	Yeast + hosts (7.5 ml + 200 larvae)	Yeast (7.5 ml)	Control (2 fields)
First choice (No.)	0	37 a	38 a	7 b
Second choice (No.)	5	38 a	33 a	6 b
Final choice (No.)	14	38 a	28 a	2 b
Longest time in odor field (No.)		42 a	34 a	6 b

TABLE 2. Continued

D. Test with dead yeast and water as control, 43 ♀♀ tested.

	No choice	Dead yeast (5 ml, 2 fields)	Water (5 ml, 2 fields)
First choice (No.)	0	27 a	16 b
Second choice (No.)	0	33 a	10 b
Final choice (No.)	3	36 a	4 b
Longest time in odor field (No.)		35 a	8 b

E. Test with living and dead yeast. The dead yeast is a yeast solution that has been boiled for 5 min before the experiment, 36 ♀♀ tested.

	No choice	Living yeast (5 ml)	Dead yeast (5 ml)	Control (2 fields)
First choice (No.)	0	22 a	6 b c	8 d
Second choice (No.)	0	23 a	5 b c	8 d
Final choice (No.)	1	25 a	8 b c	2 d
Longest time in odor field (No.)		26 a	8 b c	2 d

F. Test with living yeast and living yeast to which sugar is added 30 min before the experiment, 57 ♀♀ tested.

	No choice	Yeast (5 ml)	Yeast + sugar (5 ml + 2.5 g)	Control (2 fields)
First choice (No.)	0	20 a	25 a	12 b
Second choice (No.)	1	23 a	25 a	8 b
Final choice (No.)	15	19 a	20 a	3 b
Longest time in odor field (No.)		26 a	26 a	5 b

[a]Different letters next to figures in C–F mean that (pairs of) figures in the same row differ significantly ($P < 0.05$).

In the following experiment the same materials were offered as in the climate-room experiment. The data of 200 individually tested females are presented in Table 2B. Significantly more females (chi square test for random distribution, P values in table 2B) entered the odor fields of either the yeast or the yeast + hosts. The remaining females went, in about the same numbers, to the odor field of hosts or the field without a specific host-related smell (control). One hundred twelve females made a final choice for the fields with yeast odor, a significantly lower number (57) chose the control field and the

field with host odor. For the choice between fields for yeast and yeast + hosts, no significant differences are found; the same holds for the choice between the control field and the field with host odor. We may conclude that parasites did not distinguish between yeast odor only and yeast + host odor: long-range orientation seems to depend on factors of fermenting yeast only, and host odors do not play a role.

An objection to this conclusion can be that the test materials were prepared shortly before the experiment. A more natural habitat is one in which host larvae had been present since the time they had hatched from the egg. An experiment was therefore done in which larvae had been introduced in the yeast spots 20 hr before the test. The results in Table 2C clearly show that here, too, the parasites did not distinguish yeast + host odor fields from yeast odor fields. The previous conclusions can thus be maintained.

To determine whether the attraction to yeast is mainly caused by the fermentation products, two experiments were performed. In the first, only dead baker's yeast (*S. cerevisiae* siccum, Brocacef, Maarssen, Netherlands) was offered, and the data show that the parasites are attracted by this dead yeast (Table 2D). However, when the parasites were offered both living baker's yeast and baker's yeast that has been boiled for 5 min they preferred living yeast (Table 2E). This indicates that, indeed, the factors causing attraction are mainly present in the products of metabolism. The nutritive situation of the yeast does not seem to influence the attraction of the parasites: when a yeast suspension in water was offered along with a yeast suspension in water to which sugar was added (5 g of sugar per 10 ml yeast suspension, 30 min before the start of the experiment), the same number of parasites was found in both odor fields (Table 2F).

A mass-spectrometric analysis was made to identify the products which evaporate from a living yeast suspension. The result of this analysis is a number of fermentation products on the basis of mass spectra. Combining these results with knowledge of fermentation processes gives an indication of the possible presence of some fermentation products. According to this analysis it appears that methanol, ethanol, formaldehyde, acetaldehyde, and formic acid are produced. At the same time we did our mass-spectrometric analysis, Williams et al. (1981) published a paper in which 80 fermentation products identified from baker's yeast were mentioned. The eight most abundant of these are: ethanol, acetic acid, 2 and/or 3 methyl butanol, *n*-propanol, ethyl acetate, 2-phenethanol, isobutanol, and acetaldehyde. The information from these two sources was used to test the (possible) fermentation products (which are mentioned below) in the olfactometer for their influence on *L. heterotoma*.

The results (Table 3A–C) show that the parasites are attracted by solutions of ethanol (5%), acetaldehyde (1%) and ethylacetate (10^{-2}, 10^{-3}%).

TABLE 3. REACTION OF *Leptopilina heterotoma* TO CHEMICAL COMPONENTS OF HOST HABITAT IN AN OLFACTOMETER[a]

A. Test with ethanol (5% solution) and water as control, 48♀♀ tested.

	No choice	Ethanol (2 fields)	Control (2 fields)
First choice (No.)	0	34 a	14 b
Second choice (No.)	2	36 a	10 b
Final choice (No.)	12	25 a	11 b
Longest time in odor field (No.)		31	17

B. Test with acetaldehyde (1% solution) and water as control, 55♀♀ tested.

	No choice	Acetaldehyde (2 fields)	Control (2 fields)
First choice (No.)	0	41 a	14 b
Second choice (No.)	14	32 a	9 b
Final choice (No.)	39	12	4
Longest time in odor field (No.)		41 a	14 b

C. Test with ethyl acetate (10^{-2}% and 10^{-3}% solution) and water as control, 50♀♀ tested.

	No choice	Ethyl acetate 10^{-2}%	Ethyl acetate 10^{-3}%	Control (2 fields)
First choice (No.)	0	17	12	21
Second choice (No.)	5	14 a	18 a	13 b
Final choice (No.)	23	11 a	11 a	5 b
Longest time in odor field (No.)		17 a	21 a	12 b

D. Test with acetaldehyde (1%) + yeast, yeast, acetaldehyde (1%), and water as control, 63♀♀ tested.

	No choice	Acetaldehyde + yeast	Yeast		Acetaldehyde	Control
First choice (No.)	0	38 a	20 b	c	5 d	0 d
Second choice (No.)	0	37	20	c	5 d	1 d
Final choice (No.)	15	32 a	16 b	c	0 d	0 d
Longest time in odor field (No.)		38 a	20 b	c	5 d	0 d

TABLE 3. Continued

E. Test with ethanol (5%) + yeast, yeast, ethanol (5%), and water as control, 87 ♀♀ tested.

	No choice	Ethanol + yeast	Yeast	Ethanol	Control
First choice (No.)	0	32 a	43 a	7 b	5 b
Second choice (No.)	8	32 a	39 a	5 b	3 b
Final choice (No.)	32	24 a	25 a	4 b	2 b
Longest time in odor field (No.)		30 a	45 a	8 b	4 b

F. Test with acetic acid (3% solution) and water as control, 50 ♀♀ tested.

	No choice	Acetic acid (2 fields)	Control (2 fields)
First choice (No.)	0	30	20
Second choice (No.)	3	33 a	14 b
Final choice (No.)	14	18	18
Longest time in odor field (No.)		23	27

G. Test with formaldehyde (1% solution) and water as control, 53 ♀♀ tested.

	No choice	Formaldehyde (2 fields)	Control (2 fields)
First choice (No.)	0	27	26
Second choice (No.)	9	20	24
Final choice (No.)	27	17	9
Longest time in odor field (No.)		25	28

H. Test with methanol ($\frac{1}{4}$% solution) and water as control, 50 ♀♀ tested.

	No choice	Methanol (2 fields)	Control (2 fields)
First choice (No.)	0	26	24
Second choice (No.)	0	27	23
Final choice (No.)	8	19	23
Longest time in odor field (No.)		24	26

TABLE 3. Continued

I. Test with diacetyl (10^{-1}% and 10^{-2}% solutions) and water as control, 59 ♀♀ tested.

	No choice	Diacetyl, 10^{-1}%	Diacetyl, 10^{-2}%	Control (2 fields)
First choice (No.)	0	19	15	25
Second choice (No.)	5	21	12	21
Final choice (No.)	21	13	10	15
Longest time in odor field (No.)		21	15	23

J. Test with ethanol (5% solution) and yeast as control, 23 ♀♀ tested.

	No choice	Ethanol (2 fields)	Control (2 fields)
First choice (No.)	0	5 a	18 b
Second choice (No.)	0	1 a	22 b
Final choice (No.)	0	1 a	22 b
Longest time in odor field (No.)		1 a	22 b

K. Test with a mixture of ethanol (5%), acetaldehyde (1%), and ethyl acetate (10^{-2}%), yeast and water, 40 ♀♀ tested.

	No choice	Mixture	Yeast	Water (2 fields)
First choice (No.)	0	8 a	26 b	6
Second choice (No.)	0	3 a	32 b	5
Final choice (No.)	7	2 a	28 b	3
Longest time in odor field (No.)		3 a	33 b	4

[a]The chemical solutions were offered in quantities of 5 ml that were renewed every 30 min. Different letters after figures mean that (pairs of) figures in the same row differ significantly ($P < 0.05$).

Parasites even distinguish between yeast and yeast in combination with acetaldehyde (Table 3D), but do not do so between yeast and yeast in combination with ethanol (Table 3E). Parasites are not attracted by acetic acid (3%), formaldehyde (1%), methanol (0.25%), and diacetyl (10^{-1}, 10^{-2}%) (Table 3F–I). In all these experiments the control was water. Tables 3J and K show the results of two experiments in which solutions of chemical compounds were tested with yeast as control. In the second of these two experiments, in which the three attractive compounds were tested in combination, they were present in the same vial of the olfactometer. In both cases yeast was significantly more attractive.

TABLE 4. HOST-HABITAT LOCATION IN *Leptopilina heterotoma* REARED IN LABORATORY FOR ONLY ONE GENERATION[a]

A. Test with host and habitat material prepared 20 hr before the experiment, 51 ♀♀ tested.

	No choice	Yeast (7.5 ml)	Yeast + host (7.5 ml + 200 larvae)	Control (2 fields)
First choice (No.)	0	21 a	21 a	9 b
Second choice (No.)	4	24 a	19 a	4 b
Final choice (No.)	11	23 a	16 a	1 b
Longest time in odor field (No.)		26 a	21 a	4 b

B. Test with acetic acid (3% solution) and water as control, 50 ♀♀ tested.

	No choice	Acetic acid (5 ml, 2 fields)	Control (2 fields)
First choice (No.)	0	25	25
Second choice (No.)	6	23	21
Final choice (No.)	33	8	9
Longest time in odor field (No.)		14 a	36 b

[a] Different letters after figures mean that figures in the same row differ significantly ($P < 0.05$).

In all the above-described experiments, parasites were used that belong to a strain that has beeen reared in the laboratory for 12 years. It is possible that the searching behavior has changed because selection pressures are different under laboratory and field conditions. Therefore we carried out two experiments identical to two experiments described above (Tables 2C and 3F) but with parasites that form a first generation in the laboratory. From the results (Table 4) it appears that the field and laboratory strains do not differ in their choices.

DISCUSSION

A number of chemical stimuli play a role in host-habitat location by *Leptopilina heterotoma.* Host-habitat location is a long-range chemoreception process which Weseloh (1981) says can be demonstrated if it can be shown that an organism responds behaviorally to an obviously airborne chemical.

Leptopilina heterotoma is attracted by both living and dead baker's yeast, one of the food sources of its host in laboratory rearings and in experiments. However, living baker's yeast is much more attractive than dead

yeast. This indicates that fermentation products of yeast play an important role, although attraction of the parasite by dead yeast suggests that compounds in the yeast itself are of importance as well.

Drosophila larvae not only use yeast cells as food (Baumberger, 1919), but also its fermentation products like ethanol (McKenzie and McKechnie, 1979; Parsons and Spence, 1981), primary alcohols in general (Van Herrewege et al., 1980), and acetic acid (Parsons, 1980). Fermentation products stimulate the development of larvae and adults of *Drosophila* (Parsons et al., 1979).

Ethanol, ethyl acetate, and acetaldehyde, all metabolic products of baker's yeast (Williams et al., 1981), play a role in host-habitat location by *L. heterotoma*. The reaction of the parasites towards acetaldehyde is one of orthokinesis because the parasites slow down their walking speed and seldom make a second or final choice, towards ethanol and ethyl acetate one of taxis because of the high percentage of final choices (at the offered concentrations) as argued on p. 699. These three compounds are volatile and have a low sensory threshold, two characteristics of importance in long-range orientation-mediating chemicals (Suomalainen and Lehtonen, 1979). These three compounds, among others, also attract adult *Drosophila* (see Table 5) (Barrows, 1907; Hutner et al., 1937; Fuyama, 1976).

Leptopilina boulardi is attracted also by ethanol (Carton, 1976). Ethanol is a toxic compound to which *Drosophila* larvae have evolved a

TABLE 5. CHEMICAL STIMULI (AND CONCENTRATIONS) ATTRACTING *Drosophila* ADULTS AND/OR LARVAL PARASITES OF *Drosophila* (+ = attraction, 0 = neutral)

	Drosophila			*Leptopilina*	
	D. melanogaster		*D. ampelophila*	*L. heterotoma*	*L. boulardi*
Chemical stimulus	Hutner et. al. (1937)	Fuyama (1976)	Barrows (1907)	This report	Carton (1976)
Ethanol		+ (8%)	+	+ (5%)	+ (0.5–10%)
Pentanol			+		
Acetic acid		+ ($\frac{1}{8}$%)	+	0 (3%)	
Lactic acid		+ (2%)	+		
Ethyl ether			+		
Diacetyl	+			0 (10^{-1}, 10^{-2}%)	
Acetoin	+				
Acetaldehyde	+			+ (1%)	
n-Butyraldehyde		+ ($\frac{1}{32}$%)			
Indole	+				
Ethyl acetate		+ ($\frac{1}{4}$%)		+ (10^{-2}, 10^{-3}%)	

detoxification system (Deltombe-Lietaert et al., 1979). Likewise parasites of *Drosophila* are adapted to high ethanol concentrations, especially females, as they contact fermenting substrates during host location and parasitization. Female *L. heterotoma* tolerate an ethanol concentration of 5% and female *L. boulardi* 6.5–10% (Boulétreau and David, 1981; *L. heterotoma* = *Ganaspis mundata; L. boulardi* = *Cothonaspis boulardi*).

The fermentation products found to attract *L. heterotoma* are not specific metabolic products of *Saccharomyces cerevisiae* but occur generally during fermentation. They also attract other insects, e.g., *Biosteres longicaudatus* (Greany et al., 1977) and *Hylemya antiqua* (Ikeshoji et al., 1980, 1981). This would mean that (parasitic) insects could reach many different habitats if they would simply respond to the presence of one or some chemical compounds, instead of reacting to a special blend of such compounds, and we expect a more complex situation to occur. Chemical stimuli could be active in several ways: their presence alone, a specific concentration, a specific combination of compounds (in specific concentration), attractive and nonattractive ones combined. In our tests, a combination of the three attractive fermentation products could not compete with a yeast suspension for attractivity for *L. heterotoma.*

Future research will have to concentrate on testing of other (concentrations and combinations of) fermentation products for attractivity for *L. heterotoma.*

daCunha et al. (1957) report that different *Drosophila* species each use a specific set of yeast species as nutrition source and are attracted to a specific set of yeast species. Some yeast species produce very specific spectra of metabolic products in which, for example, one product dominates or is nearly absent. The genus *Brettanomyces,* e.g., can be recognized by its specific aroma in which relatively high concentrations of isobutyric and isovaleric acids are present (Van der Walt, 1970). The ability of *Drosophila* larvae to develop is dependent on many factors, such as growth period of the yeast, larval density, and the chemical composition of the basal growth medium (Kearny and Shorrocks, 1981).

Only in a very few cases have the yeast species of *Drosophila* breeding sites been identified (Carson et al., 1956; Begon, 1975; Kearny and Shorrocks, 1981). Of these yeast species there is often no detailed knowledge of their fermentation products. *Saccharomyces cerevisiae* is the most studied yeast species and many of its fermentation products are known (e.g., Suomalainen and Keränen, 1967; Suomalainen and Lehtonen, 1979; Suomalainen and Ronkainen, 1963; Nykänen and Nykänen, 1977; Williams et al., 1981). Factors like yeast species and basal growth medium might affect host-habitat location too. Further, not only may stimuli originating from the host habitat influence host-habitat location, but volatile stimuli originating from the host or from interaction of host and host-habitat may also be important (Weseloh,

1981; Vinson, 1981). However, no influence of host presence on host-habitat location by *L. heterotoma* has been observed.

Experiments with a *L. heterotoma* strain that was reared for only one generation in the laboratory do not show a difference from the Storrs strain in olfactory reaction to two attractants. This may indicate that the results of our experiments are valid for *L. heterotoma* in general.

In this paper we have not discussed whether preimaginal and/or imaginal conditioning plays a role in host-habitat searching. That this does occur with respect to host-habitat searching is the topic of a paper by Vet (1983).

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ANALYSIS OF TRICHOME EXUDATE FROM MITE-RESISTANT GERANIUMS

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Abstract—Trichome exudate from mite-resistant geraniums (*Pelargonium hortorum*) was analyzed, principally by mass spectrometry and NMR spectroscopy. The exudate was found to consist of two anacardic acid derivatives, *o*-pentadecenylsalicylic acid and *o*-heptadecenylsalicylic acid. Bioassays established a moderate toxicity of these compounds to the two-spotted spider mite, *Tetranychus urticae*. The production of these compounds in geraniums was correlated with the two complementary dominant genes previously reported for host resistance to spider mites.

Key Words—Trichome, geraniums, *Pelargonium* mite resistance, *Tetranychus urticae*, *o*-pentadecenylsalicylic acid, *o*-heptadecenylsalicylic acid, anacardic acids, dominant genes.

INTRODUCTION

Plants are known to possess a large and varied arsenal of weapons with which to battle insect pests. As genetic knowledge and techniques improve, it becomes possible to use these natural defenses as alternatives to costly and often hazardous synthetic pesticides. As an example, consider the economic potential of crop plants which could produce toxic pyrethrins like the chrysanthemum or ensnare insects like the sundew. Morphological, physiological, and chemical defense mechanisms have been found in virtually every plant species which has been investigated.

All of these defense mechanisms have been shown to exist in geraniums (*Pelargonium* × *hortorum*), ranging from thick cuticles and epidermal cells (Chang et al., 1972) to glandular trichomes (leaf hairs) which trap and kill

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invading mites (Stark, 1975). It was the purpose of the present study to analyze the trichome exudate and characterize its components. Additionally, since mite resistance in geraniums is governed by two complementary dominant genes (Winner, 1975), the trichome exudates of several intermediate genotypes were analyzed and compared to those of resistant and susceptible homozygotes.

METHODS AND MATERIALS

Plant Culture. Geraniums were grown from seed in a greenhouse maintained at 16–27° C. Standard cultural practices were followed, except that no pesticides were applied to the plants. Seeds for each cultivar were provided by one of the authors (R.C.), along with records of parentage and prior data on mite resistance.

Collection of Exudate. A clean glass microscope slide was pressed to the upper and lower surfaces of several leaves until a sticky yellow film was observed. The slide was then rinsed with several drops of ethyl acetate which were collected in a vial. Insoluble debris was removed from this solution with a 1-μm-pore filter in a Swinney filter holder (Millipore Corp., Bedford, Massachusetts). Following solvent evaporation under nitrogen, the exudate was weighed, redissolved in ethyl acetate, and stored in a freezer at −32° C.

Chromatographic Analyses. Gas–liquid chromatography (GLC) was performed using a Tracor 222 instrument equipped with a flame ionization detector. A 122-cm × 4-mm-ID column was used with 3% SP-2100 packing and a nitrogen carrier flow of 50 cc/min. Thin-layer chromatography (TLC) was performed with a diethyl ether–benzene–ethanol–acetic acid (40:50:2:1, by volume) solvent system on Silica-gel 60 F_{254} plates (EM Reagents, MC/B Manufacturing Chemists, Inc.). Visualization was performed by exposure to iodine vapors, charring with 1:1 H_2SO_4–MeOH, and observation under shortwave ultraviolet (UV) light. High-pressure liquid chromatography (HPLC) was performed on a Waters model ALC/GPC 244 instrument, equipped with model 6000A pumps, a model 660 solvent programmer, WISP 710A injector, model 480 LC spectrophotometer, and a data module. A 4 × 300-mm μ Bondapak C_{18} column was used with a 90% acetonitrile–10% 0.1 N acetic acid–water mobile phase.

Spectrometric Analysis. UV spectra were obtained with a Gilford model 250 spectrophotometer and infrared (IR) spectra were obtained on a Perkin-Elmer model 421 spectrophotometer. Mass spectra (MS) were taken on a AEI model MS-950 and a Finnigan model 3200 GLC-MS mass spectrometer. Nuclear magnetic resonance (NMR) analyses were obtained in deuteriochloroform on a Bruker WM 360 (360-MHz) instrument.

Bioassay Procedure. Two 1.2-cm-diameter filter paper disks (Whatman No. 540) were each treated with 226 μg (200 $\mu g/cm^2$) of exudate in ethyl acetate, and the solvent was allowed to evaporate. Two more disks were similarly treated with ethyl acetate as controls. After wetting each of the four disks with 15 μl of 5% Tween 20 detergent–water, six adult female spider mites (*Tetranychus urticae*) were transferred onto each disk from bean leaves using a fine camel-hair brush. Each disk was centered beneath a 1-cm-diameter hole in a piece of Teflon (1 × 3 × 0.02 in.), which was then sandwiched between two glass microscope slides and held in place by two metal clips. These "bioassay chambers" were placed together in a 20–25° C incubator in the dark. After 24 and 48 hr, the filter paper disks were remoistened with 15 μl of water. At 72 hr, living and dead mites were counted. Bioassays were repeated three times using a total of 72 mites.

RESULTS

Chromatographic Analyses. GLC of the ethyl acetate extract on a 3% SP-2100 column at 210° C yielded two principal peaks, henceforth referred to as A and B and present in approximately a 35:65 ratio (Figure 1). Peaks A and B corresponded to theoretical carbon numbers of 24.45 and 26.40 carbons, respectively, when compared to straight-chain hydrocarbons. GLC separation on a more polar column (OV-17) yielded the same two peaks.

Two mite-susceptible and two mite-resistant geranium strains of known genotypes were grown and compounds collected from their glandular trichomes were analyzed by GLC. Identical peaks (A and B) were found in both double-dominant (resistant) strains ($Ts_1Ts_1Ts_2Ts_2$ and $Ts_1ts_1Ts_2ts_2$), whereas no GLC-detectable material was found in the two susceptible strains ($Ts_1ts_1ts_2ts_2$ and $ts_1ts_1ts_2ts_2$), suggesting that compounds A and B account for the mite resistance studied by Winner (1975) (Table 1).

Thin-layer chromatography of the exudate gave one principal spot and a minute quantity of yellow–orange pigment which faded away several minutes after exposure to air. Exposure of the TLC plate to iodine vapors (brown) or charring with 1:1 H_2SO_4–MeOH (orange–brown) revealed one spot of R_f 0.58. This spot fluoresced light blue under short-wave UV light and intensified upon spraying with 5% KOH, suggesting the presence of phenolic compounds. Elution of the spot from the TLC plate with ethyl acetate and subsequent GLC analysis showed the presence of both compounds A and B. HPLC analysis of the exudate extract with 90% acetonitrile–10% 0.1 N acetic acid–water (1.5 ml/min) on a reverse-phase column (μBondapak C_{18} at 208 nm) revealed two major peaks (9.7 and 13.9 min). Collection of the HPLC peaks and subsequent GLC analysis of these peaks showed them to be

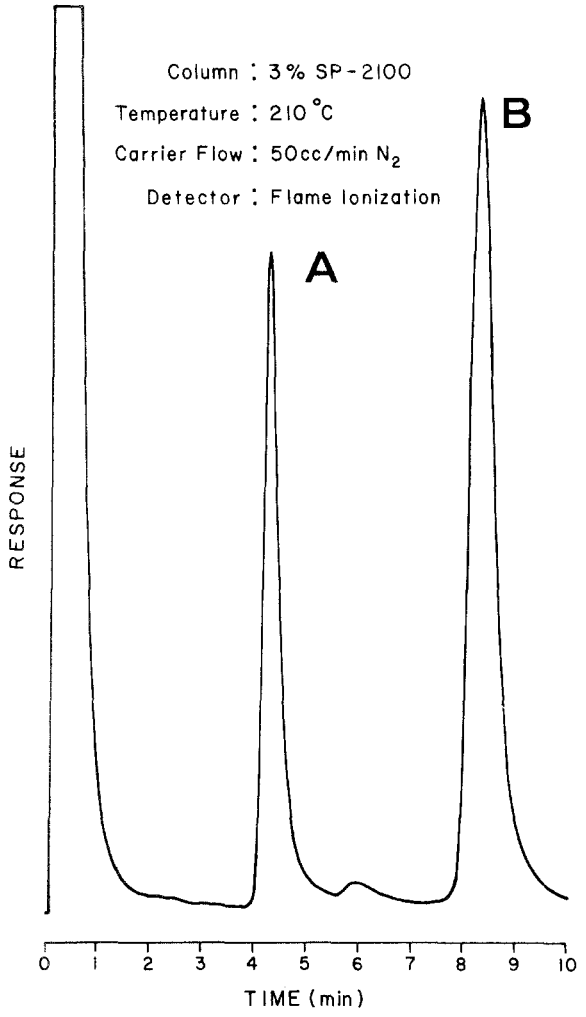


FIG. 1. GLC of the exudate from geranium trichomes.

compounds A and B. Therefore, HPLC was used to separate compounds A and B for subsequent spectroscopic analyses.

Spectroscopic Analyses. An ultraviolet scan of the crude exudate, or of purified compounds A or B in ethanol, showed absorption maxima at 208 nm and 302 nm, shifting to 228 nm and 296 nm upon addition of 0.2 N KOH. IR analysis of the crude exudates revealed the presence of a conjugated carbonyl absorbing at 1665 cm^{-1}. The GLC-mass spectra of compounds A and B were nearly identical (Figure 2A and B), differing only by an additional 28 mass

TABLE 1. RESULTS OF GLC ANALYSES OF GERANIUM TRICHOME EXUDATE FROM SUSCEPTIBLE AND RESISTANT GENOTYPES

Genotype	Phenotype[a]	GLC of exudates
$ts_1ts_1ts_2ts_2$	mite-susceptible	No peaks
$Ts_1ts_1ts_2ts_2$	mite-susceptible	No peaks
$Ts_1ts_1Ts_2ts_2$	mite-resistant	Peaks A and B
$Ts_1Ts_1Ts_2Ts_2$	mite-resistant	Peaks A and B

[a] As described by Winner (1975).

units (C_2H_4) in the molecular ion of compound B (m/z 330) as compared to compound A (m/z 302). Mass spectroscopy of the exudate employing a solid inlet system gave a fragmentation pattern similar to GLC-mass spectrometry, except that it yielded molecular ions of m/z 346 and m/z 374, an increase of 44 mass units for each compound (Figure 3). This discrepancy is explained by the heat-induced loss of CO_2 from compounds A and B (confirmed by high-resolution mass spectrometry), a process which occurs upon GLC.

High-resolution mass spectra were then obtained to elucidate structural details of compounds A and B. The molecular ions of species A and B have

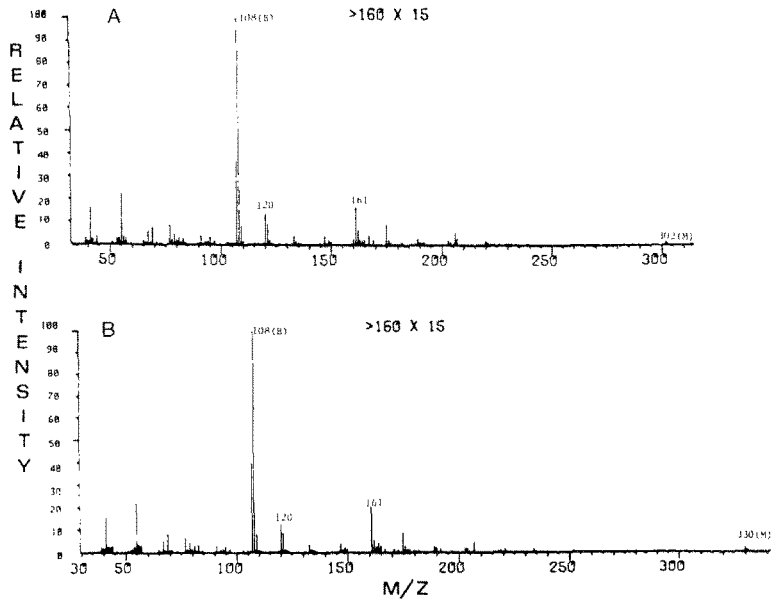


FIG. 2. GLC-mass spectra of Compounds A (A) and B (B).

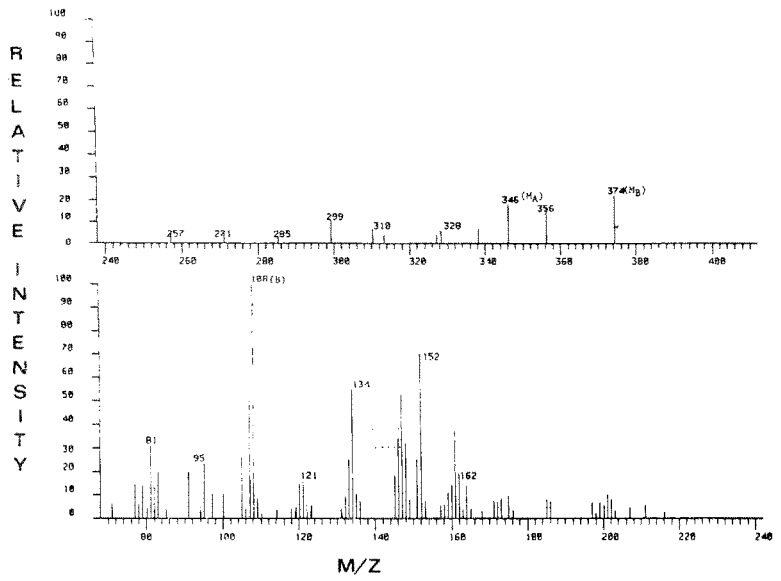


FIG. 3. Mass spectra of exudate from geranium trichomes with a solid inlet probe.

the empirical formulas: $C_{22}H_{34}O_3$ (m/z 346) and $C_{24}H_{38}O_3$ (m/z 374), respectively. The m/z 108 base peak represents the moiety C_7H_8O, an ion with a high C/H ratio indicative of a substituted benzene ring. Fragment m/z 152, which was only present in the solid inlet samples, corresponds to $C_8H_8O_3$ and shows all three oxygen atoms to be closely associated with the aromatic ring. Moreover, it is known that a carboxyl group ortho or para to a phenolic hydroxyl group is unstable when heated, suggesting a mechanism for the observed loss of CO_2 (Royals, 1954). On this basis, the structure proposed for the m/z 152 fragment is methyl-hydroxyl-benzoic acid. This interpretation is supported by the bathochromic shift which occurred in the UV spectra upon addition of base, similar to the shift evidenced by salicylic acid. Mass spectrometry indicated that the remainder of each molecule is a long hydrocarbon chain which is two carbons longer in B than in A.

NMR spectroscopy of the exudate was employed to determine the geometry of groups around the rings and to provide details of the hydrocarbon chain's structure (Figure 4). The three aromatic peaks at δ 6.77 (*d*, $J = 7.74$), 6.87 (d, $J = 8.17$) and 7.36 (*t*, $J = 8.17$ and 7.74) are consistent with that of a 1,2,3-trisubstituted benzenoid ring. The more deshielded triplet absorption at δ 7.36 indicates the carboxyl is para to this proton. The vinylic absorption at δ 5.35 (t, $J = 4.45, 4.73$) is indicative of a cis double bond. The benzylic absorption occurs at δ 2.97 (t, $J = 7.31$).

On the basis of these data, compound A is *o*-pentadecenylsalicylic acid

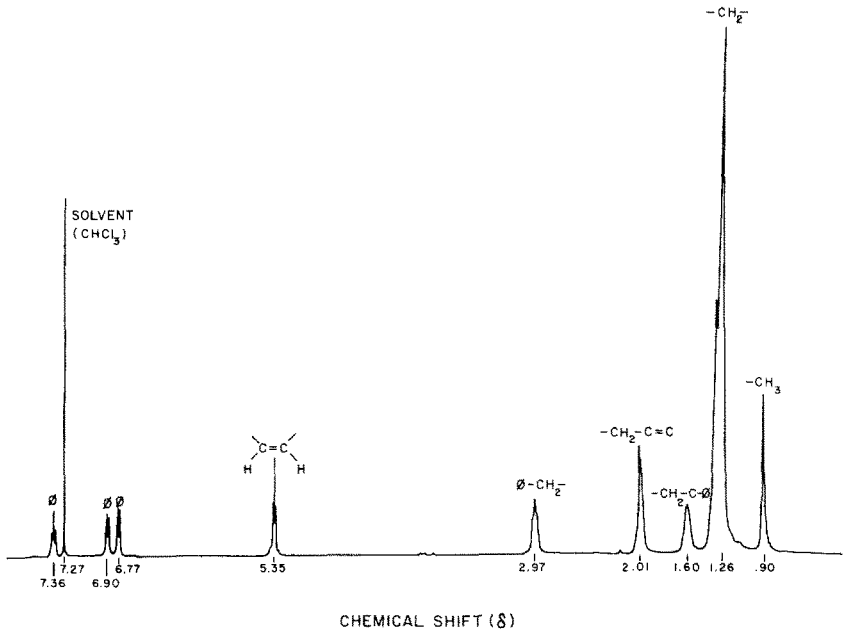
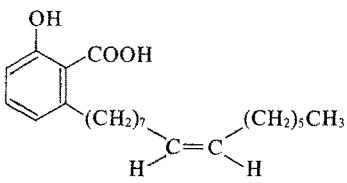


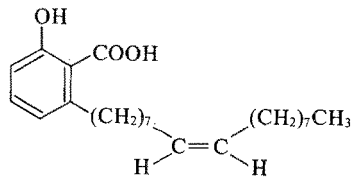
FIG. 4. NMR spectrum of exudate from geranium trichomes.

and compound B is *o*-heptadecenylsalicylic acid; we propose the names romanicardic acid and geranicardic acid, respectively. Figure 5 presents an interpretation of the mass spectral fragmentations of compound B. The m/z 108 (base peak) may also be structurally represented as a tropylium ion. Anacardol, a mixture of components of decarboxylated anarcardic acids which were isolated from cashew nutshell oil by preparative TLC (R_f = 0.62), gave peaks on GLC identical with the retention times of peaks obtained from GLC of compounds A and B and gave NMR and GLC-mass spectra similar to compounds A and B.

To verify that the exudate was toxic to mites, a bioassay procedure was



Compound A
(romanicardic acid)



Compound B
(geranicardic acid)

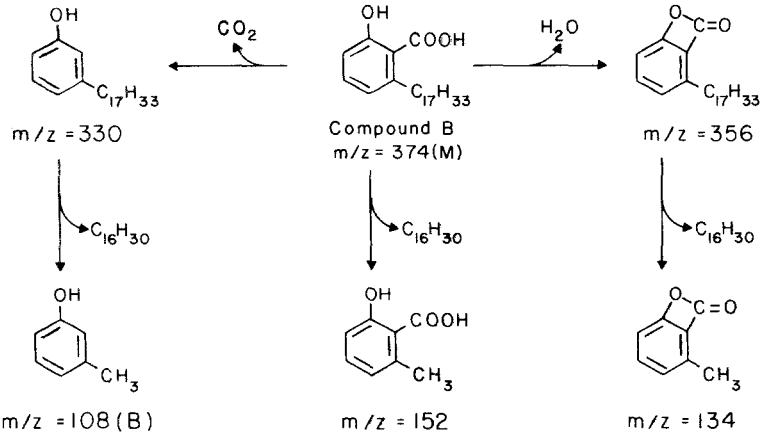


FIG. 5. Mass spectral fragmentation scheme of compound B.

performed in triplicate. Mites exposed to the exudate at the rate of 200 $\mu g/cm^2$ for 72 hr showed 75% mortality, as compared to 14% mortality in the controls. Chi-square analysis of the pooled data indicated that mortality means were significantly different at the 0.01 level.

DISCUSSION

Analysis of the exudate has shown it to be comprised of two anacardic acid derivatives bearing 15-carbon and 17-carbon monounsaturated side chains. All analytical methods showed the exudate to be suprisingly pure, although a probe mass spectrum suggested the presence of traces of anacardic acids with 19-carbon and 21-carbon monounsaturated side chains. The only other detectable material was a yellow–orange pigment, present in trace amounts, which colored the exudate. Anacardic acids have been previously reported (Wasserman and Dawson, 1945) as occurring in members of the family *Anacardiaceae* (cashew nut tree, Japanese lac tree, poison ivy, etc.), but were not previously reported outside this family.

Although side-chain unsaturation is clearly evidenced by both MS and NMR spectroscopy, there is some uncertainty concerning the position of the double bond in each molecule. The biosynthetic scheme suggested by Geissman (1963) for anacardic acid shows the side chain to be of fatty acid origin. Geismann's scheme further hypothesizes the addition of three acetyl-CoA groups to the fatty acid, and their subsequent cyclization and reduction to form the unsaturated salicylic acid-type ring. Knowledge of the positions

of the double bonds in palmitoleic and oleic acids permits postulation of the double bond position in compounds A and B.

While collecting exudate from geraniums, it was noted that glandular hairs are not only present on leaves, but also petioles, stems, and even cotyledons. The exudate was apparently produced by the bulbous terminal gland cell of each multicellular trichome. The exudate formed a sticky, viscous coating around each gland cell, an arrangment which applied exudate to mites very efficiently. The 61% mortality above the controls measured in the bioassays from exposure to 200 $\mu g/cm^2$ exudate in the chambers compares well with the 70% mortality above controls obtained with leaf disks by Stark (1975). In running bioassays, very shallow chambers were devised in order to maximize exposure of mites to the exudate. Tween 20 was used because the exudate is insoluble in water but is readily soluble in the presence of this mild detergent.

By studying the defensive role of the glandular trichomes, it becomes possible to partially explain the expression of the two genes which control resistance. Apparently, dominant alleles are necessary at both loci in order for a plant to produce exudate. No biochemical differences were observed between homozygous recessive ($ts_1ts_1ts_2ts_2$) and single-locus dominant ($Ts_1ts_1ts_2ts_2$) genotypes, or between homozygous dominant ($Ts_1Ts_1Ts_2Ts_2$) and doubly heterozygous ($Ts_1ts_1Ts_2ts_2$) genotypes, confirming that the Ts_2 locus is involved in formation of exudate. The genetic studies of Winner (1975) suggest that the Ts_1 locus is also involved in glandular trichome formation. This would be an interesting system to study genetically, to see whether several enzymes involved in exudate synthesis are controlled by the Ts_1 and Ts_2 loci, or whether one locus controls morphological characters and one codes for biochemical factors. Further work on this problem could add to our knowledge of gene expression on an apparently simple model and would likely facilitate use of these loci in future host-resistance breeding.

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ATTRACTION OF BARK BEETLES (COLEOPTERA: SCOLYTIDAE) TO A PHEROMONE TRAP
Experiment and Mathematical Models

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Abstract—The movement of bark beetles near an attractive pheromone source is described in terms of mathematical models of the diffusion type. To test the models, two release experiments involving 47,000 marked spruce bark beetles [*Ips typographus* (L.)] were performed. The attractive source was a pheromone trap, surrounded by eight concentric rings with eight passive trap stations on each ring. Captures were recorded every 2–10 minutes for the pheromone trap and once for the passive traps. The models were fitted to the distribution in time of the central pheromone trap catch and to the spatial distribution of catch among the passive traps. The first model that gives a reasonable fit consists of two phases: Phase one—After release the beetles move according to a diffusion process with drift towards the pheromone trap. The strength of the drift is inversely proportional to the distance from the traps. Phase two—those beetles attracted to, but not caught by, the pheromone trap are no longer influenced by the pheromone, and their movement is described by a diffusion process without drift. In phase two we work with a loss of beetles, whereas the experiment seems to indicate that the loss of beetles in phase one is negligible. As a second model, the following modification of phase one is considered: After release the beetles move according to a diffusion process without drift, until they start responding to the pheromone (with constant probability per unit time), whereafter they start moving according to a diffusion process with

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drift. This study, like other release experiments, shows that the efficiency of the pheromone trap is rather low. What is specific for the present investigation is that we try to explain this low efficiency in terms of dynamic models for insect movement. Two factors seem to contribute: Some beetles do not respond to pheromone at all, and some beetles disappear again after having been close to the pheromone trap. It also seems that the motility of the beetles decreased after they ceased responding to the pheromone. Furthermore, the data lend some support to the hypothesis that flight exercise increases the response of the beetles to pheromone.

Key Words—*Ips typographus,* pheromone, release, recapture, diffusion, model, Coleoptera, Scolytidae, trap, marking, dispersal.

INTRODUCTION

The present investigation concerns the movement of flying *Ips typographus* (L.) when attracted towards a synthetic pheromone source, i.e., a pheromone trap of the same type as used in the recent campaign against this species in Scandinavian forests (Bakke, 1981).

There are great practical difficulties involved in tracing the individual beetles in such a situation, and even if these difficulties could be resolved, the question remains how the motion of a large number of beetles can be described quantitatively. In this paper we try an indirect approach: From mathematical models of the movement of the individual beetles, we deduce the expected capture in passive traps at various positions near the pheromone trap, and these model predictions are compared with the observed capture in two release–recapture experiments. By fitting the models and testing which models that fit the data best, we aim to learn more about the behavior of the individual beetles.

The approach of connecting release experiments to models of the behavior of the beetles is not common in chemical ecology. Most experiments deal only with recapture percents at various release distances and with qualitative investigations of the beetles' response to pheromones (Hertel et al., 1969; Borden, 1974, 1977; Sauerwein, 1981). Models of the spread of pheromones from a source have been made (Fares et al., 1980; Okubo, 1980; Murlis and Jones, 1981) as well as more general models for insect dispersal (Ludwig et al., 1979; Okubo, 1980). The role of pheromones in the biology of bark beetles was recently reviewed by Wood (1982), where further references can be found.

The present paper describes a simple experimental situation, and it is only in such situations that it is possible to find explicit expressions for the quantities of interest in the models. However, if we can find models that compare reasonably well with reality in such simple situations, and use such

simple situations to estimate the parameters of the models, then it may be possible to approach more complex situations by means of computer simulations. Thus, it should be possible to describe the dispersal of bark beetles near a group of several pheromone traps and also situations where trees are attacked by bark beetles. [See Johnson and Coster (1978) for empirical fits of the attack intensity by southern pine beetle as a function of the distance from a pheromone source.]

The mathematical discussion will be fairly superficial in this paper, but a detailed account is given in Helland (1982, 1983).

METHODS AND MATERIALS

To test the models and estimate parameter values, a release–recapture experiment for the spruce bark beetle *Ips typographus* was designed. The beetles were caught in olfactometers in Lardal, southern Norway, during the end of May and beginning of June 1981, and kept in a refrigerator at 4° C until about 1 h before release. The olfactometers were loaded with strips of Ipslure® containing 1500 mg methylbutenol, 70 mg *cis*-verbenol, and 15 mg ipsdienol per meter. These substances have been identified from male beetles initiating boring in trees (Bakke, 1976; Bakke et al., 1977), and methylbutenol and *cis*-verbenol were suggested to be the primary aggregation pheromones of *I. typographus* (Bakke et al., 1977).

Release was performed when weather conditions allowed for flight, i.e., air temperature above 20° C and little wind. These conditions were fulfilled on June 23 and July 8, 1981, and one release experiment was done each day.

Experimental Site. The experiment was performed in an open plain near Stensoffa Ecological Research Station outside Lund, southern Sweden. The distance to the nearest spruce tree was more than 1 km and to the nearest spruce forest several kilometers. This means that the released beetles were not influenced by any natural pheromone source.

Experimental Setup. The experimental setup consisted of one central plastic cylinder trap baited with a 1 m Ipslure® dispenser. The trap is of a type that has been used in the campaign against *I. typographus* in Scandinavia during recent years (type N80 in Regnander and Solbreck, 1981, where a picture can be found). Passive trap stations were placed in eight concentric rings around the central pheromone trap. The distance between the rings was 3 m, and the stations were placed on eight equally spaced radial lines, giving a symmetric setup. A passive trap station consisted of two window traps made of transparent plastic attached to a wooden pole, each capturing beetles separately from both sides. The top of each trap was 2 or 3.2 m above the ground, one oriented tangentially to the

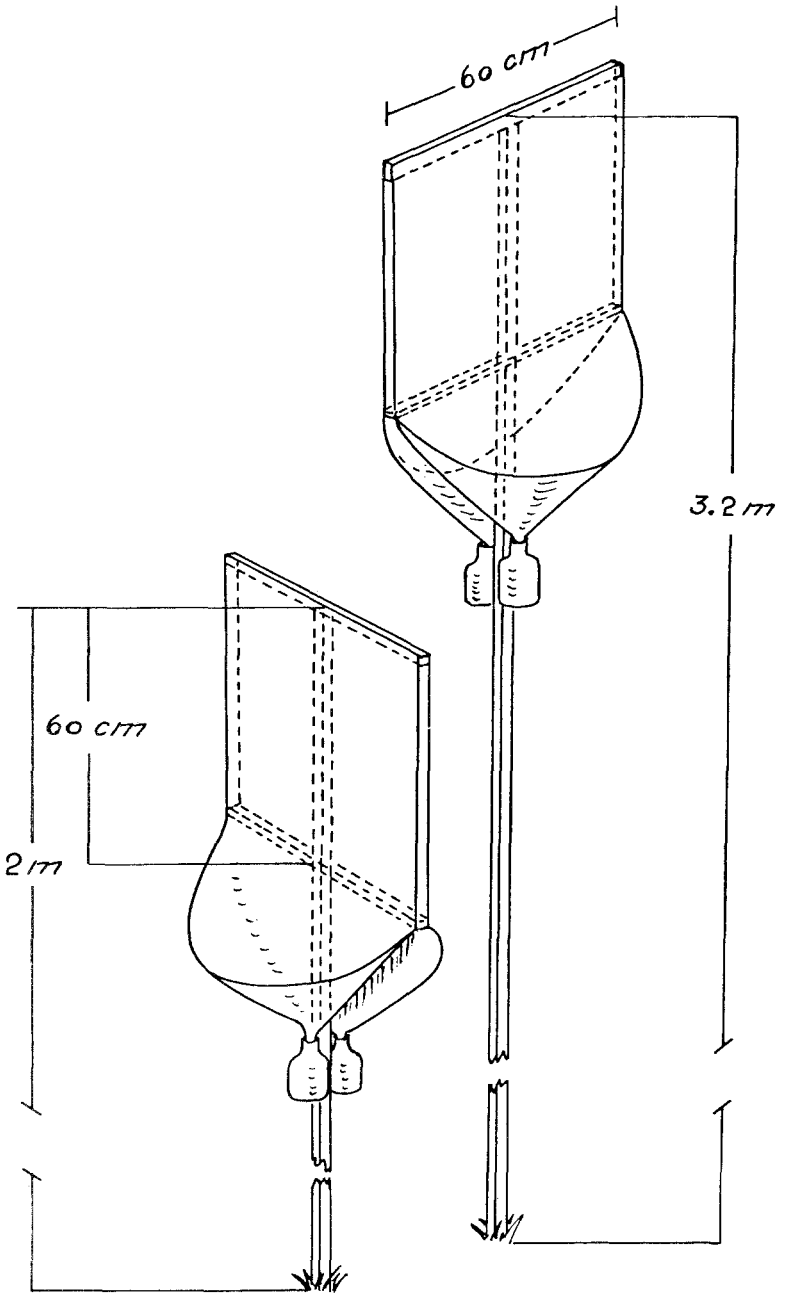

FIG. 1. A passive trap station catching beetles from all four directions.

ring and the other radially. The directions of the traps were alternated so that a trap station with a tangential high trap was surrounded (inside, outside, and both sides) by four stations with the high trap oriented radially. The station thus captured beetles from all four directions, at two heights. This enabled us to differentiate between beetles caught while flying towards the pheromone trap, and those caught while flying outwards (Figure 1).

In addition we placed a pheromone trap, of the same type as the central one, 500 m from the experimental area in each of four directions to check if any long-distance dispersal occurred.

Releases were performed from four platforms, 1 m above the ground, placed symmetrically around the central trap, at 12 m distance on June 23, and at 20 m distance on July 8. The number of beetles used in each experiment was estimated volumetrically by the ratio 40/ml and divided in four equal groups. They were marked with a minimum amount of fluorescent powder (from Swada, London) (Wollerman, 1979), with a different color for each release platform, and released simultaneously. After each experiment, the number of beetles remaining on the platforms was estimated and the difference between the two estimates gave the number of released beetles (Table 1). Seventy-eight and 39% of the beetles left the platforms on June 23 and July 8, respectively. The catch in the central trap was registered every 2, 5, or 10 min, and the passive trap catch was registered 3.5 h after the release, when the swarming activity had ceased and the experiment was terminated.

TABLE 1. RECAPTURE DATA FROM RELEASE EXPERIMENT

			Passive traps		
	Released	Central trap	Total	Inside[a]	Outside[a]
June 23					
NE	6,920	243	228	23	121
NW	6,160	204	177	27	82
SW	6,360	326	324	40	119
SE	5,680	244	272	35	126
Total	25,120	1513[b]	1001	125	448
July 8					
NE	5,920	209	148	20	59
NW	5,320	132	103	10	44
SW	5,160	186	144	31	42
SE	6,240	459	270	31	111
Total	22,640	986	668[b]	92	256

[a]Inside and outside refer to the catch in the tangentially oriented passive traps.
[b]Some of the beetles were color contaminated in the traps so their origin could not be determined.

The four distant pheromone traps were checked 4 h after release. To check the persistence of the marking, the catch in the central traps was registered 1, 2, and 6 days after the first experiment. The beetles caught were counted and their origin was detected by color examination under ultraviolet light. The setup is shown in Figure 2.

The wind direction and speed were measured with a combined vane and anemometer, type 1462, Wilh. Lambrecht, Göttingen.

The main meteorological differences between the two release dates were higher air temperature (25.6–29.9° C) on July 8 than on June 23 (20.8–25.6° C), and a higher mean wind speed on July 8 (2.2 m/sec compared to 1.5 m/sec).

This experiment gave us a temporal distribution of catches in the central pheromone trap and a spatial distribution of the passive trap catches. From our models of how the beetles move around a pheromone trap, these distributions can be expressed mathematically and fitted to the data to see how well they describe the empirical results.

RESULTS

The recapture data on the day of release are presented in Tables 1 and 2. The recapture percent is low: 6.02 and 4.36% for the central trap on June 23 and July 8, respectively, and 10.01 and 7.31% for the central plus the passive traps. Significantly more beetles were caught in the low passive traps than in the high ones (1059 compared to 610, $P < 0.001$). More beetles were trapped on the outside than on the inside of the tangential traps, 704 compared to 217, $P < 0.001$, indicating a drift of beetles towards the central trap. The four distant traps caught 89 beetles on June 23 and 127 beetles on July 8. The catch in the central trap 1, 2, and 6 days after release was 18, 14, and 12 beetles, respectively, and the origin (color) of all, except on the last day, could be determined.

The distribution of captures among the passive traps is also shown in Figure 2, together with the wind direction during the experiment. There was a tendency toward larger captures in the downwind direction from the pheromone trap. This may be so because the pheromone plume is most concentrated here, and when the beetles enter the plume, they start to fly against the wind and remain within the plume for longer periods than outside in adjacent areas.

As an attempt to eliminate the effect of wind on the drift of beetles, we symmetrize the catches as follows. The capture point of a beetle is identified by the distance from the central trap and the angle between the release point and the capture point, as seen from the central trap (Figure 3). Adding

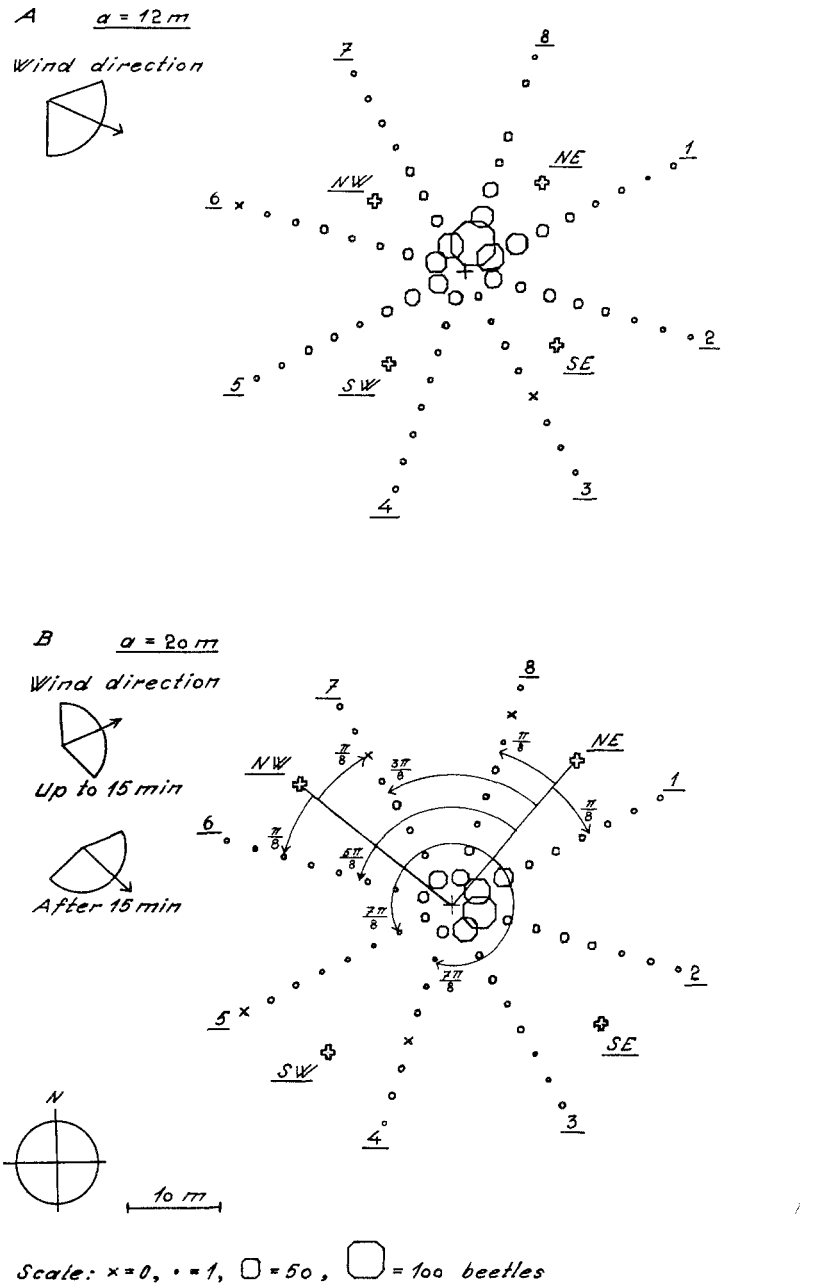

FIG. 2. Setup of the two replicates of the experiment and total catch in passive traps on June 23 (A) and July 8 (B). + = central pheromone trap, ✣ = release point.

TABLE 2. RECAPTURE IN PASSIVE TRAPS SHOWING RELEASE PLATFORM, DISTANCE *r* FROM PHEROMONE TRAP, AND POSITION IN RING OF RADIUS *r*

Release platform	Trap position	Radius (m)							
		3	6	9	12	15	18	21	24
June 23[a]									
NE	1	22	11	5	3	3	0	0	0
	2	7	3	3	0	1	1	0	0
	3	2	0	0	0	0	0	0	0
	4	1	0	1	0	0	0	0	0
	5	11	5	2	1	0	0	0	0
	6	16	0	2	0	0	0	1	0
	7	22	2	1	4	2	3	1	0
	8	34	24	13	7	8	1	4	1
	Total	115	45	27	15	14	5	6	1
NW	1	8	5	5	2	0	3	1	0
	2	4	1	1	1	0	0	1	0
	3	1	0	0	1	0	0	0	0
	4	1	0	0	0	0	0	0	0
	5	6	3	1	1	0	0	0	0
	6	12	6	4	5	9	7	0	0
	7	21	10	10	9	2	3	0	1
	8	5	6	8	3	5	2	3	0
	Total	58	31	29	22	16	15	5	1
SW	1	19	13	1	6	1	2	0	1
	2	13	8	4	1	3	0	3	0
	3	2	1	5	0	0	1	0	1
	4	18	3	4	2	2	2	0	0
	5	20	22	16	3	7	7	2	2
	6	16	6	2	3	3	0	2	0
	7	11	5	3	2	2	0	1	1
	8	47	11	8	1	2	2	1	0
	Total	146	69	43	18	20	14	9	5
SE	1	18	20	14	6	2	4	1	4
	2	17	8	18	16	10	3	0	1
	3	6	3	5	5	0	3	1	1
	4	7	1	1	0	0	1	3	2
	5	6	4	0	2	3	3	0	0
	6	4	5	0	0	2	0	0	0
	7	8	4	2	1	0	1	0	0
	8	28	9	3	1	2	2	1	0
	Total	94	54	43	31	19	17	6	8
July 8[b]									
NE	1	14	8	7	6	0	3	2	3
	2	17	4	1	2	0	1	2	0
	3	9	2	1	1	1	0	0	2
	4	11	1	0	0	0	0	0	1
	5	2	1	0	1	0	0	0	0
	6	6	2	0	0	0	0	0	0

TABLE 2. Continued

Release platform	Trap position	Radius (m) 3	6	9	12	15	18	21	24
July 8[b]									
NE	7	13	1	1	2	0	0	0	1
	8	5	4	1	2	3	1	0	3
	Total	77	23	11	14	4	5	4	10
NW	1	5	7	1	3	0	0	2	1
	2	14	4	2	1	1	0	0	1
	3	11	2	1	2	0	0	0	0
	4	2	0	0	0	0	1	0	0
	5	2	0	1	0	0	0	0	0
	6	3	0	3	0	1	2	1	1
	7	5	1	0	5	1	0	2	4
	8	3	2	1	1	2	0	0	1
	Total	45	16	9	12	5	3	5	8
SW	1	12	10	3	1	5	2	0	2
	2	13	5	1	1	4	0	0	1
	3	13	3	3	1	1	0	0	2
	4	5	0	2	3	0	2	4	1
	5	4	1	0	0	2	4	4	0
	6	0	0	0	2	1	0	0	1
	7	2	2	3	2	1	0	0	0
	8	8	3	1	0	2	1	0	0
	Total	57	24	13	10	16	9	8	7
SE	1	30	17	2	3	4	2	1	1
	2	34	5	10	12	9	4	5	1
	3	22	1	5	1	5	2	2	1
	4	6	1	0	0	0	1	1	0
	5	5	0	0	0	0	0	0	0
	6	10	0	2	0	0	0	0	0
	7	21	2	1	2	1	0	0	0
	8	20	8	4	3	2	1	0	0
	Total	148	34	24	21	21	10	9	3

[a]Release distance $a = 12$ m; see Figure 2A.
[b]Release distance $a = 20$ m, see Figure 2B.

captures with the same distance and angle from the four release points, the total captures will consist of four components, each exposed to wind from different directions. The catches as a function of angle and distance are given in Figure 4. This indicates a stronger tendency to move towards the central trap than to diffuse outwards from the release point. This tendency is not so profound close to the central trap, indicating that the beetles are not moving straight into the pheromone trap, but are swarming around it. This is also what is seen at pheromone traps in nature on days with heavy swarming.

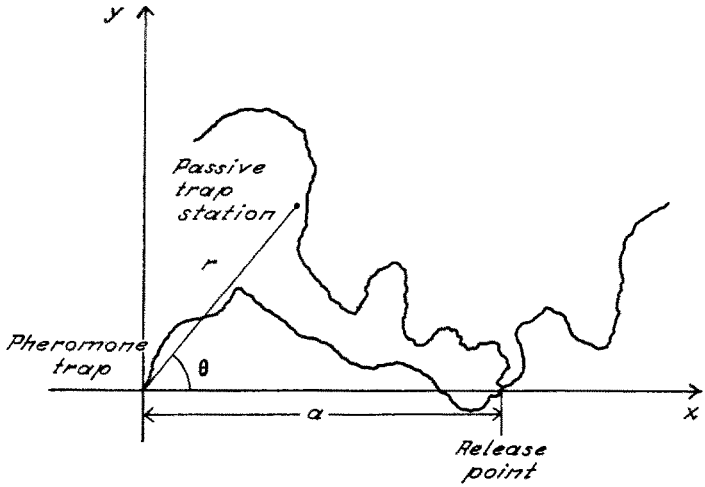

FIG. 3. Tracks of three hypothetical beetles at a time t after release at a distance a from the pheromone trap. The beetles are diffusing with drift k'/r towards the pheromone trap in the origin. k' = constant, r = distance to pheromone trap. A passive trap station in position (r, θ) is shown.

Mathematical Models

Background. Following Freeman (1977) and Stinner et al. (1983), mathematical models of insect dispersal can be classified as descriptive, explanatory, or predictive. In this terminology, our models will be of the explanatory type: A dynamic description of the insects' movement by a diffusion processes is used to explain the observed recapture of beetles. By contrast, in a purely descriptive model, data are just fitted to some regression equations. The models that we will use here are of the diffusion type. As noted by Stinner et al. (1983), such models have so far been of limited applicability, but even so, we feel that they are worth a closer examination.

The use of diffusion models in this context goes back to Dobzhansky and Wright (1943) and to Skellam (1951), who were modeling dispersal over larger areas by a simple diffusion process (Brownian motion) without drift. Corresponding to the present experiment, we will concentrate on modeling insects attracted towards a point, and this is most naturally done by including a drift term in the diffusion equations.

The drift is given by a direction and a magnitude, and the magnitude of the drift coefficient can be defined as the mean displacement per unit time in a small time interval, given the position of the beetle. Note that we use the term "drift" to denote the net (expected) displacement in space, combining the

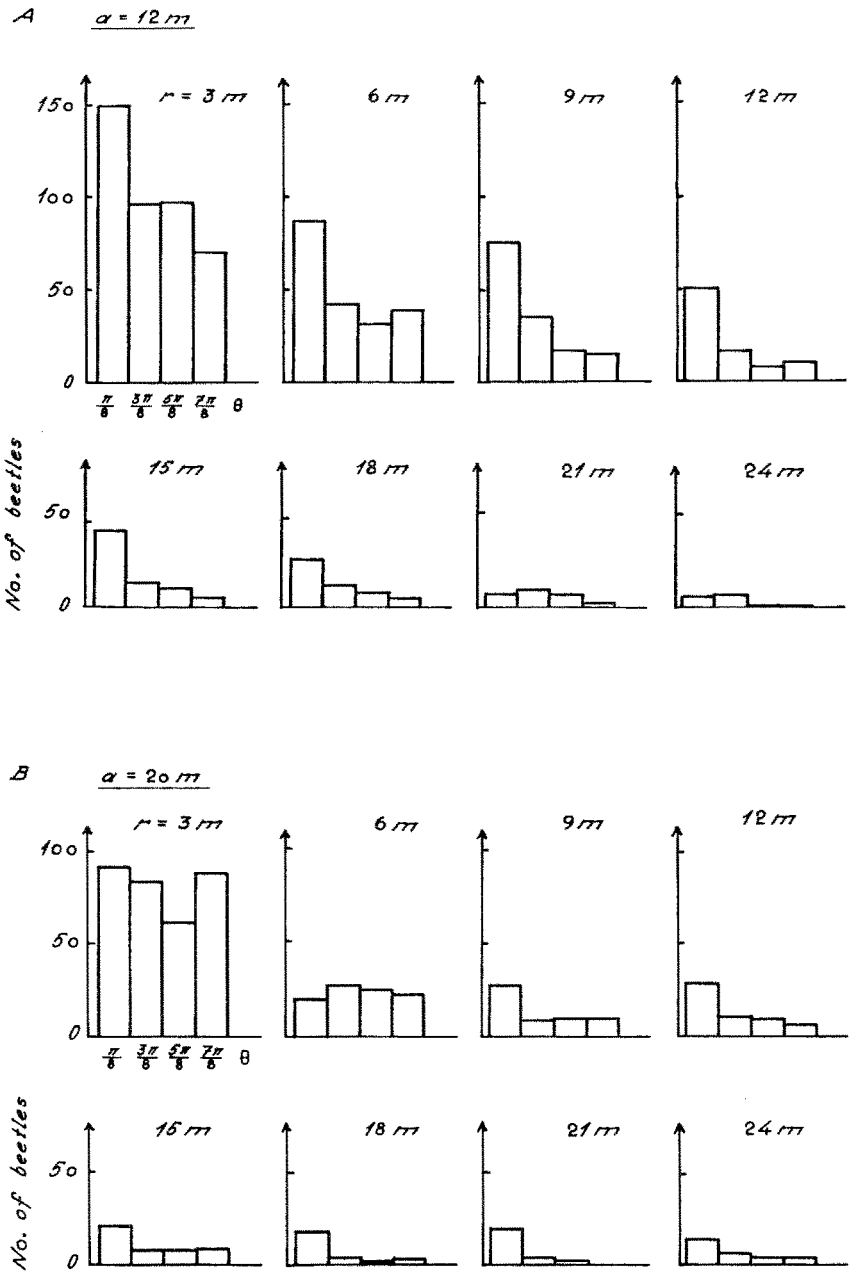

FIG. 4. Observed catch in passive traps as a function of angle θ (in radians) and distance r to pheromone trap on June 23 (A) and July 8 (B).

"wind" aspect and the "beetle flight" aspect of the motion. It is natural to suppose that the drift is directed towards the pheromone source and that it is stronger the closer the beetle is to the source, since the pheromone concentration on the average is much higher near the pheromone source (Murlis and Jones, 1981). In this paper we assume that the drift coefficient is given by $\mu(r) = \text{constant}/r$, where r is the distance from the pheromone source to the beetle. The equation of motion also involves the diffusion coefficient D, which is defined as one half the mean square displacement per unit time over a small time interval in any direction. For convenience, we redefine the constant in the drift term as $2kD$, so that $\mu(r) = 2kD/r$. The new constant, k, characterizing the strength of the drift will be dimensionless.

In the model equations we neglect the vertical motion of the beetles, since they tend to fly near the ground and their altitude usually does not exceed 15 m above the ground (Solbreck, personal communication). Also, as shown above, the lower traps caught significantly more beetles than the higher ones.

Now, consider a passive trap at position (x,y). Let the expected density of beetles at this position be $N \cdot f_t(x,y)$ at time t, where N is the total number of beetles, and $f_t(x,y)$ is the probability density function. Suppose that when a beetle is within an area R around the passive trap, it has a probability q per unit time of being caught. Then the expected number of beetles caught in the trap will be

$$\int_0^\infty Nf_t(x,y) \cdot Rqdt = Ne \int_0^\infty f_t(x,y)\, dt \tag{1}$$

where the parameter $E = Rq$ measures the efficiency of the trap. The dimension of E will be cm^2/sec or m^2/min as for D.

In a simple diffusion model without drift, $f_t(x,y)$ will be a two-dimensional Gaussian probability density with mean zero and with variance $2Dt$. In this case the integral (1) turns out to be infinite, which is a consequence of the ideal nature of the model. A more realistic model would result by assuming that each beetle has a constant probability c per unit time of dying or of leaving its random walk behavior, that is, landing on a tree or on the ground, being trapped, caught by a predator, or moving into higher layers of the air and blown away from the region. Then the Gaussian density function should be multiplied by a factor e^{-ct}, and the expected catch (1) turns out to be

$$\frac{NE}{2\pi D} K_0\left(r\sqrt{\frac{c}{D}}\right) \tag{2}$$

where r is the distance from the release point of the beetles to the trap, and K_0 is a modified Bessel function of the second kind. [See, for instance, Abramowitz and Stegun (1964) for details about these functions.]

In Stinner et al. (1983) a number of experiments are described, the data of which in principle could be fitted to equation (2), but this is outside the scope of the present paper. A priori, the parameters c and D can depend upon environment, meteorology, condition of the beetles, and other factors, which may complicate the fitting. In analogy with the argument in Dobzhansky and Wright (1943) and in Okubo (1980), if the observed catch is greater than what is given by (2) for large distances, this can, to some extent, be explained by heterogeneity in the population of insects.

Diffusion Model with Drift and Loss. Corresponding to the experiment treated in the present paper, we will now consider a situation where beetles are released at a distance a from a pheromone trap. As the basic model we will assume that all the beetles move independently of each other and that the motion of each beetle can be described by a diffusion process with drift of magnitude $2kD/r$ towards the pheromone trap. Suppose, first, that all beetles are captured when reaching the trap. In this model—admittedly unrealistic, but the one from which all the more realistic models below are derived—it is also possible to determine $f_i(x,y)$ (see Helland, 1983). Placing the origin at the pheromone trap, it turns out that the expected number (1) of beetles caught by a trap at the position (r, θ) in polar coordinates (Figure 3) will be

$$\frac{NE}{2\pi D}\left(\frac{a}{r}\right)^k \sum_{m=-\infty}^{\infty} K_0\left(k\sqrt{(\theta+2\pi m)^2+\left(\ln\frac{a}{r}\right)^2}\right) \tag{3}$$

where a is the distance from the pheromone trap to the release point. Since K_0 is a rapidly decreasing function, the most important term in the sum will be the one with $m = 0$. The other terms correspond to beetles that have circulated around the trap one or several times before they are caught at the position (r,θ).

Now the recapture percent in the present experiment was only 6.02% and 4.36% for the central pheromone trap (12 and 20-m release). Similar or smaller percentages have been reported elsewhere for comparable experiments (Hain and Anderson, 1977; Anderson, 1977). In the idealized model described above, all the beetles will arrive at the pheromone trap sooner or latter. Thus we must incorporate a loss of beetles in the model in order to explain the observed data. Such a loss will, in principle, consist of the following three components, all leading to different modifications of the simple diffusion model: (I) Some of the beetles released immediately fly into higher air layers and migrate away from region. (II) Some of the beetles are lost during the movement towards the pheromone source, e.g., they land on the ground, are caught by a predator, or are trapped in a passive trap. (III) Some of the beetles that arrive at the pheromone trap are not caught, but spread out again. One of the objectives of the present paper is to compare our

experimental data with the predictions from models where one or more of these modifications are taken into account. This may contribute to our understanding of the rather low efficiency of pheromone traps.

It is clear that the first modification above has to be included. However, the effect of this modification will only be to reduce the number (N) of beetles that enter into the equations. In particular, if this was the only modification needed, the prediction (3) of the distribution of catches among the passive traps would continue to hold. The second modification will change this pattern somewhat and will be discussed at the end of this section.

The third modification seems to be an important one. One way of thinking of this is to link it to the so-called modified behavior threshold, which has been discussed by other authors (Roelofs, 1978; Mankin et al., 1980):

Close to the pheromone trap, the concentration of pheromone in the air is very high, and this causes a large portion of the beetles to modify their behavior (cf. Byers, 1983). They are no longer attracted to the trap, but disperse in a way that can be described by a simple diffusion model without drift and maybe by a new diffusion coefficient D_2. This will be called phase two. The previously described movement of the beetles under the influence of pheromones will be called phase one. As can be seen by combining (2) and (3), in such a model the expected number of beetles caught by a passive trap at the position (r,θ) (Figure 3) is given by

$$AK_0(b_2 r) + C\left(\frac{a}{r}\right)^k K_0\left(k\sqrt{\theta^2 + \left(\ln\frac{a}{r}\right)^2}\right) \tag{4}$$

or more precisely, the last term should be replaced by a sum of terms with angles θ, $\theta \pm 2\pi$, $\pi \pm 4\pi$, $\theta \pm 6\pi$, etc., as in (3). Here

$$C = \frac{N_1 E}{2\pi D_1},\ A = \frac{N_2 E}{2\pi D_2},\ b_2 = \sqrt{\frac{c_2}{D_2}} \tag{5}$$

where N_1 is the number of beetles released minus the number that fly out of the region at once, and N_2 is the number of beetles that enter phase two near the pheromone trap. Furthermore, D_1 is the diffusion coefficient during the first phase (diffusion with drift towards the pheromone trap), E is the efficiency parameter for the passive trap, D_2 is the diffusion coefficient in phase two, and c_2 is the loss of beetles per unit time during this phase.

Now suppose that there is a continuous loss of beetles during the first phase, say at a rate c_1 per unit time (modification II). Then the expected catch (4) should be replaced by a formula involving a fairly complicated integral which has to be computed numerically (Helland, 1982, Equation I.7). If $k > 1$ and if $b_1 = \sqrt{c_1/D_1}$ is small, a Taylor expansion gives that the

predicted catch should be given by Equation (4) minus a loss term of the form

$$\frac{b_1^2 C}{4}\left(\frac{a}{r}\right)^k \sum_m \int^\infty \cos\left[(\theta + 2\pi m)s\right] \cdot \frac{e^{-|\ln\frac{a}{r}|\sqrt{k^2+s^2}}}{\sqrt{k^2+s^2}} \cdot \frac{(a^2+r^2) + |a^2 - r^2|\sqrt{k^2+s^2}}{k^2+s^2-1}\, ds \quad (6)$$

The expected total loss in the first phase can also be found, which gives an approximate relation between the numbers N_1 and N_2 in (5). When b_1 is small and $k > 1$, we find from a Taylor expansion

$$A \approx C\frac{D_1}{D_2}\left[1 - \frac{(ab_1)^2}{8(k-1)} - \frac{F}{N_1}\right] \quad (7)$$

where F is the total catch in the central pheromone trap. In particular, if $D_1 = D_2$ we see that A should be less than C.

As will be shown below, the expression (4) gives a fairly good fit to the data, but the parameter A appears to be as large as or larger than C.

A Two-State Model. The catch in passive traps seems to consist of two components: one that is symmetric around the pheromone trap and another that has an overweight of beetles in the region between the point of release and the pheromone trap (Figure 4). The symmetric component is explained by the term $AK_0(b_2 r)$ in (4) and is attributed to the beetles that reach the pheromone trap, escape, and start a new dispersion. In this second phase of the beetles' movement, we assume a diffusion without drift. Even if this model fits the data reasonably well, the explanation of the symmetric part may not be the right one, at least not the only one, and it becomes important to look for alternative/additional explanations.

One mechanism that will be investigated here is the following: Suppose that the released beetles first spend some time in a "free" state before they are affected by the pheromones from the central trap. This is what sometimes is called "flight exercises" (Graham, 1959; Wollerman, 1979). The dispersion of beetles in this free state is modeled by a diffusion without drift and with diffusion coefficient D_1. A free beetle has a probability c_0 per unit time of entering a "responsive" state, and in this latter state it gets a drift $k'/r = 2kD_1/r$ towards the pheromone trap. Both "free" and "responsive" beetles have a probability d_1 per unit time of getting lost. The states and the transitions of this model are summarized in Figure 5.

Under the assumptions of the two-state model, the expected catch in a passive trap at the position (r,θ) will be given by

$$AK_0(b_2 r) + C\left[H(r,\theta) + K_0\left(b_0\sqrt{r^2 + a^2 - 2a\cos\theta}\right)\right] \quad (8)$$

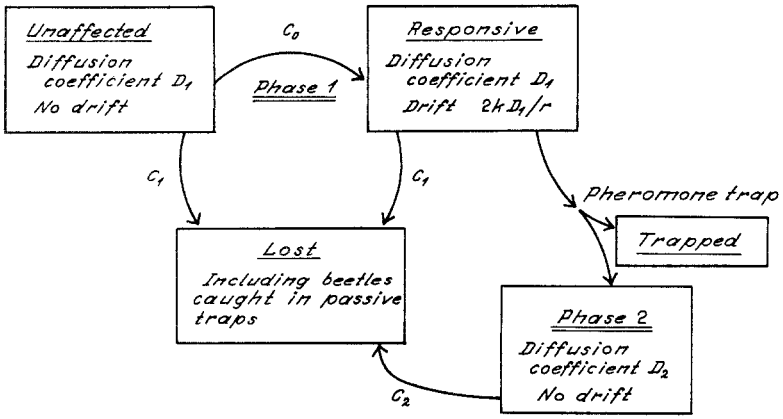

FIG. 5. The states of the beetles according to the two-state model. c_0 is the probability of entering the responsive state per unit time, c_1 and c_2 are the probabilities of getting lost in phase one and two, respectively; r = distance to pheromone trap.

where

$$H(r,\theta) = \frac{b_0^2}{2\pi r^k} \int_{x=0}^{\infty} \int_{u=-\lambda}^{\infty} x^{k+1} \cdot K_0\left(k\sqrt{(\theta-u)^2 + \ln\left(\frac{x}{r}\right)^2}\right) K_0\left(b_0\sqrt{x^2 + a^2 - 2ax\cos u}\right) du\, dx$$

and

$$b_0 = \sqrt{\frac{c_0}{D_1}}$$

see Helland (1982).

Here we have neglected the loss of beetles during the entire first phase ($c_1 = 0$). The double integral is calculated by a Monte Carlo simulation method (A detailed description can be obtained from the authors.). If we also want to account for losses during the first phase, a triple integral results, which in principle may be calculated by a similar method. The first term in (7) is the contribution from the beetles diffusing outwards from the pheromone trap after being attracted to it (phase 2), the $H(r,\theta)$ is the contribution from beetles affected by pheromones (phase 1), and the last K_0 term is the contribution from the beetles that have never been under the influence of pheromones (unaffected state).

The model considered in this section is only one of several possible "two-state models" for dispersal of insects. Here we have assumed a constant

probability c_0 per unit time of transition from the free to the responsive state, i.e., to the state with a drift towards the pheromone source. One can also imagine models where $c_0 = c_0(r)$ depends upon the distance r to the pheromone source. However, this is somewhat difficult to handle analytically [except for the special case $c_0(r) = \text{constant}/r^2$]. Another simple mechanism is when the transition takes place at a fixed distance from the pheromone source.

Pheromone Trap Catch. In all the models considered up to now, we can also find the probability distribution of the flight time used by the beetles before they reach the pheromone trap. This distribution is simplest in the first (one state) model. Here, if we assume that after the release there is a flight initiation time which is exponentially distributed with parameter λ, then the expected number of beetles per unit time that reach the pheromone trap will be

$$N\phi(t) = \frac{N\lambda}{\Gamma(k)} \int_0^t \frac{1}{s} \left(\frac{a^2}{4D_1 s}\right)^k e^{-\frac{a^2}{4D_1 s}} e^{-\lambda(t-s)}\, ds \tag{9}$$

where $\Gamma(k)$ is the gamma function.

When loss of beetles is taken into account, the distribution (9) is unaffected by the modifications (I) and (III) discussed above, except that under modification (I) (loss immediately after release) the number N that enters into (9) will be smaller than the number of released beetles. Under modification (II) (loss during flight), the formula (9) should be multiplied by $e^{-c_1 t}$.

Again the two-state model leads to more complicated expressions. When $c_1 = \lambda = 0$ (no flight initiation delay, no loss during flight), the expression (9) should be replaced by

$$N\phi(t) = N\frac{c_0 k}{t^{k+1}} \int_0^t u^k e^{-c_0 u - \frac{a^2}{4D_1 k}} g\left(\frac{a^2}{4D_1 t} \cdot \frac{t-u}{u}\right) du, \tag{10}$$

where

$$g(z) = \sum_{j=0}^{\infty} \frac{\Gamma(k+j+1) \cdot z^j}{\Gamma(k+1)(j!)^2}$$

is a degenerate hypergeometric function. For alternative expressions, see Helland (1982).

Fitting Models; Discussion of Fit

Passive Trap Catch Distribution. We first consider the one-state model with its modifications. In particular we include the possibility of a second phase, i.e., a free dispersal for some of the beetles after they have been close to the pheromone trap (modification III). Then the expected catch in a passive

trap at position (r,θ) is given by (4). If we allow for loss in phase one, the expression (6) should be subtracted. In (4) there are four unknown parameters: k, b_2, A, C, and in (4) minus (6) there is one in addition: b_1. The number of parameters can be reduced by fixing k equal to the mean value of the two estimates of k from the pheromone trap catch distributions (see below). Thus, we have to estimate from three to five parameters, depending on the assumptions incorporated into the model.

Parameters are estimated by maximum likelihood estimation using Fisher's score method and assuming the captures to be Poisson distributed. General descriptions of this method can be found in the appendix of Bailey (1961) and in Rao (1952). The estimates, together with their estimated standard deviations, and the fit to the data are given in Table 3. In Table 4 we have given the estimated variance–covariance matrix for A, C, b_2 in the estimation (ii) (no loss in phase one), and the fit to data is shown in Figure 6.

In the estimation where k is fixed, the standard errors as given in the tables are too low, since they do not take into account the uncertainty in the estimate of k.

In Table 3, the last two sets of parameter estimates, (iv) and (v), concern the two-state model and will be commented on later. In the simple one-state model, all the parameters k, C, b_1, and b_2 are estimated simultaneously by the set (i). The fit, as measured by a chi-square test, seems reasonable, even though the test shows significant deviations on the 5% level. It should be kept in mind that any biological model is an approximation, and thus the chi-square test will nearly always given significance when the total number of units observed is large enough. In this connection the chi-square should only be interpreted as a descriptive statistic, measuring the fit on a familiar scale.

However, two other difficulties are apparent in estimation (i). First, the parameter b_1^2 gets a negative estimate in the data set from June 23 and secondly the k estimate from July 8 is less than one, producing divergence in the integral of the equation (6). Both difficulties are solved by putting $b_1 = 0$, which results in the set of estimate (ii). The estimates for k, A, C, and b_2 do not change much, and the chi-square is about the same.

The loss of beetles in phase one is thus not significant. Allowing for a deviation from the estimate of about two standard deviations, it is seen that b_1^2 can be at most about 0.0035 (m^{-2}), i.e., b_1 about 0.06 (m^{-1}). The loss in phase two, as measured by b_2, seems to be considerably greater. Now this need not reflect any difference in the loss rates per unit time, but may be due to a difference in the diffusion coefficients of the two states, since $b_i = \sqrt{c_i/D_i}$, $i = 1,2$, where c_1 and c_2 are the loss rates and D_1 and D_2 are the diffusion coefficients of the two states. With b_1 about 0.06 and b_2 about 0.15, we find $D_2 \approx D_1/6$ if the loss rates are equal in the two phases. Thus the diffusion rate seems to be considerably slower in the second phase.

TABLE 3. MAXIMUM LIKELIHOOD ESTIMATES AND STANDARD DEVIATIONS OF PARAMETERS IN FIVE MODEL FITTINGS OF EXPECTED CATCH IN PASSIVE TRAPS[a]

	Date	k[b]	A	C	$b_1^2(m^{-2})$	$b_2(m^{-1})$	χ^2	df[d]	P
(i) Loss in both phases, k	June 23	1.52 ± 0.27	80.0 ± 9.7	60.7 ± 10.1	−0.0005 ± 0.0004	0.162 ± 0.016	38.8	23	0.021
estimated simultaneously	July 8[c]	0.72 ± 0.09	194.5 ± 42.0	13.7 ± 2.1	0.0016 ± 0.0009	0.368 ± 0.045	35.2	23	0.050
(ii) Loss in phase two, k	June 23	1.20 ± 0.13	89.9 ± 12.7	51.7 ± 6.5		0.184 ± 0.021	39.1	23	0.019
estimated simultaneously	July 8	0.66 ± 0.08	185.8 ± 38.6	12.3 ± 1.8		0.355 ± 0.043	38.2	24	0.033
(iii) Loss in phase two	June 23	2.26	60.4 ± 5.9	91.8 ± 7.6		0.121 ± 0.008	44.5	25	0.010
	July 8	2.26	67.0 ± 7.2	26.6 ± 3.3		0.160 ± 0.012	74.5	21	<0.001
(iv) Two states, loss in phase	June 23	2.26	67.9 ± 6.8	22.2 ± 2.0		0.134 ± 0.010	123.7	25	<0.001
two	July 8	2.26	67.6 ± 7.5	7.3 ± 0.9		0.164 ± 0.013	104.6	22	<0.001
(v) As (iv) but with k as	June 23	1.20	105.4 ± 13.5	11.3 ± 0.9		0.204 ± 0.019	57.7	25	<0.001
estimated in the	July 8	0.66	148.2 ± 26.3	2.7 ± 0.2		0.303 ± 0.033	53.3	22	<0.001
analogous one-state model (ii)									

[a]The estimations (i)–(iii) are based on the one-state model, (iv) and (v) on the two-state model.
[b]k estimated by pheromone trap catch distribution when value is 2.26.
[c]k estimated less than 1, giving divergence of the integral in (7).
[d]The degrees of freedom (df) in the χ^2 test vary, due to differences in the number of traps combined in the test.

TABLE 4. ESTIMATED VARIANCE–COVARIANCE MATRIX FOR A, b_2, AND C IN ESTIMATION (i) (NO LINKAGE BETWEEN A AND C, LOSS IN PHASE TWO)

	A	C	$b_2(m^{-1})$	
June 23	34.77	−8.15	0.04328	A
		57.68	0.00418	C
			0.00007	$b_2(m^{-1})$
July 8	51.37	1.72	0.07498	A
		10.66	0.01087	C
			0.00014	$b_2(m^{-1})$

This is also supported by the fact that parameter A is estimated larger than the parameter C. From (7) we get that a lower bound of the ratio D_1/D_2, is given by A/C, which seems to be greater than one. If we fit the data with A and C related by (7) with $D_1 = D_2$, a considerably poorer fit is obtained. We therefore conclude that D_1 is larger than D_2. This can be interpreted to mean that the flight activity of the beetles is larger in phases where the beetles are responding to pheromone, giving a larger diffusion coefficient in these phases.

What is more difficult to understand is that the ratio A/C seems larger on July 8 ($a = 20$ m) than on June 23 ($a = 12$ m). One part of the explanation may be that F/N_1 is closer to one on June 23, i.e., that a larger percent of the dispersing beetles are caught in the pheromone trap, but to account for the differences in A/C, we must assume that 80–90% of the released beetles leave the region at once, which seems like a high figure.

In any case it seems clear that a large part of the beetles leave the region at once. This may be interpreted as the beetle's need for "flight exercise" before they respond to pheromones, which will be discussed below in connection with the two-state model. The fact that we used pheromone to collect beetles for the experiment may also have an influence on their later response to pheromone. Earlier studies have, however, shown that beetles that have responded to pheromones once (as in our experiment) more readily respond a second time (Hain and Anderson, 1976; Anderson 1977), so this should rather increase the recapture.

The sole fact that A is not negligible shows that it is indispensable to include a second phase in the model. This means that only a portion of the beetles that arrive at the pheromone trap are caught by the trap. Regnander and Solbreck (1981) have shown that other types of cylinder traps are more efficient than the one used in this experiment.

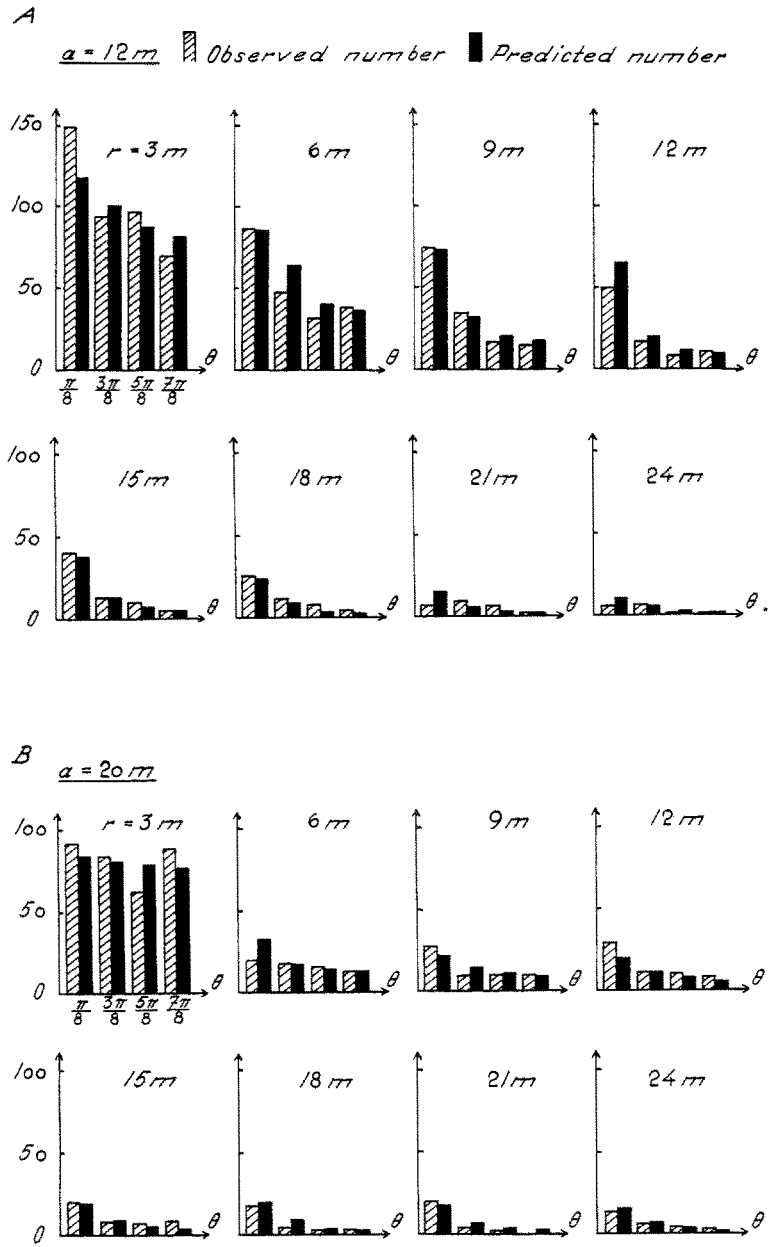

FIG. 6. Predicted and observed distribution of the passive trap catch distribution by estimation (i), Table 3. [Simultaneous estimation of A, b_2, and C on June 23 (A) and July 8 (B).] Angle θ and distance r as in Figures 3 and 4.

The estimates of the drift constant from the passive trap catch distribution are somewhat smaller than the estimate (2.26) from the pheromone trap data. By setting k equal to 2.26, we get a poorer fit, in particular for the data set from July 8 (set iii) of estimates, but note that visually the fit still seems to be relatively good (Figure 6).

The fact that k has a smaller estimate for the release distance 20 m than for release distance 12 m, may mean in reality that the drift falls off faster than constant/r as the distance increases. Another possible explanation is given by the two-state model. This explanation is also consistent with the fact that the recapture percentage was largest when the release distance was 12 m. Furthermore, the two-state model seems to reduce the difference between the two estimates for the loss b_2 in the second phase.

Pheromone Trap Catch Distribution. The diffusion coefficient D_1 and the drift factor k can be estimated from the pheromone trap catch distribution given by (9) with unknown parameters λ, k, and D_1. By observation, about half of the beetles had flown after 10 min, giving $\lambda = 0.0693$ min^{-1}, or $1/\lambda = 14.4$ min.

Using this and assuming $k > 2$, we arrive at the moment estimators

$$\hat{k} = 2 + \frac{(\overline{T} - 14.4)^2}{s^2 - 14.4^2} \qquad \hat{D}_1 = \frac{a^2}{4(\overline{T} - 14.4)(\hat{k} - 1)} \tag{11}$$

where $\overline{T}$ and s^2 are the mean and empirical variance of the observed times from release until catch in the pheromone trap. We have disregarded times larger than 140 min when calculating $\overline{T}$ and s^2.

The estimates are given in Table 5. The standard errors are rough estimates taking into account contributions from three independent sources of error: (1) the sampling error calculated from a Taylor expansion of (13), (2) the uncertainty in the flight initiation halving time, roughly put equal to 4 min, (3) the uncertainty due to too long observation periods at the pheromone trap on June 23.

We see that the two estimates of k are consistent and that they have about the same standard error. Their mean value is $\hat{k} = 2.26 \pm 0.12$. The estimate of D_1 from June 23 has a too large standard error to give any information about

TABLE 5. ESTIMATES OF k AND D_1 WITH APPROXIMATE STANDARD ERROR[a]

	k	D_1 (m^2/min)
June 23	2.02 ± 0.16	13.96 ± 37.50
July 8	2.50 ± 0.19	3.46 ± 1.59

[a]Pheromone trap catch distribution truncated at $t = 140$ min. $\lambda = 0.0693$ min^{-1}.

the size of the parameter. In the fit below we therefore use the value from July 8, namely, $\hat{D} = 3.46 \pm 1.59$ (m^2/min) in both experiments.

In Figure 7 we have fitted the time distribution (4) with these common values of k and D_1 and also with the separate estimates from the two dates.

If k is less than 2, the variance of the time of arrival at the pheromone trap will be inifinite in the theoretical model, which is consistent with the observed fact that the time distribution has very heavy tails. This implies that the moment estimators used in Figure 7 break down. An alternative is to use Fisher's scoring technique again. With $k = 0.66$, which is the estimate from the passive trap catch distribution on July 8, the parameter D_1 is estimated as (8.8 ± 3.7) m^2/min from the corresponding distribution of catches over time in the pheromone trap. The fit is poorer than what is shown on Figure 7, and this remains true if we truncate the observations (at 140 min) before estimating D_1. This may indicate that the true value of k for release distance 20 m should be somewhat higher than 0.66.

The distribution of catches over time in the pheromone trap shows heavy tails compared to the expected distributions (Figure 7). Since we did not measure the take-off frequency over time, we have assumed an exponentially distributed time for flight initiation with a half-life time of 10 min. However, it is reported from other experiments that this half-time increases when the individuals most eager to fly have left (Hain and Anderson, 1976), and this may be part of the explanation. Another release experiment (Hoff, unpublished) showed that the flight initiation was very sensitive to wind. It seemed as if the beetles preferred a weak wind to fly, whereas very few flew when there was absolutely no wind, and none when the wind was too strong. This tendency seemed to be stronger the fewer beetles were left on the platform. The heavy tails in the pheromone trap catch distribution may, in part, be due to favorable flight conditions just before the late peaks.

The fit in Figure 7 is about the same whether we use separate estimates for the two dates or use the same (mean) estimate of k and D_1. The mean gives a more accurate estimate, provided that the parametric values are equal in the two releases. The main physical difference between the two releases was higher air temperature and stronger mean wind speed on July 8. It is reasonable to believe that temperature and wind affect the diffusion coefficient D_1, since this reflects the flight activity of the beetles. *I. typographus* needs a temperature above approximately 20° C to fly (Annila, 1969). It is not known how the flight speed varies with temperature, but it might increase within reasonable limits. The higher the temperature, the more pheromone is evaporating from the trap, and this might increase the activity level and/or the drift coefficient. Increased activity or "flight speed" should increase the diffusion coefficient D_1. A complicating factor in this experiment is that the beetles were in worse condition on July 8 than on June 23, due to

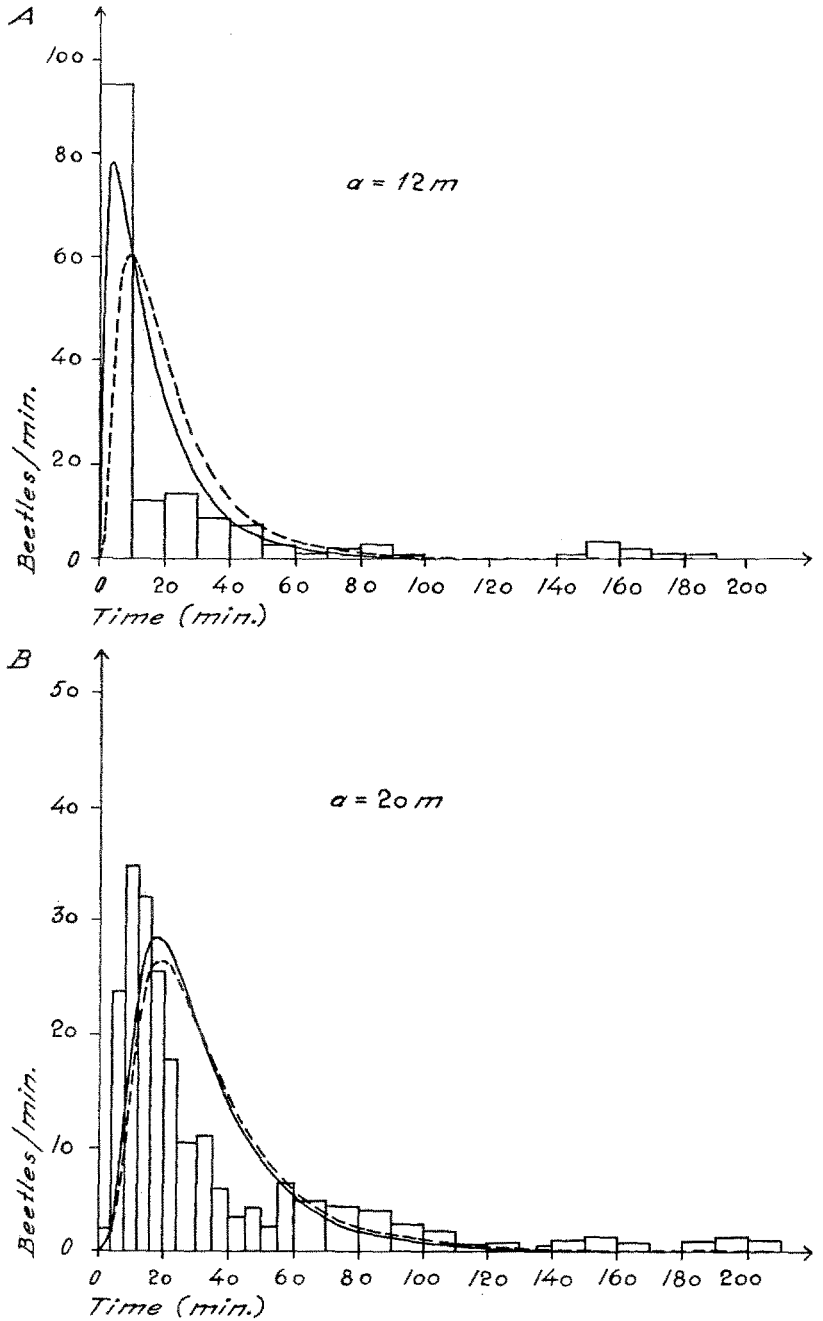

FIG. 7. Fitted time distribution of pheromone trap catch with time delay in flight initiation on June 23 (A) and July 8 (B). In estimating parameters, the observed catch is truncated at $t = 140$ min. Dashed line is the distribution when using same estimates of the drift and diffusion coefficients in the two experiments.

two more weeks in the refrigerator. This was indicated by the lower proportion of beetles that left the platforms on July 8.

Two-State Model. Consider now the model where the beetles first spend some time in an unaffected state, as shown in Figure 5. The expected time before the beetles are caught in the pheromone trap will be larger in this model, and this increase can be calculated explicitly in terms of the transition rate of c_0. If $\overline{T}$ is the (untruncated) mean time until catch, then this leads to the moment estimator of c_0 determined by

$$\overline{T} = \frac{1}{\lambda} + \frac{a^4}{4\hat{D}_1(\hat{k}-1)} + \frac{1}{\hat{c}_0} \cdot \frac{\hat{k}}{\hat{k}-1}$$

As an approximation, we take the same estimates $\hat{D}_1$ and $\hat{k}$ as before, since these are primarily determined by the leftmost part of the time curve. This leads to the c_0 estimates 0.202 min^{-1} and 0.196 min^{-1} on June 23 and July 8, respectively. The expected time distribution of the pheromone trap catch is calculated as $F \cdot \phi(t)$, where $\phi(t)$ is given by (9) and F is the total pheromone trap catch.

The result given in Figure 8 shows a better fit to data than the one-state model. However, note that the heavy tails are still not explained. Maximum likelihood estimates of the unknown parameters A, b_2, and C of (8) are given in Table 3. We see that the parameter A is still estimated larger than C in both experiments (Table 3). As for the one-state model, this is most likely due to a higher diffusion coefficient in the first phase. The fit to the observed passive trap catch distribution is, however, much more difficult to calculate due to the double integral in (8). The poor fit, as measured by the χ^2 statistic, is partly due to numerical inaccuracies in the Monte Carlo technique used for calculating the integral.

Comparing the estimations (iv) and (v), we see that the fit is somewhat better when we use the value of k as estimated from the same data set (estimation ii) than if we use $k = 2.26$ (from the distributions of catches over time in the pheromone trap). However, the ratio A/C seems to have unreasonably high estimates when using the lowest k values. The true value of the drift constant k is probably somewhere between 0.66 and 2.26.

The fit to data of the distributions of catches over time in the pheromone trap is better with the two-state model. This strengthens the hypothesis that flight enhances the response of the beetles to pheromone, which has received support from laboratory studies on scolytids of at least three genera: *Trypodendron* (Graham, 1959; Bennet and Borden, 1971), *Dendroctonus* (Bennet and Borden, 1971), and *Scolytus* (Choudhury and Kennedy, 1980). It seems reasonable to assume that this is valid for the genus *Ips* too, although Borden (1967) showed that brood adults of *Ips paraconfusus*, without any flight exercise, respond equally well to attractants as control beetles from the

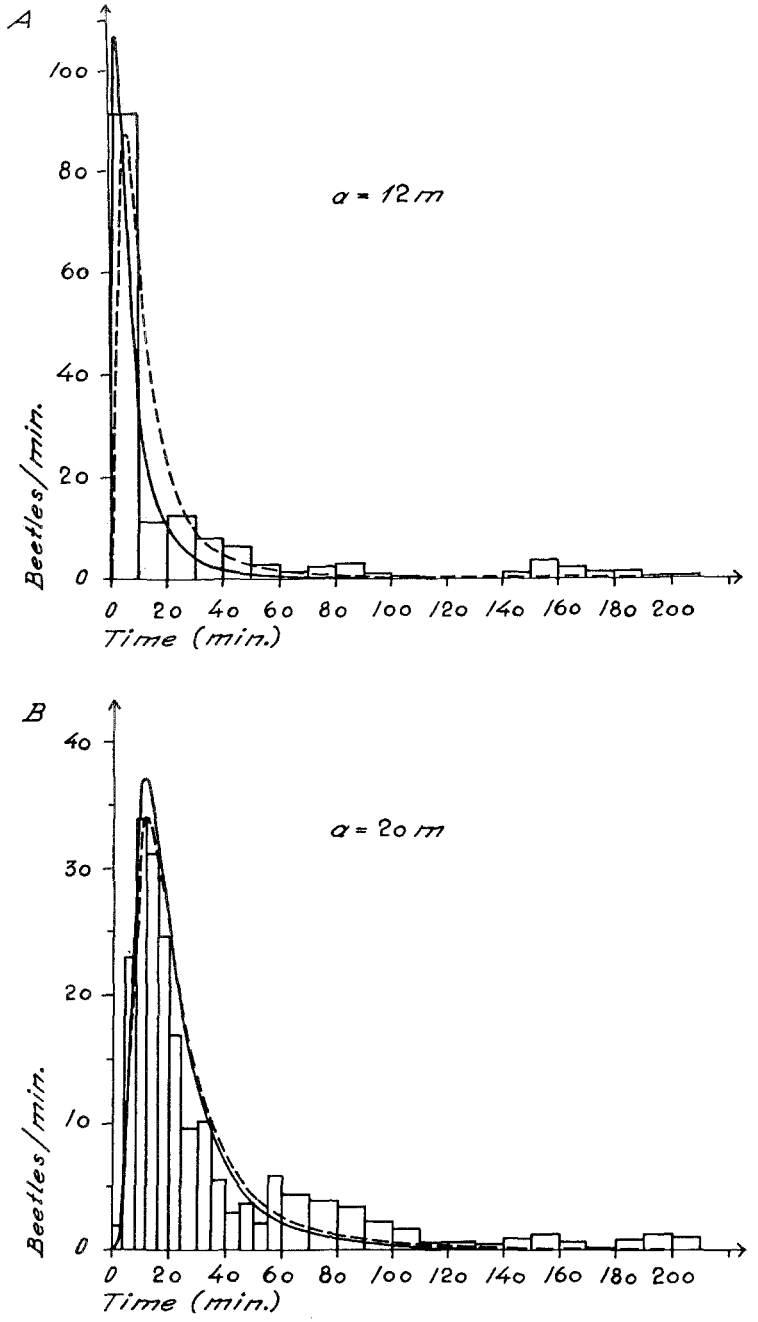

FIG. 8. Fitted time distribution of pheromone trap catch for the two state model, with time delay in flight initiation. In parameter estimation, the observed catch is truncated at $t = 140$ min. Dashed line is the distribution when using common estimates of the drift and diffusion coefficients in the two experiments.

overwintering population. The flight exercise hypothesis was also used by Wollerman (1979) to explain his low overall recapture rate in a large field study (3–8%) even though the hypothesis to some degree is contradicted by the fact that beetles he allowed to fly before release were recovered at an even lower rate. The present two-state model, with a constant rate of transition from an unaffected to a responsive state (see Figure 5), may, to a certain extent, describe results caused by difference in response of the beetles.

DISCUSSION

Referring to our first simple diffusion model with drift constant/r, and to the proposed modifications, it is seen that several modifications had to be taken into account to explain the data. It seems clear that the modification (II) (loss during dispersion towards the pheromone trap) can be neglected, but that both the modifications (I) and (III) are important: Some beetles disappear from the experimental area at once, and some beetles arrive at the pheromone trap but fly away again (phase two).

It seems difficult to arrive at definite conclusions regarding the relative importance of these two modifications. Ideally the ratio N_2/N_1 of beetles in the two phases can be found by using (6) and the observed ratio A/C [cf. (9)]. However, this is complicated by the lack of definite information about the ratio between the diffusion coefficients in the two phases (D_1/D_2). As discussed above, it seems that D_1 is much greater than D_2. Hence the ratio N_2/N_1 can still be relatively small even if A/C is considerable. This is strongly supported by the fact that the passive traps caught many more beetles on the outside than on the inside (see Table 1).

In any case, it is clear that to account for the symmetric part of the passive trap capture, one is forced to include a second phase in the model, i.e., to assume that a certain percentage of the beetles that arrive at the pheromone trap are never caught. This is so even if we try a more complicated model for the first phase (the two-state model, taking the need for "flight exercise" into account). Intuitively, it seems likely that these qualitative conclusions also will be retained if we use other basic models, for instance diffusions with other functional forms of the drift term.

In all models of this kind that have been investigated, the average expected catch on a ring of radius r, looked upon as a function of r, has a derivative which is discontinuous at $r = a$ (the release distance). The observed average catch per ring in this experiment seems to be a smooth function of r, which may be due to the fact that we have no observations for the angle $\theta = 0$. If this experiment is to be repeated, one should also try to place passive traps along the line between the release point and the pheromone trap, so that this phenomenon can be studied more closely.

As for the results of the present experiment, the average catch per ring shows good fit to the simple Bessel function (2), which reflects the fact that the first (symmetric) term in (4) plays a dominant role.

The fit to the data of the present model with all its modifications seems to be relatively good, judged qualitatively. For instance, Figure 6 is drawn for a set of parameter estimates that formally gives significant deviations, as judged by a chi-square test, but the qualitative form of the data on the one hand and the model predictions on the other hand are still about the same. If we had used the estimation (ii), the fit would have been better, but the rough form of the predictions would not have changed much.

Thus it appears that the diffusion model used in this paper can be used to give a rough description of data from experiments of the type considered here. This does not mean that the model described reality completely. The way we have used the model here has been to look at the deviations from the model and try to understand what these deviations tell us about the biology behind insect dispersal and about the pheromone traps. We hope that it will be possible to use the same method to derive more information about the dispersal of insects, either from this experiment or from other related experiments. It is clear that the wind will have to be included if we want to go into more details. In the present paper we have only symmetrized with respect to wind direction. A more satisfying solution would have been to include wind in the models. However, this seems to require even more complicated mathematical models. Any diffusion model taking the wind into account will, by necessity, have to include a nonsymmetrical drift term.

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GYPSY MOTH (*Lymantria dispar* L.) ATTRACTION TO DISPARLURE ENANTIOMERS AND THE OLEFIN PRECURSOR IN THE PEOPLE'S REPUBLIC OF CHINA[1]

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Abstract—Pheromone traps baited with disparlure, *cis*-7,8-epoxy-2-methyloctadecane, captured males of *Lymantria dispar*, the gypsy moth, at two widely separated locations in the People's Republic of China. The (+) enantiomer of disparlure attracted significantly more males than the racemate; addition of olefin reduced captures. The duration of the flight period was longer (eight weeks) and peaked earlier near Beijing than farther north near Dunhua (five weeks).

Key Words—*Lymantria dispar*, gypsy moth, Lepidoptera, Lymantriidae, pheromone, disparlure.

INTRODUCTION

A team of United States entomologists traveled to the People's Republic of China from May 13 to July 9, 1982, under a scientific agreement between the

[1] Lepidoptera: Lymantriidae.

Chinese Academy of Forestry and the U.S. Department of Agriculture to investigate the natural enemy complex, biology, and distribution of the gypsy moth, *Lymantria dispar* L. The gypsy moth is commonly found in the northeastern provinces of Heilongjiang and Jilin as well as Beijing. East Asian gypsy moths differ from North American ones in a number of characteristics, including wide variations in larval coloration. However, the major behavioral difference is the ability to fly; East Asian and some Eurasian females fly, whereas North American females are flightless (Leonard, 1974). Miao (1939) reported that, in Nanjing, female gypsy moths flew only when disturbed. We observed that females flew readily and saw evidence in the field that females had flown to lights and laid egg masses on nearby objects.

Disparlure (*cis*-7,8-epoxy-2-methyloctadecane) is widely utilized for monitoring gypsy moth populations in the U.S. (Elkinton and Cardé, 1980; Schwalbe, 1981; Elkinton and Childs, 1983). The (+) enantiomer of disparlure is ca. 10 times as attractive as the racemate (Yamada et al., 1976; Cardé et al., 1977; Plimmer et al., 1977; Miller et al., 1977). We report here some results of studies to determine the attractiveness of (+)- and (±)-disparlure and its olefin precursor to gypsy moth males at two widely separated locations in the People's Republic of China.

METHODS AND MATERIALS

Two sites were selected and traps were deployed on May 19, 1982, at the Ming Tombs, ca. 45 km northwest of Beijing (40° 12′N, 116° 6′E) in an 8-hectare persimmon (*Diospyros* spp.) orchard, and on July 1, 1982, at Ma Lu Gou Forest Farm (43° 12′N, 128° 18′E) ca. 60 km northeast of Dunhua, Jilin Province, in a forest of 20 to 25-cm-dbh *Quercus mongolica* Fisch. trees. In each location, 36 standard milk-carton traps (Elkinton and Childs, 1983) were placed 15 m apart in a 6 × 6 randomized complete block design. Five of the six traps in each treatment were then baited with cotton wicks (1 cm long × 1 cm diam) impregnated with the test chemicals, each in 100 μl hexane. Fresh disposable rubber gloves were used while baiting traps for each treatment. Treatments evaluated were: (1) (+)-disparlure (Farnum et al., 1977) (1 mg), (2) (+)-disparlure (1 mg) plus olefin 2-methyl-(*Z*)-7-octadecane (1 mg), (3) olefin (1 mg), (4) (±)-disparlure (2 mg), (5) (±)-disparlure (2 mg) plus olefin (1 mg), and (6) for the control, hexane (100 μl). A 3 × 3 × 0.03-cm piece of Vapona Peststrip[7] (dichlorvos) was placed on the bottom of each trap to kill

[7] The use of trade, firm, or corporation names in this publication is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Agriculture or the Forest Service of any other product or service to the exclusion of others that may be suitable.

captured insects. Traps were examined weekly, the numbers of *L. dispar* males captured were recorded, and the insects were removed.

RESULTS AND DISCUSSION

Gypsy moth males in the People's Republic of China were attracted to both (+)- and (±)-disparlure in both locations. (+)-Disparlure and (+)-disparlure and olefin captured significantly more ($P < 0.01$) males in Beijing than all other treatments. However, in Dunhua (+)-disparlure caught significantly more ($P < 0.01$) males than all treatments including (+)-disparlure and olefin (Table 1). The addition of olefin to either (+)- or (±)-disparlure did not increase captures and significantly reduced them at Dunhua. We cannot explain the reason for this variability, but it suggests varietal or specific biotype differences. Olefin alone had minimal attraction; the hexane control captured nothing.

No gypsy moth females or other Lepidoptera were captured by any treatment although *Lymantria mathura* Moore larvae were observed feeding in the Dunhua site when traps were deployed. Adult *L. mathura* activity was not actually observed, but rearing tests indicated that its flight activity would coincide with that of *L. dispar*. Thus, *L. mathura* was not attracted to disparlure (or if it was, was not captured) as has been reported for its congeneric species *Lymantria monacha* L. in the U.S.S.R. (Kovalev et al., 1980; Klimetzek et al., 1976; Vité et al., 1976).

Adult flight began earlier at Beijing than at Dunhua but the flight period

TABLE 1. TOTAL *L. dispar* MALES CAPTURED[a] DURING 1982 AT PHEROMONE TRAPS BAITED WITH DISPARLURE ENANTIOMERS AND OLEFIN PRECURSOR IN THE PEOPLE'S REPUBLIC OF CHINA[b]

Treatment/trap	Beijing	Dunhua
(+)-Disparlure (1 mg)	55a	32a
(+)-Disparlure (1 mg) and olefin (1 mg)	44a	2b
Olefin (1 mg)	1b	2b
(±)-Disparlure (2 mg)	3b	3b
(±)-Disparlure (2 mg) and olefin (1 mg)	5b	6b
Hexane (100 μl) (control)	0b	0b

[a] All counts were compared and those with different letters are significantly different at the $P < 0.01$ level for the χ^2 test.

[b] Randomized block design of five milk carton traps per treatment.

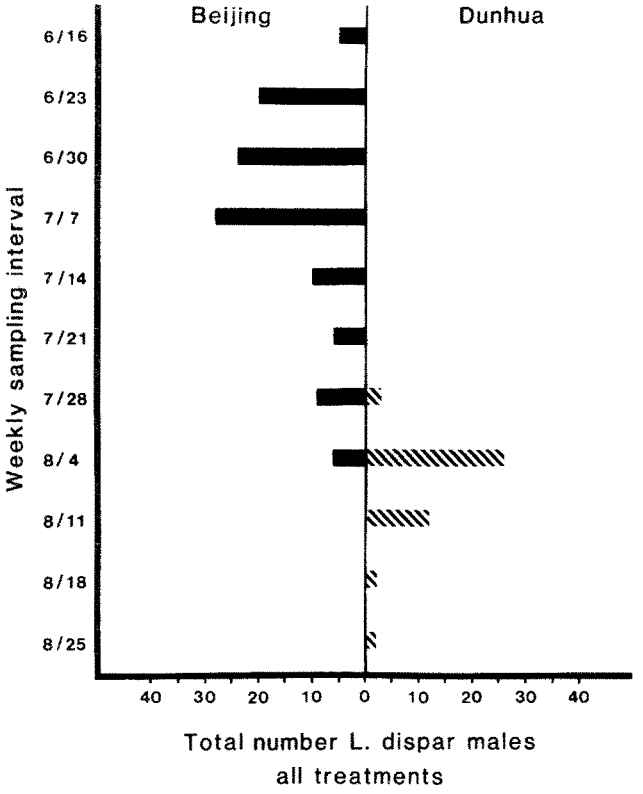

FIG. 1. Total weekly capture of *L. dispar* from all pheromone trap treatments at Beijing and Dunhua, People's Republic of China.

was longer at Beijing (eight weeks) than at Dunhua (five weeks) (Figure 1). Since we could not determine egg mass or larval numbers in each area, comparison of total male captures between Beijing and Dunhua is not realistic, but peak flight periods are comparable. Peak adult male activity was 1 month earlier at Beijing than at Dunhua, which may reflect geographic location, hosts upon which larvae fed, biotype differences, or other factors. The weekly proportion of total capture by treatment did not differ significantly ($P > 0.05$, Beijing $G^2 = 26.00$; Dunhua $G^2 = 9.80$) from one week to another by a log likelihood ratio test.

The greater attraction of (+)-disparlure than its racemate and the effect of the olefin analog of disparlure in reducing attraction in these tests are consistent with the response of male gypsy moths to these chemicals in northeastern North America (Cardé et al., 1977; Plimmer et al., 1977) and in the eastern U.S.S.R. (Kovalev et al., 1980). This suggests that pheromone

production and response of Eurasian gypsy moths are similar to those of North American gypsy moths, despite other behavioral differences between these populations.

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RECEPTOR CELLS IN *Ips typographus* AND *Dendroctonus micans* SPECIFIC TO PHEROMONES OF THE RECIPROCAL GENUS

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Abstract—Olfactory receptor cells were studied electrophysiologically in *Ips typographus* and *Dendroctonus micans*. The investigation revealed cells which were keyed to pheromone compounds characteristic of the reciprocal genus. Thus, cells keyed to *exo*-brevicomin were found in *I. typographus*, whereas cells keyed to (+)-ipsdienol were present in *D. micans*. Laboratory behavioral tests indicated an attractive effect of the two compounds on beetles of the reciprocal genus. In *I. typographus* the effect of *exo*-brevicomin predominantly concerned males and enhanced their response to the pheromone "ipslure." It is suggested that *exo*-brevicomin serves as an interspecific attractant for *I. typographus*, which may be guided by pheromone compounds of the reciprocal genus in finding suitable breeding material. The function of (+)-ipsdienol in *D. micans* is more uncertain. It may be either a pheromone or an interspecific messenger.

Key Words—*Ips typographus*, *Dendroctonus micans*, Coleoptera, Scolytidae, *exo*-brevicomin, (+)-ipsdienol, single-cell recordings, interspecific attraction.

INTRODUCTION

Aggregation pheromone components that are specific for the two genera *Ips* and *Dendroctonus* are known as ipsdienol, ipsenol and brevicomin, frontalin, respectively (cf. Bordon, 1974), and are produced by males in species of *Ips* and by females and males in species of *Dendroctonus*. It has recently been found in field tests that ipsdienol also influences the behavior of beetles belonging to the genus *Dendroctonus*. Catches of *D. brevicomis* in phero-

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mone traps were reduced when ipsdienol was added (Byers, 1982). It was concluded that ipsdienol plays a role in the interspecific interruption between the two sympatric species, *D. brevicomis* and *I. paraconfusus*, the latter using (+)-ipsdienol as one compound of its aggregation pheromone (Silverstein, 1966). Byers (1982) also showed that the males of *D. brevicomis* produce a small amount of (+)-ipsdienol which increases by exposing the beetles to myrcene, found to be the precursor of ipsdienol in *Ips* species (Hughes, 1974). Byers suggested that (+)-ipsdienol, produced by the males, has the function of interrupting the attraction of *I. pini*, another species competing with *D. brevicomis*. Accordingly, it was previously shown that the attraction of *I. pini* to its pheromone, (−)-ipsdienol, is interrupted when (+)-ipsdienol is added (Birch et al., 1980).

In the European species *I. typographus* a mixture of ipsdienol, *cis*-verbenol, and 2,3,2-methyl-butenol (named ipslure) serves as aggregation pheromone (Bakke et al., 1977). *I. typographus* and *D. micans* are sympatric in some areas. Both infest the Norwegian spruce *Picea abies*, but, at different parts of the trunk. *D. micans* prefers the lowest part where the bark is thickest, while *I. typographus* generally breeds above this level. The behavior of *D. micans* differs from that of *I. typographus* by not involving aggregation (cf. Gregoire, 1983). The females of *D. micans* attack healthy trees without killing them, while *I. typographus* mainly attack enfeebled trees. Thus, the two species living on the same host tree generally do not seem to be competitors.

In the present study we have investigated the responses of sensory cells of the two species to pheromones existing in both genera. The results show that specific receptor cells for ipsdienol and *exo*-brevicomin are present both in *I. typographus* and in *D. micans*. Behavioral tests performed in the laboratory suggest that the reciprocal pheromone compounds, ipsdienol and *exo*-brevicomin, play a role in the attraction of the two species.

METHODS AND MATERIALS

The beetles used were cultured in the laboratory. *I. typographus* originated from Klaebu, Trondheim, and *D. micans* from Lozere, France.

Electrophysiology

Recording. The beetles were mounted vertically in a Plexiglas holder and fixed for recordings as described by Angst and Lanier (1979). Tungsten microelectrodes (tip diameter about 0.3 μm) were used for recording. The recording of impulses from single olfactory cells was carried out as previously described (Boeckh, 1962; Mustaparta et al., 1979).

Stimulation. The "syringe-olfactometer" was used for stimulation (Kafka, 1971). The same procedure as described previously (Mustaparta et al.,

1980) was employed, i.e., stepwise dilution (1:10) of each compound in hexane, performed in two series. Control of the concentrations was made by gas–liquid chromatography and application of the samples on filter paper for evaporation of hexane. Stimulation was carried out with five intensities of each compound—compound amounts of 0.1, 1, 10, 100, and 1000 μg, (or 0.5, 5, 50, and 500 μg) inside each syringe.

Substances. Optical isomers of ipsdienol were kindly supplied by Dr. G. Ohloff, Firmenich, Geneva, Switzerland (Ohloff and Giersch, 1977). Each sample contained about 8% of the opposite enantiometer. *Exo*-brevicomin (racemic mixture) was purchased from Chemical Samples (purity about 98%). In addition, a number of other bark beetle pheromones and host volatiles (cf. Mustaparta, 1979) were used for electrophysiological classification of the olfactory receptor cells.

Behavioral Tests

An open arena (30 × 30 cm) was used for studies of the behavioral responses to the compounds tested. The beetles were allowed to enter the arena, one by one, through a narrow channel at the middle of one side of the arena. The channel emanated from a Petri dish where the beetles originally were placed. Two airstreams were blown through glass capillaries from the opposite side of the arena and crossed at an angle of 50°, 1 cm in front of the channel outlet. The two airstreams had the same flow rate, 250 ml/min (controlled by a flowmeter). To prevent adaptation, the streams were applied by the same pump in synchronous pulses with 2-sec durations and intervals. Odorless air was led directly through Teflon tubes into the glass capillaries, while air to be odorized was first led through a glass bottle (2 liters) containing the odorous medium.

I. typographus. Seven millimeters of the Borregaard dispenser, containing 1 mg of 2,3,2-methyl-butenol, 0.5 mg of *cis*-verbenol, and 0.1 mg of ipsdienol, served as pheromone medium for *I. typographus.* Addition of *exo*-brevicomin to this odor was accomplished via a separate capillary system. The beetles were taken from the laboratory culture logs and were kept on moistened filter paper in Petri dishes for about 20 hr before the tests. Since the beetles were of different ages and thus might respond differently, we performed a selection test before each experiment. In the selection test the beetles were allowed to choose between an airstream containing the odor from host materials added to the pheromone mixture and an odorless airstream. Only individuals showing positive reaction to the odorous airstream were selected for the experiments, while those choosing the odorless airstream, or any other direction, were discarded. In order to test the influence of *exo*-brevicomin on the behavior of *I. typographus*, the selected beetles were then allowed to choose between the odor of the pheromone mixture alone and that of the same

mixture to which *exo*-brevicomin (0.8 mg) was added. Preliminary tests showed no effect of *exo*-brevicomin alone.

D. micans. The beetles of *D. micans* were of the same age and were tested directly without any preceding selection test. Each beetle was used in two tests, one allowing a choice between ipsdienol and pure air and the other a choice between *exo*-brevicomin and pure air. Since preliminary tests did not show an enhanced effect, the mixture of the two were not tested systematically.

RESULTS

Receptor Responses. Like olfactory cells previously described in other *Ips* species (Mustaparta et al., 1979), the cells studied in *I. typographus* and *D. micans* could be classified in separate groups, each responding to one key compound. In *I. typographus* 80 olfactory cells were classified based on their responses to different odors, of which 56 were found specialized to bark beetle pheromones. Twelve cells responded specifically to ipsdienol, and six were specialized to *exo*-brevicomin. In *D. micans* 32 olfactory cells were classified. Five different types were found. Of these, one type (five cells) was specialized to ipsdienol, and another (eight cells) to *exo*-brevicomin. Only the cells specialized to ipsdienol and to *exo*-brevicomin in the two species are further considered here.

The cells keyed to ipsdienol were tested with the optical isomers and in *I. typographus* could be separated further as (+)- and (−)-ipsdienol types, according to the most potent enantiomer (cf. Mustaparta et al., 1980). However, the five ipsdienol cells obtained in *D. micans* were all keyed to (+)-ipsdienol. Figure 1A and B shows results consistently obtained in *I. typographus* and *D. micans*, respectively. These dose–response curves demonstrate that (+)-ipsdienol is the key compound for cells of both species. Furthermore, the roughly 10 times lower effect of (−)-ipsdienol can be ascribed to the optical impurity of the ipsdienol enantiomer used. All other test compounds had only minor effects on these cells. In Figure 1C the dose–response curves show that (−)-ipsdienol is the key substance for another cell type in *I. typographus*. The low effect obtained here for (+)-ipsdienol can also be ascribed to optical impurity, and again here the stimulation effect of other test compounds was minimal.

Since optical isomers of *exo*-brevicomin were not available, all cells keyed to that compound were placed in one group. Typical examples of the dose–response relationships of these cells in each species are shown in Figure 2A and B. The curves clearly demonstrate that *exo*-brevicomin is the key substance.

Behavioral Responses. Upstream walking (zig-zag or with frequent stops) together with antennal movements were regarded as positive behavioral reactions to the applied odors. Walking either ended with a flight

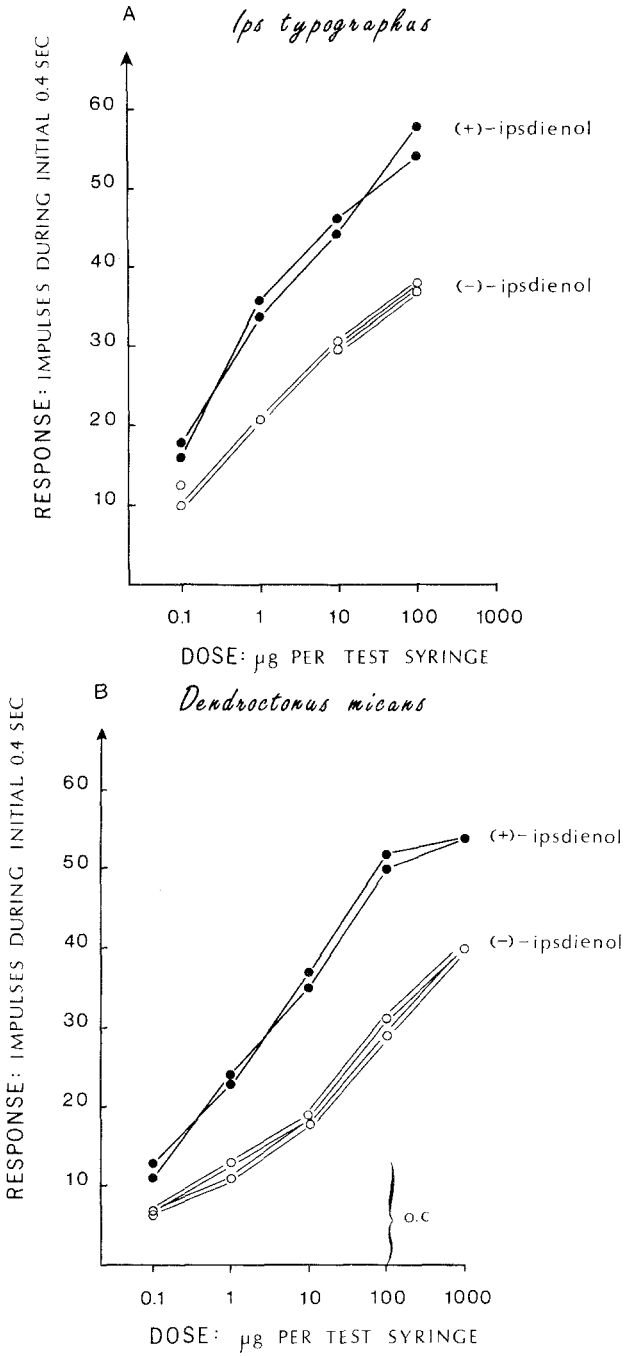

FIG. 1. Dose–response curves for olfactory receptor cells specialized in ipsdienol in *Ips typographus* and *Dendroctonus micans:* (A) cell keyed to (+)—ipsdienol in *I. typographus;* (B) cell keyed to (+)-ipsdienol in *D. micans;* (C) cell keyed to (−)-ipsdienol in *I. typographus.*

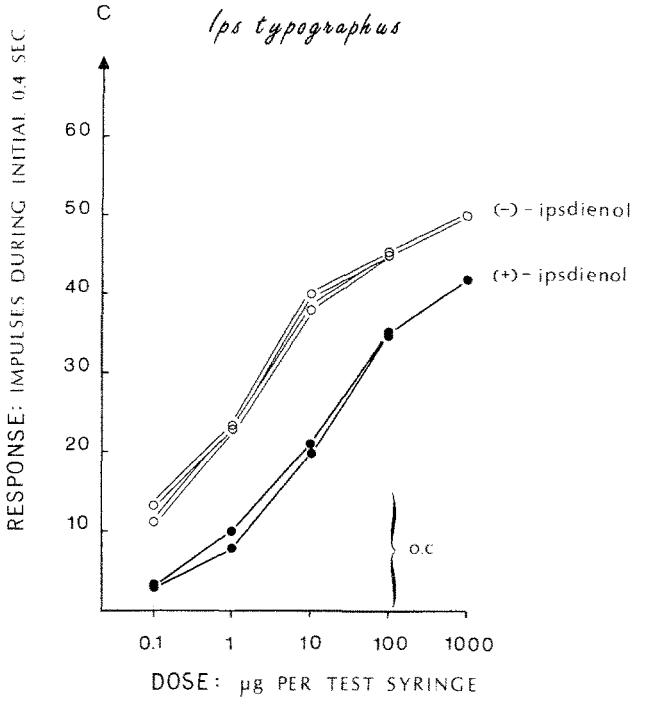

FIG. 1. Continued

reaction or continued until the glass capillaries were reached, around which the beetles displayed searching behavior.

For the *I. typographus* experiments, 333 fresh beetles responded positively to odors in the selection test. In the subsequent test, allowing the beetles, one by one, to choose between ipslure and the mixture of ipslure and *exo*-brevicomin, 72 beetles chose ipslure and 118 the *exo*-brevicomin mixture (Figure 3); 143 did not react. After the tests the beetles were dissected for sex determination. The results are shown in Figure 3. In the group which chose ipslure alone, the sex ratio was 25 ♂♂ : 47 ♀♀. In the group which chose the ipslure–*exo*- brevicomin mixture the ratio was 67 ♂♂ : 51 ♀♀. The sex ratio of all 333 selected beetles was 154 ♂♂ : 179 ♀♀. Thus, the accentuated attraction of beetles to the *exo*-brevicomin–ipslure mixture, compared to the ipslure alone, was due to increased attraction of males, while females were about equally attracted to the two odors. The sex ratios of the two beetle groups choosing the *exo*-brevicomin–ipslure mixture and ipslure alone were significantly different ($\chi^2 = 8.71$; $P < 0.005$).

Only 119 beetles of *D. micans* were available for behavioral tests. In the first test (choice between ipsdienol and pure air), 41 chose ipsdienol, no beetles chose pure air, and 78 did not respond. In the second test (choice between

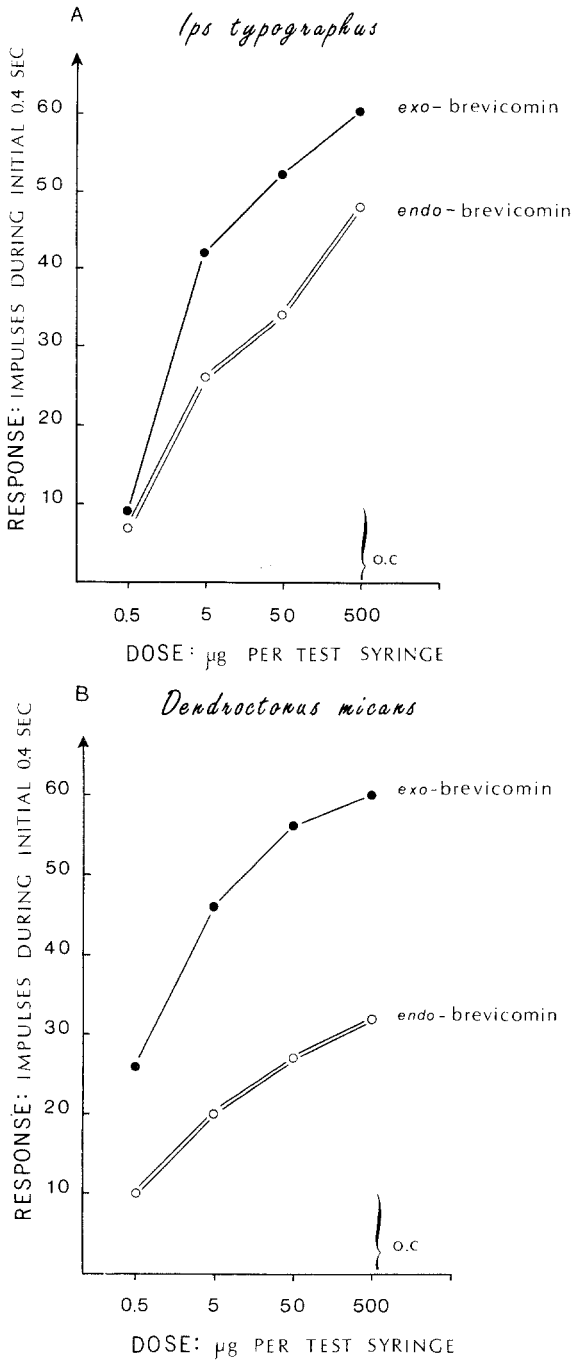

FIG. 2. Dose-response curves for olfactory receptor cells specialized to *exo*-brevicomin in: (A) *Ips typographus* and (B) *Dendroctonus micans.* Optical isomers were not tested.

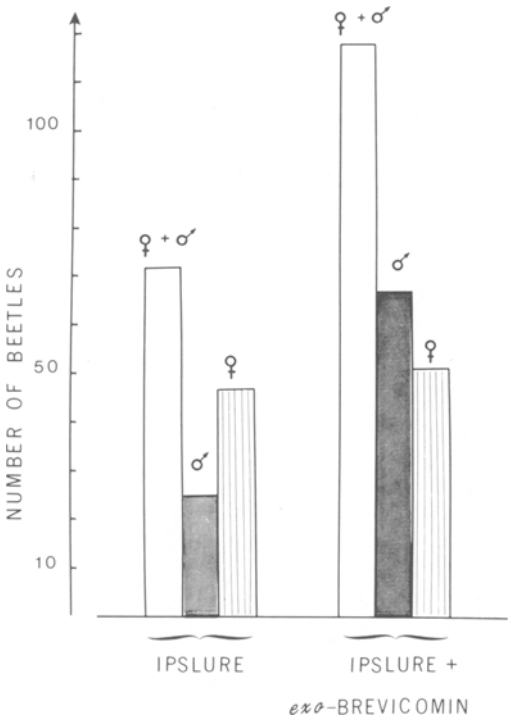

FIG. 3. Histograms showing the number of beetles of *Ips typographus* which chose an *exo*-brevicomin–ipslure mixture vs. ipslure alone in a choice experiment where the beetles were exposed to both odors. The sex ratio is shown to the right in each category.

exo-brevicomin and pure air), 36 of the beetles chose *exo*-brevicomin, no beetles chose pure air, and 83 did not respond. Twelve of the beetles responding to ipsdienol in the first test also responded to *exo*-brevicomin in the second test.

DISCUSSION

The electrophysiological recordings of this study demonstrate the presence of receptor cells in one species of *Ips*, and in one species of *Dendroctonus* which are specific for pheromones characteristic of the reciprocal genus. Since the number of these cells was similar to the number of cells keyed to pheromones of the species, it appears that they are of functional importance for the beetles. The relative numbers obtained here seem well correlated to the results of our preliminary electroantennogram (EAG) recordings, showing that (+)-

ipsdienol and *exo*-brevicomin have the highest effect on the antennal receptor cells of *D. micans* females, and that *exo*-brevicomin is almost as effective as the ipsdienol enantiomers in *I. typographus* (unpublished). Byers and Wood (1981) have discussed tentative acceptors to explain the influence of ipsdienol on the behavior of *D. brevicomis*. Since the present results show that specific ipsdienol receptor cells are present in *D. micans*, it appears likely that such cells also exist in *D. brevicomis*.

In the behavioral tests, performed as a complement to the electrophysiological studies, it was of interest to find out whether *exo*-brevicomin influences the behavior of *I. typographus*. The results suggest that *exo*-brevicomin enhances the attraction to the pheromone ipslure: *exo*-brevicomin alone did not seem to attract *L. typographus*, but when added to ipslure, it enhanced the attraction of that pheromone by about 50% (Figure 3). Extensive chemical analysis of pheromones in *I. typographus* (Bakke et al., 1977) as well as in various other *Ips* (Silverstein et al., 1966) have not revealed the presence of *exo*-brevicomin. Therefore, it appears likely that *exo*-brevicomin acts interspecifically in *I. typographus*. Interspecific pheromone action is established in the case of interruption of competing species of *D. brevicomis* and *I. paraconfusus* (Byers and Wood, 1980). However, the present results indicate an attractive function. The most likely sympatric species producing this signal is *D. micans* (see below). Therefore, one possibility is that *I. typographus* uses *exo*-brevicomin (produced by *D. micans*) as one cue in finding areas with suitable host materials. Since the male is responsible for selecting the colonization site, it is reasonable that *exo*-brevicomin enhances their attraction. This assumption is supported by the present sex determination which revealed that *exo*-brevicomin significantly enhanced the attraction of males but had no effect on females. In contrast, more females than males were attracted to the ipslure alone in the present competition test. However, this test cannot answer the question of whether ipslure is more attractive to females in a non-competing situation. Field studies have shown that the ratio between females and males in ipslure traps is between 1.5 and 3 (Bakke and Riege, 1982). Therefore, on the basis of the present results, it is tempting to suggest that *exo*-brevicomin should be used in the field (either added to ipslure traps, or in their vicinity) in order to increase catches of *I. typographus* males.

An interspecific attraction between beetles of *Ips* and *Dendroctonus* has been observed by Byers and Wood (1981). It was found that the pheromone of *I. paraconfusus* attracted *D. brevicomis* at a low release rate, whereas higher release rates caused interruption. Byers and Wood suggested that *D. brevicomis* might exploit weakened host materials by responding to the less aggressive tree-killer *I. paraconfusus*. A similar relationship may exist for *I. typographus* and *D. micans*; the latter being the less aggressive tree-killer whose pheromone compounds may guide *I. typographus* to suitable host

materials. We did not find, however, that *exo*-brevicomin at high concentrations reduced the attraction of *I. typographus.*

The pheromones of *D. micans* have not been identified so far. However, it is suggested that this species only uses sex pheromones (and not aggregation pheromones) where females attract males (Gregoire 1983). In preliminary field tests, Vité (personal communication) found that *exo*-brevicomin attracted males of *D. micans*, and our preliminary EAG recordings (see above) indicate that this compound belongs to the most important of chemical signals in *D. micans.* As mentioned above, the characteristic *Ips* pheromone, (+)-ipsdienol, had a pronounced EAG effect in *D. micans* females (males were not tested). This raises the question of whether (+)-ipsdienol is used as a pheromone in *D. micans* or is acting as an interspecific signal. Byers (1982) reported that males of *D. brevicomis* produce a small amount of ipsdienol and suggested that it functioned both intraspecifically to interrupt attraction at close range and interspecifically to interrupt attraction of a competing species, *I. pini.* In our behavioral tests only 40% of the *D. micans* beetles chose ipsdienol. However, the individual responses were clearly positive. It may well be that *D. micans* use (+)-ipsdienol, produced by species of *Ips*, as an additional cue to find breeding materials. Their different host requirement would, however, prevent *D. micans* from settling on the same host tree as *Ips* species. The suggestions made here have to be tested in the field before any definite conclusions about the ecological importance of *exo*-brevicomin and (+)-ipsdienol can be drawn.

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GENERAL APPROACH TO SYNTHESIS OF CHIRAL BRANCHED HYDROCARBONS IN HIGH CONFIGURATIONAL PURITY

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Abstract—Configurationally pure (>99.6%) α-methylalkanoic acids have been employed to prepare chiral hydrocarbon semiochemicals. The stereoisomers of the following compounds were synthesized: 13-methylhentriacontane, 15-methyltritriacontane, and 15,19-dimethyltritriacontane. The first compound was identified earlier as a kairomone of the corn earworm while the other two are sex excitants of the stable fly. The methods described have broad applicability in asymmetric synthesis.

Key Words—Chiral hydrocarbons, synthesis, branched hydrocarbons, dicestereomeric amides, resolution, enantiomers, optical purity.

INTRODUCTION

A number of hydrocarbons have been isolated from insects that have been implicated as kairomones or sex excitants and that have one or more centers of asymmetry in their molecular structures. At the present time, assignment of absolute configuration for such natural products rests upon successful asymmetric synthesis of the requisite steroisomers and subsequent assessment of their biological activity. To date, only a few chiral hydrocarbon semiochemicals have been synthesized and evaluated. The set of stereoisomers of 17,21-dimethyl-heptatriacontane, a component of the sex pheromone of a species of tsetse fly, *Glossina morsitans morsitans* (Westwood), was synthesized employing preparative high-performance liquid chromatography (HPLC) and a novel method of asymmetric induction employing alkylation of a chiral enolate (Ade et al., 1980). Although several other chiral aliphatic

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hydrocarbon structures have been reported (Carlson et al., 1978; Jones et al., 1971; McDowell et al., 1981; Rogoff et al., 1980; Sonnet et al., 1977, 1979; Uebel et al., 1976; Vinson et al., 1975), none has been synthesized configurationally pure. Probably the weak pheromonal activity of the natural product coupled with projected arduous synthetic effort had dissuaded chemists from the task.

Recently we described a method for obtaining α-alkylalkanoic acids in high configurational purity that was facilitated by a high-yield fractional crystallization of diastereomeric amides based on readily available and very pure (*R*)- and (*S*)-α-methylbenzylamines (Sonnet, 1982). The target acids were converted to the stereoisomers of the sex pheromone components of southern corn rootworm, *Diabrotica undecimpunctata howardi* Barber, and the lesser tea tortrix, *Adoxophyes* sp.; the required methyl branch was generated from the carboxyl group of the chiral acid. Herein is described the alternative strategy that generates an α-methylalkanoic acid and builds the required chain from the carboxyl group in order to prepare dimethyl—as well as monomethylalkanes. The route described here functions as an alternative to the method of Ade et al. (1980) in that HPLC is not used for resolution, and the one chiral auxiliary employed is much less expensive than those described in the tsetse fly pheromone synthesis. It should be noted also that both the routes of Ade et al. (1980) and this one could be successfully employed for the synthesis of compounds with branch substituents other than methyl and are therefore very general.

METHODS AND MATERIALS

Gas–liquid chromatography (GLC) was performed with Varian 2400 instruments (flame ionization detection, He carrier) using the following columns: column A, OV-1 4% (3.2 mm × 1.5 m) and column B, Durabond I (0.25 mm × 31 m) operated at temperatures as indicated. Infrared (IR) data were obtained with a Perkin Elmer model 467 spectrophotometer (3% solutions in either CCl_4 or $CHCl_3$ as indicated, and nuclear magnetic resonance (NMR) data were obtained with a Nicolet 300 mHz FTNMR spectrometer (1% solutions in $CDCl_3$). Mass spectral data were obtained with a Finnegan model 3200 chemical-ionization mass spectrometer (isobutane) that was equipped with a GLC inlet (Varian model 1400®) served by an OV-101 column (0.25 mm × 31 m).

2-Methylhexadecanoic and 2-Methyleicosanoic Acids. These acids were synthesized by alkylation of the dianion of propionic acid with the appropriate *n*-alkyl bromide by the general method of Pfeffer and Silbert (1970). The yields of the racemic acids were 85–86%; mp (α-methylpalmitic acid) 108–110°C (MeOH); IR (CCl_4) 1710 cm^{-1}; NMR δ 0.88 (bt, 3H, $C\underline{H}_3R$), 1.18

(d, 3H, C<u>H</u>$_3$CH), 2.45 (m, 1H, C<u>H</u>CO$_2$H) ppm; CIMS of the methyl esters (*m/e*) 285 (P + 1) and 342 (P + 1), respectively.

Preparation and Resolution of Diastereomeric Amides, I. Both (*R*)- and (*S*)-α-methylbenzylamines (Hexcel Specialty Chemicals) were purified by recrystallization of monotartrate salts [d-tartaric acid with (*S*)-amine] from aqueous ethanol. The recovered amines were analyzed by GLC as amides of methoxytrifluoromethylphenylacetyl chloride on column A (Dale and Mosher, 1973). Each amine was judged to be ≥99.9% pure. The α-methylalkanoic acids were converted to acid halides using $SOCl_2$ and DMF catalyst in anhydrous ether; the acid halides were then allowed to react in CH_2Cl_2 with each chiral amine using triethylamine as an acid scavenger (Figure 1). Details for analogous reactions and the resolutions of product amides by recrystallization from ethanol have been reported (Sonnet, 1982). The yields of pure *R*S**-diastereomer were 64–66% of theoretical and ≥99.6% diastereomeric purity after four crystallizations. Amide Ia: mp 121–122° C; IR ($CHCl_3$) 3460, 1600 cm^{-1}; NMR δ 0.88 (bt, 3H, C<u>H</u>$_3$R), 1.11 (d, 2H, *J* = 7, C<u>H</u>$_3$CHC=O), 1.49 (d, 2H, *J* = 7, C<u>H</u>$_3$CHN), 2.13 (m, 1H, CH$_3$C<u>H</u>C=O), 5.13 (m, 1H, CH$_3$C<u>H</u>N) ppm; CIMS (*m/e*) 430 (P + 1). Amide Ib: mp 119–120° C, spectral data in accord with assigned structure. GLC data are tabulated below (the R*S*-diastereomer that was being purified emerged second).

	α Col A (°C)	α Col B (°C)
Amide Ia	1.12 (230)	
Amide Ib	1.13 (220)	1.08 (250)

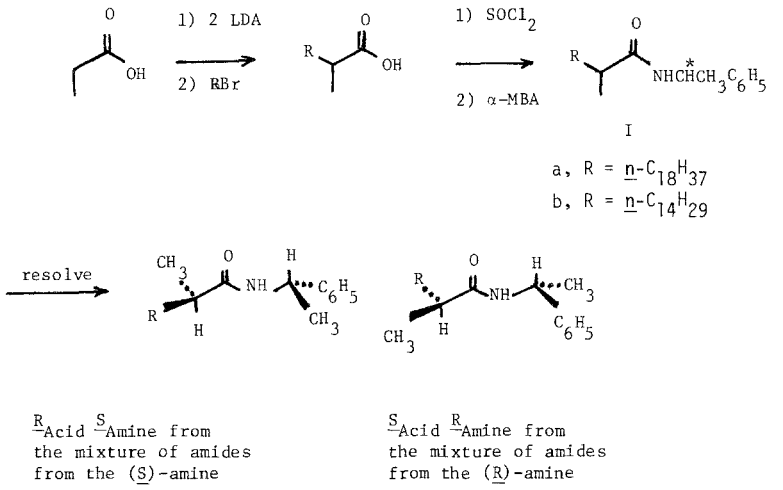


FIG. 1. Resolution of carboxylic acids.

Preparation of Carbinols, II, from Amides, I. The amide (36.4 mmol) was added from an attached flask via Gooch tubing to a vessel containing a solution of lithium diisopropylamide (LDA) (54.5 mmol) in 80 ml of tetrahydrofuran (THF) stirred under nitrogen and cooled to $\leqslant 0°C$. The resulting mixture was stirred without external cooling for 0.25–0.5 hr. After the homogeneous solution had been cooled again to ca. $-30°C$, ethylene oxide (3.7 ml, 73 mmol) and hexamethylphosphoric triamide (HMPT) (10 ml) were injected. The resulting mixture was allowed to come to ambient temperature and stand overnight. The mixture was then worked up with 1 N HCl and extracted with ether. The products of these reactions were the *N*-β-hydroxyethylated amides (Figure 2) that provided the following spectral data: mp (Ia adduct) 69–72°C; IR (CCl_4) 3640, 1650 cm^{-1}; NMR δ 0.88 (bt, 3H, $C\underline{H}_3R$), 1.04 (d, 3H, $J = 7.0$, $C\underline{H}_3CHC{=}O$), 1.49 (3H, d, $J = 6.6$, $C\underline{H}_3CHN$), 1.63 (m, 2H, $CH_2C\underline{H}_2N$), 3.48 (m, 2H, $CH_2C\underline{H}_2OH$), 5.30 (m, 1H, $C\underline{H}N$) ppm.

The crude hydroxyethylated amide was heated under reflux for 16 hr in a solution of 250 ml of THF, 30 ml $HClO_4$, and H_2O (to saturation). The solution was concentrated on a rotary evaporator to $\leqslant 1/2$ volume, diluted

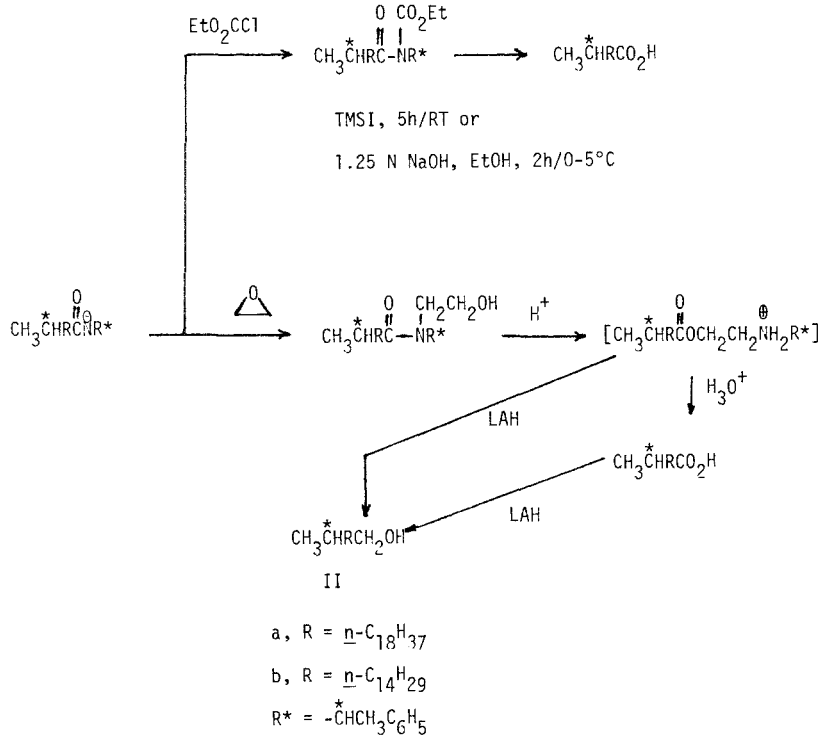


FIG. 2. Recovery of the acid residue.

with brine, and extracted with CH_2Cl_2. In this manner both aminoester HCl and completely hydrolyzed acid are jointly collected. The extract was dried ($MgSO_4$) and stripped of its solvent with added hexane. The residue was dissolved in ca. 30 ml of THF and added to a slurry of excess LAH (1.25 g) in 30 ml of THF that was kept at 0–10°C. The resulting mixture was heated under reflux overnight and then worked up with 1.25 N NaOH, celite filtration (ether dilution and rinses), and extraction of the filtrate with 2 N HCl to remove amines. Removal of organic solvent provided the carbinols II in 75–85% yield: mp (IIa) 43–46°C, (IIb) 38–40°C, IR (CCl_4) 3640 cm^{-1}; NMR δ 0.88 (bt, 3H, $C\underline{H}_3R$), 0.91 (d, 3H, $J = 6.6$, $C\underline{H}_3CH$), 3.45 (d of d, 2H, $C\underline{H}_2OH$) ppm; CIMS (m/e) (IIa) 314 (P + 1), 296 (M + 1-H_2O), (IIb) 255 (M + 1), 239 (M + 1-H_2O).

A complete amide hydrolysis was performed in one instance, namely the amide Ia (R_{acid}, S_{amine}) whereby a sample of configurationally pure acid was obtained: $[\alpha]_D^{25}$—12.7° (c, 8.61, $CHCl_3$). The sign of rotation is in agreement with the assignment of *R* configuration (Jacques et al., 1980).

Preparation of Bromides, III. Triphenylphosphine dibromide was prepared from triphenylphosphine (M&T Chemicals, Inc.) (10.5 g, 40 mmol) and bromine (6.4 g, 40 mmol) in CH_2Cl_2 (50 ml). The carbinol II (9.24 g, 36 mmol) was added in one portion and the resulting solution was allowed to stand overnight. Methanol (5 ml) was added and the solvent stripped. The residue was triturated several times with hexane and filtered from triphenylphosphine oxide. The hexane was removed, and the crude product (liquid) was filtered through a column of silica gel (30 g) with hexane (100 mL). In this manner were obtained the bromides III (Figure 3, R = *n*-$C_{14}H_{29}$, *n*-$C_{18}H_{37}$) in near

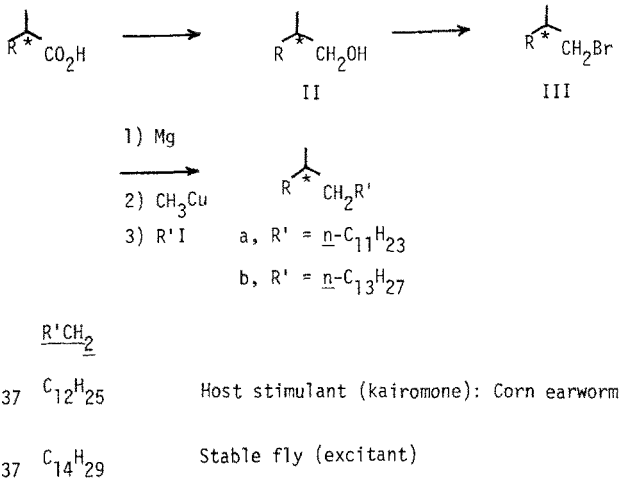


FIG. 3. Building on the carboxyl function.

quantitative yields from the carbinols II or in ca. 70% yield directly from the amides I: bp (III, R = n-$C_{14}H_{29}$) 132–134°C (0.01 mm); NMR 0.88 (bt, 3H, $C\underline{H}_3R$), 1.01 (d, 3H, J = 6.6, $C\underline{H}_3CH$), 3.4 (m, 2H, CH_2Br) ppm; CIMS (m/e) 240 (M + 1-Br). Spectral data from bromide III (R = n-$C_{18}H_{37}$) were in accord with the assigned structure.

Preparation of Methyl Branched Alkanes, IV. A Grignard reagent was prepared from the bromide III (R = n-$C_{18}H_{37}$) (0.94 g, 2.5 mmol) and magnesium (0.24 g, 10 mmol) in dry THF (7 ml) that had been distilled from Na-benzophenone. In another vessel under nitrogen were placed purified CuI (Kleinberg, 1963) (0.48 g, 2.5 mmol) and 5 ml THF. Following the procedure of Tamura and Kochi (1971), the slurry was cooled to −78°C, CH_3Li (1.7 ml of 1.5 M in ether) was injected, and the resulting mixture was stirred for 1 hr cold. The mixture was allowed to reach 0°C and was then returned to −78°C. The solution of magnesio derivative was injected, and the mixture was stirred for 0.75 hr at −78°C, then allowed to warm till nearly homogeneous. The mixture was cooled again to −78°C and the alkyl iodide (IVa; n-undecyl iodide; IVb; n-tridecyl iodide) (3.0 mmol) was injected. An oversized flask and a large stirring bar were helpful. Coupling in the reverse sense, namly n-alkyl magnesium halide with cuprate derived from bromide (iodide) III was a much slower, less successful process. The final reaction mixture was stirred for 1 hr at −78°C, then 2 hr without cooling, and finally worked up with aqueous NH_4Cl and hexane. The crude products could be recrystallized from acetone. Purer samples were obtained by preparative GLC using a 0.64-mm × 2.7-m column packed with OV-1 (2.5%) on Chromosorb-GHP 80–100 mesh. The yields of alkanes from the coupling were 50–60%: IVa: mp 38–39°C; CIMS (m/e) 452 (M + 1), 281 (M + 1-$C_{12}H_{25}$), 197 (M + 1-$C_{18}H_{37}$); IVb: mp 35–37°C; CIMS (m/e) 478 (M + 1), 225 (M + 1-$C_{18}H_{37}$).

Preparation of (R)- and (S)-*Carbinols, V.* The bromide, III (4.0 g, 12.5 mmol) was converted to a Grignard reagent with magnesium (0.6 g, 25 mmol) in anhydrous ether in the usual manner (Figure 4). Paraformaldehyde (0.75 g, 25 mmol) was added, and the mixture was heated under gentle reflux overnight. The product was worked up with aqueous NH_4Cl, and the crude alcohol was freed of any residual halide or hydrocarbon by passage through silica gel (30 g) eluting first with hexane (100 ml) and then ether–hexane (100 ml 1 : 1). The alcohol was obtained from the latter cut: 2.87 g, 84.9%; IR (CCl_4) 3640 cm^{-1}; NMR δ 0.88 and 0.89 (ca. 6H, $C\underline{H}_3R$ and a doublet for $C\underline{H}_3CH$), 3.7 (m, 2H, $C\underline{H}_2OH$) ppm; CIMS (m/e) 270 (M), 253 (M + 1 − 18).

Preparation of (R) *and* (S)-*Aldehydes, VI.* The carbinol, V, (2.85 g, 10.6 mmol) was added to a slurry of pyridinium chlorochromate (3.5 g, 21.4 mmol) in 25 ml of CH_2Cl_2 using 5 ml of CH_2Cl_2 as a rinse (Figure 4). After 2 hr of vigorous stirring, the mixture was diluted with 100 ml of ether, and the mixture was filtered through a column of Florisil (20 g). Two 25-ml portions

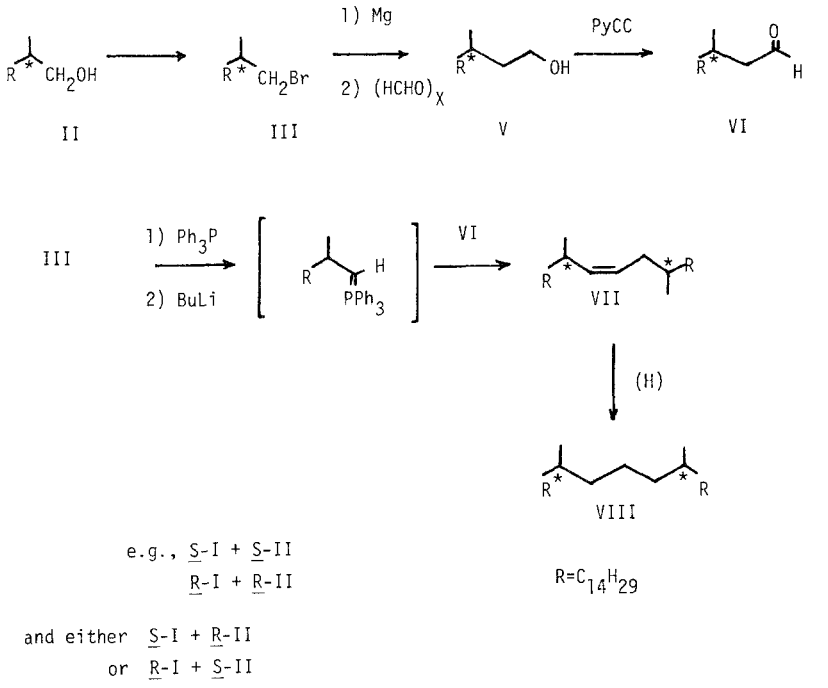


FIG. 4. Synthesis of dimethyl alkanes.

of ether were employed as a rinse. The crude aldehyde, obtained by removing the solvent, was then chromatographed on silica gel (20 g) eluting with 60 ml of hexane, 60 mol of 5% ether–hexane, and 60 ml of 10% ether–hexane. The second two fractions were combined to give 2.38 g (82.9%) of aldehyde that could be used directly in the subsequent condensation reactions: IR (CCl_4) 2825, 1730 cm^{-1}; NMR δ 0.88 (bt, 3 H, $C\underline{H}_3R$), 0.96 (d, 3H, J = 6.6, $C\underline{H}_3CH$), 9.76 (s, 1H, CHO) ppm; CIMS (m/e) 269 (M + 1).

Preparation of (R)- *and* (S)-*Phosphonium Salts and their Conversions to Dimethylalkenes, VII.* Because of the slowness of bromide displacement in α-methylalkyl bromides, the following procedure was developed (Figure 4). The bromide V (4.0 g, 12.5 mmol), triphenylphosphine (4.0 g, 16 mmol), and NaI (3.8 g, 25 mmol) were heated under reflux in 20 ml of CH_3CN for 24 hr. The mixture was stripped of solvent, and the residue was partitioned between water and ethylene dichloride (EDC). The EDC layer was washed with H_2O twice, dried ($MgSO_4$), and concentrated. The residual oil was freed of EDC by stripping twice with heptane and was then made up as a 0.36 M solution in dry THF.

The THF solution of (presumably) phosphonium iodide (6.1 ml, 2.2

mmol) was placed under nitrogen and cooled to below 0°C. Butyllithium (1.0 ml of 2.3 M, 2.3 mmol) was slowly added and, after 0.25 hr, the aldehyde (0.50 g, 1.9 mmol) was added. After 1 hr at ambient temperature, the mixture was worked up with H_2O and hexane. The organic phase was dried, the solvent was removed, and the residue was chromatographed on silica gel (12 g) with 50 ml of hexane to give a configurationally pure dimethylalkene VII: 0.85 g (78.7%).

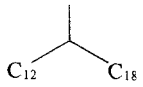
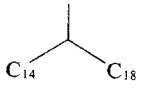
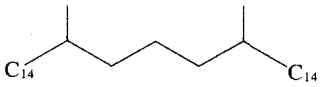
Preparation of (R,R)-, (S,S)-and meso-*Dimethylalkanes, VIII.* Each alkene was quantitatively reduced over PtO_2 in propionic acid monitoring with column A at 230°C: mp (*R,R*) 37–38°C, (*S,S*) 37–39°C (acetone–hexane), (*meso*) semisolid; CIMS (*m/e*) 492 (M + 1).

DISCUSSION

The target structures were the stereoisomers of 13-methylhentriacontane, 15-methyltritriacontane, and 15,19-dimethyltritriacontane (Table 1). The first of these is a kairomone: it serves as the chemical cue to the parasitic wasp *Microplitis croceipes* (Cresson) in its search for its host, the corn earworm, *Heliothis zea* (Boddie) (Jones et al., 1971). The other two compounds were identified along with similar compounds from cuticular washes of female stable flies, *Stomoxys calcitrans* L., and an examination of (racemic) synthetics indicated that these were most effective in producing male striking behavior (Sonnet et al., 1977).

In order to prepare the monomethylalkanes that share a C-18 chain, α-methyleicosanoic acid was synthesized. Propionic acid was converted to a

TABLE 1. STRUCTURES OF SEMIOCHEMICALS OF WHICH STEREOISOMERS HAVE BEEN SYNTHESIZED

Structure	Source, proposed function
	Corn earworm larvae, kairomone
	Stable fly female, mating stimulant
	Stable fly female, mating stimulant

dianion with two equivalents of lithium diisopropylamide using the general method of Pfeffer and Silbert (1970). Alkylation with octadecyl bromide provided the α-branched C-20 acid (Figure 1, R = n-$C_{18}H_{37}$). The acid was then converted to an amide with either (*S*)- or (*R*)-α-methylbenzylamine. Although the (*S*)-amine was found to be 99.4% pure and the (*R*)-enantiomer was 98.6% pure (see Materials and Methods), each amine was further purified by recrystallizing its monotartrate salt. The amides obtained (Ia) could be crystallized from aqueous ethanol and, after four recrystallizations, exhibited ≥99.7% diastereomeric purity as judged by GLC (see Materials and Methods). Guided by the efforts of Helmchen et al. (1972, 1977), we determined that the least soluble diastereomer of a pair of amides of this type, i.e., α-methylbenzylamine and α-alkylalkanoic acid, is *R*S** (Sonnet and Heath, 1982). Also, this diastereomer elutes earlier on HPLC and later on GLC. In addition to assignment by earlier analogy, the optical rotation of the presumed (*R*)-enantiomer was checked and found in accord with expectation, namely the (*R*)-acid has negative rotation (Jacques et al., 1980). By contrast simple recrystallization of the racemic acid as its salt with the chiral amine was much less efficient: after five crystallizations the acid had only 76% ee.

The resistance of amide linkages to hydrolysis prompted an investigation of means by which to labilize the functionality. Alkylation of the amide on nitrogen with ethyl chloroformate (Figure 2) produced an *N*-acyl urethan similar in its chemistry to that of *N*-acylated cyclic urethans (Evans et al., 1982). Brief treatment (2 hr) with ethanol containing 1.25 N NaOH at ice-bath temperatures produced the acid without significant racemization. It was found that trimethylsilyl iodide (CH_3CN, 5 hr, room temperature) likewise produced the configurationally uncompromised acid. Treatment of the acyl urethans with conc. HCl–hexane tended to produce mostly amides. The route most convenient in this case is that previously reported (Sonnet, 1982) whereby the diastereomerically pure amide is hydroxyethylated with ethylene oxide (Figure 2). The product can be treated with aq. $HClO_4$/THF under reflux for 24 hr to obtain complete hydrolysis or can be worked up after ~6 hr and treated with lithium aluminum hydride to produce the corresponding carbinols (*R*) and (*S*) II (Figure 3). The acid hydrolysis proceeds via an intermediate aminoester that undergoes a much slower hydrolytic cleavage. Since the carbinol was the next compound sought after the acid, the latter route is preferable. It should be noted, however, that neither the hydrolytic conditions nor the hydride reduction endanger the asymmetric center. Even though the point has been previously demonstrated (Sonnet, 1982), in the case of one of the current structures it was confirmed again by oxidizing a product carbinol to the acid, and the acid's configurational purity was ascertained by GLC analysis after conversion to an amide with one of the pure α-methylbenzylamines.

The carbinols II were converted to the corresponding bromides III (Fiugre 3) with triphenylphosphine dibromide in CH_2Cl_2. On a small scale this is convenient; clearly the transformation could have been effected by other less expensive reagents. The enantiomeric bromides were then coupled as cuprates to undecyl iodide to give the configurationally pure 13-methylhentriacontanes IVa. Alternatively, coupling with tridecyl iodide yielded the 15-methyltritriacontanes IVb. This copper-mediated coupling was complicated by solubility problems as well as by self-coupling of the organometallic fragment. Thus yields were modest (40–60% and recrystallizations (from acetone) still left contaminating hydrocarbons. Samples of highest purity were obtained by preparative GLC. A better route involving the dimethylalkanes is described subsequently.

In order to synthesize the stereoisomers of the symmetrically branched 15,19-dimethylalkanes, propionic acid was alkylated with tetradecyl bromide. The resulting α-methylpalmitic acid was resolved as previously described using (*R*)- and (*S*)-α-methylbenzylamines. The diastereomerically pure amides were *N*-hydroxyethylated and converted to the carbinols II (Figure 4, R = n-$C_{14}H_{29}$) by acyl transfer–reduction. The configurationally pure carbinols were then converted to the bromides III (Figure 4) as before. Reactions of the bromides as magnesio derivatives with formaldehyde gave carbinols V that were then oxidized to the adlehydes VI with pyridinium chlorochromate. The bromides III were also converted to phosphonium salts by treatment with triphenylphosphine in refluxing acetonitrile. Because this reaction was quite slow, NaI was added (2 equiv.) and the product worked up so as to obtain the phosphonium iodide. A Wittig condensation between ylids derived from these salts and the aldehydes VI led to the dimethylalkenes VII that were then reduced (PtO_2, propionic acid) to the target dimethylalkanes VIII, namely *S,S*; *R,R*; and *meso*-15,19-dimethyltritriacontanes.

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CHEMICAL COMMUNICATION DURING TANDEM RUNNING IN *Pachycondyla obscuricornis* (HYMENOPTERA: FORMICIDAE)[1]

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Abstract—Communication during tandem running in *Pachycondyla obscuricornis* is chemically mediated by a pheromone produced in the pygidial gland, which is composed of a cluster of gland cells and an associated reservoir located beween the 6th and 7th tergites of the gaster. The secretion of the gland appears to be transferred to the hindlegs of a tandem pair leader by a series of self-grooming behaviors that occur prior to the formation of a tandem pair. During tandem running, the antennae of the following ant are kept in close contact with the leader's hindlegs and therefore with the chemical signal that provides a communicative bond between individuals.

Key Words—*Pachycondyla obscuricornis*, Hymenoptera, Formicidae, chemical communication, tandem running, pygidial gland, nest emigration behavior.

INTRODUCTION

Recruitment by tandem running is employed by many ponerine ant species during nest relocations (Hölldobler, 1981). In this system scout ants recruit only one nestmate at a time to a target area. It has been demonstrated that in *Pachycondyla* (=*Bothroponera*) *tesserinoda* the signals involved in tandem running include tactile stimulation of the hindlegs and gaster of the leader ant by the antennae of the follower and an extractable surface pheromone from the gaster (Hölldobler et al., 1973; Maschwitz et al., 1974). More recently, a survey of tergal and sternal glands in ants revealed the presence of a pygidial

[1]Dedicated to the memory of Robert E. Silberglied.

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gland located on the gaster between the 6th and 7th tergites in some ponerine species (Hölldobler and Engel, 1978; Jessen et al., 1979). Subsequently, Hölldobler and Traniello (1980a) found that the pygidial gland of *Pachycondyla obscuricornis* produces a pheromone that provides a chemical bond between leader and follower ants during tandem running. We here present a detailed ethological analysis of chemical communication during tandem running in *P. obscuricornis.*

METHODS AND MATERIALS

Colonies of *Pachycondyla apicalis*, *Ectatomma ruidum*, *E. tuberculatum*, and *Odontomachus chelifer* were collected on Barro Colorado Island, Panama, in June 1979 as either queenright whole colonies or worker groups from wood or soil nests and were subsequently housed in the laboratory in test tubes which were kept moist by water trapped at the bottom by tight cotton plugs. The nest tubes were placed in covered plastic boxes 30 × 22 × 10 cm.

A single colony of *P. obscuricornis* was collected from a log in secondary growth in Frijoles, Panama, and was used in all experiments. The colony contained several wingless females which laid fertile eggs, and grew from a size of 30–40 ☿☿ to 80–90 ☿☿ in approximately eight months and produced sexuals. The colony was fed termites, cockroaches, and honeywater.

Nest emigration experiments were conducted in a 71 × 142-cm arena. Behavioral patterns associated with tandem running were studied by single-frame analysis of slow-motion films (super-8 mm; 70 frames per second). For histological investigations, live ants were fixed in alcoholic Bouin, embedded in methyl methacrylate, and sectioned 8 μm thick with a Jung Tetrander I microtome. The staining was Azan (Heidenhain).

RESULTS

Sociogram of Nest Emigration. Tandem running appears to be the most important communication mechanism in nest emigration. In the laboratory we observed tandem running only in the context of nest moving and never during food retrieval or any aspect of foraging. In fact, field and laboratory observations suggest that workers of *Pachycondyla obscuricornis* forage independently.

We experimentally induced nest emigration by removing the red glass plate that normally covered occupied nest tubes and placing a 100-W lamp approximately 25 cm above. A new nest (several darkened test tubes with moist cotton plugs) was offered 1 m away. A cardboard bridge allowed workers to leave the old nest and search in the arena.

Attempts at tandem pair formation by ants that had located the new nest typically began soon after we created adverse conditions in the old nest. Social carrying occurred early during nest emigration and continued while other individuals were forming tandem pairs. A typical example of the pattern of nest emigration is illustrated in Figure 1 in which the numbers of ants recruited by tandem running and social carrying are given for the duration of one nest moving process. Of a total of 58 individuals, 34 (59%) were led to the new nest site by tandem running; 24 (41%) were carried. Workers were recruited in tandem pairs whereas alate males and females were carried. However, occasionally we also observed sexual females being led to the new nest site by tandem running, and early in nest emigration both behaviors were also involved in the recruitment of individuals from the new nest back to the old nest. Eggs, larvae, and pupae were moved during the last stages of emigration.

Behavior during Tandem Pair Formation and Social Carrying. Soon after discovering and inspecting the new nest, scout ants returned to the old nest and contacted individuals within and outside nest tubes. Recruiting ants engaged in a striking stereotyped behavioral sequence that led to the formation of a tandem pair. Initial contact was characterized by brief antennation and a "bowing" display given by the recruiting ant. During this display the recruiting ant spread her pro- and mesothoracic legs and lowered her head and thorax in a brief series of vertical movements (Figure 2a). At times her mandibles contacted those of the individual recruited. Often the recruitee reciprocated with a similar bowing behavior. Immediately following this motor pattern, the recruiting ant engaged in a jerking motion, pulling the nestmate forward by the mandibles, then releasing her grip and quickly

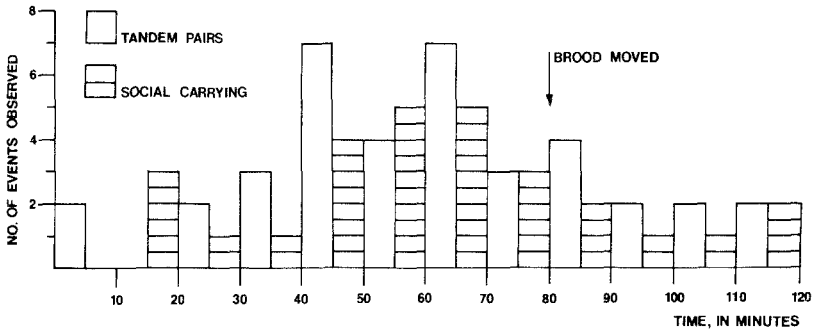

FIG. 1. Sociogram of nest emigration of *Pachycondyla obscuricornis*. Histograms represent the number of tandem pairs formed during recruitment to a new nest site (white) and the number of individuals carried (horizontal hatching). Time is relative to inital contact with the new nest.

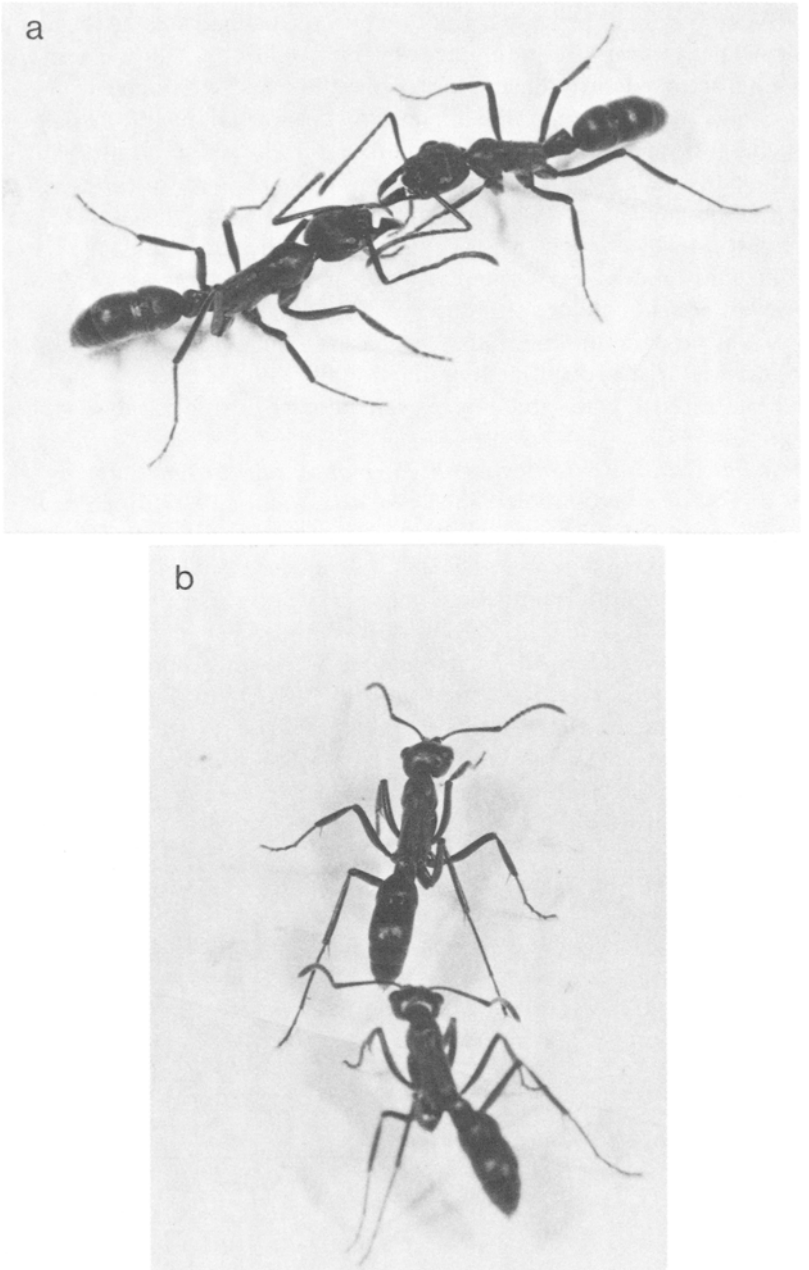

FIG. 2. (a) *Pachycondyla obscuricornis* "bowing" motor pattern of a recruiting ant (left) inducing in a nestmate (right) a tandem following response. (b) Tandem pair, the follower ant keeping close antennal contact with the leader ant.

offering her gaster. Motivated nestmates responded by contacting the recruiter's hindlegs or gaster with the antennae, and tandem running began (Figure 2b). Occasionally the sequence of "invitation behaviors" was insufficient to motivate nestmates to follow and recruiters moved away. If a tandem pair formed, nestmates were led to the new nest while keeping close contact with the hindlegs of the leader ant. Overall, tandem running appeared as a series of jerking movements as the leader periodically slowed in movement and was "pushed" forward by contact from the follower.

Recruitment by carrying did not necessarily follow unsuccessful attempts at tandem pair formation. Often the "bowing" display so prominent as an initial pattern during invitation behavior to tandem running was absent. Recruiters grasped nestmates by the mandibles and vigorously pulled them forward until the individual folded its legs tightly to the body and lowered the head to the thoracic sternum. The nestmate was subsequently transported slung under the recruiter's body (Figure 3a and b). Males were haphazardly carried by the neck or wings.

Grooming Behaviors Associated with Tandem Running. Prior to recruitment we observed scout ants that had located the new nest passing the hindlegs over the gaster and rubbing them together before showing invitational displays to nestmates. Normally, hindleg movements on the gaster were followed by hindleg rubbing. Ants showing gaster/hindleg grooming tended to arch the gaster upward. The same behavior was often shown by tandem leaders when the following ant lost contact. Although we loosely refer to this behavior as grooming, we believe that it is a sequence involved in the application of a chemical signal to the hindlegs necessary for communication during tandem running.

Pachycondyla obscuricornis has well-developed paired intersegmental glandular structures between the 6th and 7th terga (Figure 4), and we have previously demonstrated that this pygidial gland produces a pheromone responsible for maintaining the communicative bond between the leader and follower of a tandem pair of *P. obscuricornis* (Hölldobler and Traniello 1980a). We have two lines of circumstantial evidence which suggest that hindleg/gaster and hindleg/hindleg grooming are behavioral patterns involved in applying pygidial gland pheromone to the hindlegs of the leader ant, which are subsequently contacted by the follower's antennae. First, hindleg/gaster and hingleg/hindleg grooming occurred in statistically significantly higher frequencies during nest emigration. To demonstrate this we recorded all grooming acts in a 30- to 45-min period under normal conditions (an undisturbed nest), during foraging, and during nest emigration. Grooming acts that could possibly be involved in application of pygidial gland secretion to the hindlegs were significantly higher only during nest moving (Table 1). Second, the frequency of hindleg/gaster and hindleg/hindleg grooming increased during the course of nest emigration. In one study, grooming acts

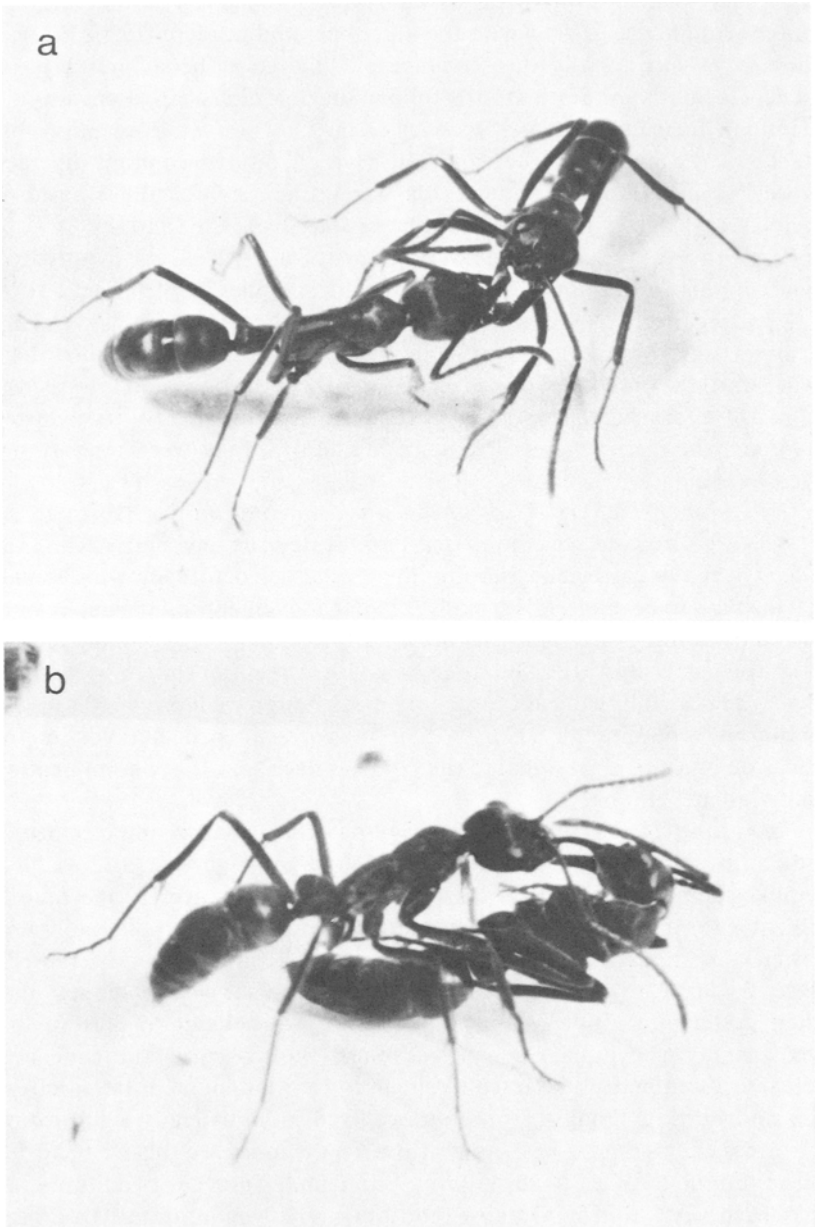

FIG. 3. (a) A recruiting *Pachycondyla obscuricornis* (left) grasps a nestmate on the mandibles and swings it under its body. (b) In this position the nestmate is carried to the new nest site.

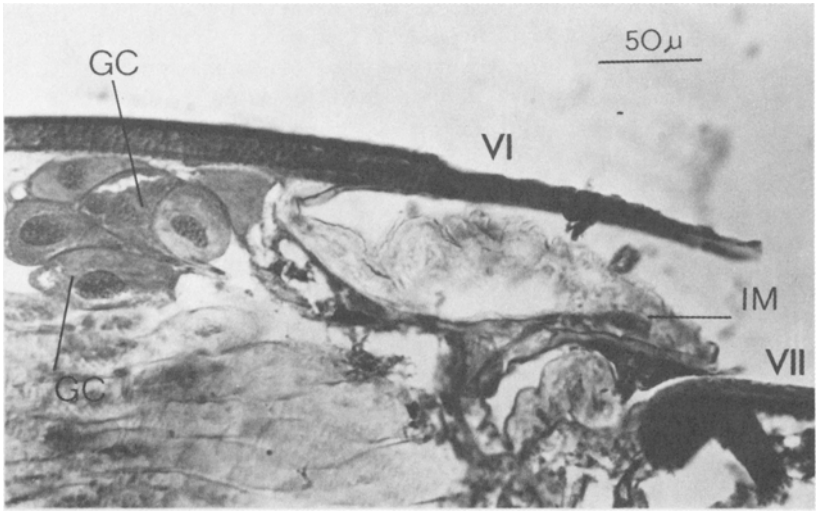

FIG. 4. The pygidial gland of a *Pachycondyla obscuricornis* worker. The cluster of gland cells (GC) have ducts which lead to the intersegmental membrane (IM) between the 6th (VI) and 7th (VII) tergites.

associated with the head, mouthparts, and pro- and mesothoracic legs were high in the initial stage of nest moving (probably due to the fact that workers were emptied from nest tubes in order to induce emigration); hindleg/gaster and hindleg/hindleg grooming were comparatively infrequent. At the beginning of the organization of the emigration, these latter grooming acts increased in frequency relative to other grooming acts.

Analysis of Communication Signals of Tandem Running. Our preliminary study suggested that pygidial gland secretion is an important chemical signal involved in tandem running (Hölldobler and Traniello, 1980a). We found that gasters of freshly killed workers are effective tandem leader dummies and that their effectiveness is lost after the gaster is extracted in acetone. Treatment of an extracted gaster with secretion from freshly dissected pygidial glands reinstated the effectiveness of the dummy as a leader. Also, the pygidial gland secretion was the only exocrine gland product that, when placed on an applicator stick, was able to act as a tandem leader. Subsequent tests support our initial results. In a series of double-blind "diversion" experiments in which crushes of sternites and tergites were offered in competition with real tandem leaders, tergal segments associated with or anatomically close to the pygidial gland were able to divert followers from their natural leader (Table 2). In a second series of double-blind experiments, followers in tandem pairs were separated from leaders and were given the choice between alternative leader models. Again, segments associated with the

TABLE 1. COMPARISONS OF FREQUENCIES OF GROOMING ACTS DURING CONTROL CONDITIONS (SUM OF ALL GROOMING ACTS INSIDE AND OUTSIDE NEST TUBES IN UNDISTURBED COLONY); DURING FORAGING (COCKROACH FOOD SOURCE), AND DURING NEST EMIGRATION[a]

	Control	Foraging	Nest emigration
N	94	118	75
Gland-unrelated grooming acts			
Antenna/foreleg	19 (20.2)	22 (18.6)	16 (21.3)
Foreleg/head	13 (13.8)	26 (22.0)	3 (4.0)
Foreleg/mouth	25 (26.6)	54 (45.8)	19 (25.3)
Pro-, mesothoracic legs	28 (29.8)	12 (10.2)	14 (18.7)
Total gland-unrelated acts	85 (90.4)	114 (96.6)	52 (69.3)
Gland-related grooming acts			
Hindleg/gaster	7 (7.4)	2 (1.7)	14 (18.7)
Hindleg/hindleg	2 (2.1)	2 (1.7)	9 (12.0)
Total gland-related acts	9 (9.6)	4 (3.4)	23 (30.7)

[a]The number of grooming acts recorded is followed in parentheses by the percent of the total number of acts scored. N = sample size. For each context, grooming acts are summarized as gland-related (hindleg/gaster and hindleg/hindleg grooming) and gland-unrelated (all other grooming behaviors). These latter frequencies were statistically analyzed by a test for the equality of two percentages after percentages were subject to arc-sine transformation. The frequency of gland-related grooming acts is significantly higher during nest emigration than during control ($ts = 3.52$, $P < 0.001$) or foraging ($ts = 5.45$, $P < 0.001$) situations, but not during control or foraging contexts ($ts = 1.87$, $0.95 < P < 0.9$).

gland or the intersegmental membrane of the 6th/7th tergites were most effective as leaders (Table 3). These results support the hypothesis that the pygidial gland is responsible for the production of a chemical signal which maintains a communicative bond between leader and follower ants in a tandem pair.

TABLE 2. RESULTS OF "DIVERSION" EXPERIMENTS[a]

Preparation assayed	Number of ants tested	Number of positive responses
6th and 7th tergites (pygidial gland)	17	13 (76%)
5th and 6th tergites	12	8 (67%)
6th and 7th sternites	11	4 (36%)

[a]Applicator sticks contaminated with various secretions obtained by crushing various body parts were carefully inserted as dummies between individuals of a tandem pair. As soon as the dummy was contacted by the follower ant, it was slowly moved away. If the dummy was followed for a distance of at least 25 cm the response was considered positive. The experimenter did not know which body parts were being tested. Three preparations were tested in each case.

TABLE 3. RESULTS OF "CHOICE" EXPERIMENTS IN WHICH INDIVIDUALS OF A TANDEM PAIR WERE SEPARATED AND FOLLOWER ANT WAS CONFRONTED SIMULTANEOUSLY WITH TWO DUMMIES WHICH WERE SLOWLY MOVED APART[a]

Number of ants tested	Paired preparations offered	
	Intersegmental membrane only of 6th and 7th tergites	Intersegmental membrane only of the 5th and 6th tergites ($N = 4$)
12	8 (67%)	0
	6th and 7th tergites (including intersegmental membrane)	5th and 6th tergites (including intersegmental membrane) ($N = 3$)
21	13 (62%)	6 (28%)

[a]A positive response was recorded if the ant followed a dummy for a distance of 25 cm. The experimenter did not know the identity of the dummy being tested. N = number of preparations tested.

We next examined the species-specificity of pygidial gland secretions by offering models made of gasters with attached hindlegs in competition with natural leaders of tandem pairs. Gasters were prepared from freshly killed workers of the ponerine species *Pachycondyla apicalis*, *Ectatomma ruidum*, *E. tuberculatum*, and *Odontomachus chelifer*. These species are known to have well-developed pygidial glands (Hölldobler and Engel, 1978). None was effective in diverting followers from tandem leaders.

DISCUSSION

Recruitment by tandem running has been discovered in ant species belonging to three different subfamilies: the Ponerinae, Myrmicinae, and Formicinae (see review in Hölldobler, 1981). The basic patterns of this recruitment technique appear to be strikingly similar in all species investigated. In all cases tandem running is maintained by the exchange of tactile and chemical signals between leader and follower ant.

The anatomical origin of the tandem running pheromone, however, appears to be different in the three subfamilies. This strongly suggests that the tandem running recruitment technique has evolved independently in the Ponerinae, Myrmicinae, and Formicinae.

In the myrmicine genera *Leptothorax* and *Harpagoxenus*, the tandem running pheromone originates from the poison gland of the sting apparatus (Möglich et al., 1974; Möglich, 1979; Buschinger and Winter, 1977). In the formicine *Camponotus sericeus* the anatomical source of the tandem running

pheromone is not yet identified, but it has been demonstrated that the pheromone does not derive from the poison gland, Dufour's gland, or hindgut (Hölldobler et al., 1974). Furthermore, no pygidial gland has been found in formicine ants (Hölldobler and Engel, 1978). In the ponerine species *Pachycondyla tesserinoda*, a tandem running pheromone appeared to be present on the surface of the whole body of the ants, but dummies prepared from gasters clearly elicited the best following response (Maschwitz et al., 1974). From our new results obtained with *P. obscuricornis* (presented in this paper), from preliminary experiments with *P. crassa* (Hölldobler, unpublished), and from experiments with *Paltothyreus tarsatus* (Hölldobler, 1983), we now conclude that in the Ponerinae the tandem running pheromone is produced in the pygidial gland.

As is the case of *P. tesserinoda*, in *P. obscuricornis* all external body parts of freshly killed worker ants were effective as leader dummies. But when only glandular secretions were tested, only applicator sticks contaminated with pygidial gland secretions evoked a tandem following response (Hölldobler and Traniello, 1980a). In the current study we have shown that dummies consisting of isolated gaster tergites or sternites can elicit a following response, but when tested in a choice experiment, the tissue between 6th and 7th tergites (where the pygidial gland is located) were significantly more effective than any other body part. Either small amounts of pygidial gland secretions are spread over the whole body surface of the ants, or a more general species specific recognition signal on the cuticle makes these dummies, constructed from different body parts, acceptable once an ant has been induced to tandem running by a recruiting nestmate. Our evidence, however, strongly suggests that pygidial gland secretions play a significant role in chemical communication during tandem running. In fact, our observations indicate that just prior to tandem running, pygidial gland secretions are spread by grooming movements of hindlegs over the gaster by hindleg/hindleg rubbing. These grooming patterns may result in the application of pheromone to the tibial and tarsal segments of the hindlegs which are frequently contacted by antennae of the follower ant of a tandem pair.

Recent anatomical and behavioral studies on formicid glands have provided significant new information on ant phylogeny and the evolution of recruitment communication (Hölldobler and Engel, 1978; Hölldobler, 1982). The pygidial gland plays a role in various aspects of communication in ponerine ant species, and the contexts in which pygidial gland secretion is used parallels those of myrmicine ants, namely tandem running, trail recruitment, and sexual calling. Now, with our comparative studies of communication in *Pachycondyla*, we are able to add an ecological component to further elucidate those factors involved in the evolution of the secretions and recruitment behaviors used by different ant species. *P. obscuricornis* has a relatively small

colony size (100–200 workers) and workers forage for prey independently of one another. This type of foraging system has been described in a closely related and ecologically similar species, *Pachycondyla* (*Neoponera*) *apicalis* (Fresneau, 1982), and appears to be correlated with food type (evenly dispersed arthropod prey). Our laboratory studies with *P. apicalis* show that this species has no food recruitment behavior, but uses tandem running communication during nest emigration. *P. obscuricornis* uses tandem running only during nest emigration, and pygidial gland secretion provides an important chemical cue in this behavior. Another species of *Pachycondyla*, *P. laevigata* (= *Termitopone laevigata*), is an obligate predator of termites, and workers have a well-developed pygidial gland that produces a trail substance (Hölldobler and Traniello, 1980b). We suggest that the shift in diet from evenly dispersed to strongly clumped food sources (termites) led to the use of the pygidial gland in trail communication and the evolution of glandular secretions with mass recruitment (stimulatory and orienting) properties. The ancestral function of the pygidial gland, however, was most likely in sexual calling behavior, a role that has been demonstrated in other ponerine species (Hölldobler and Haskins, 1977). Again there is a striking parallel with the evolution of recruitment pheromones in the Myrmicinae; in this group sexual calling appears to be an ancestral function of poison gland secretion.

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SYNTHESES OF PURE (9*Z*,11*Z*), (9*E*,11*E*), (9*E*,11*Z*), AND (9*Z*,11*E*)-9,11-HEXADECADIENALS—POSSIBLE CANDIDATE PHEROMONES

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Abstract—The title compounds were prepared by six different routes, and recommendations are given for the more convenient procedures in laboratory-scale syntheses. Modifications in the literature preparations of the 9*E*,11*E* and 9*E*,11*Z* isomers are described. Baseline separation of a prepared mixture of all four isomers of the (9*Z*,11*Z*), (9*E*,11*E*), (9*E*,11*Z*), and (9*Z*,11*E*)-9,11-hexadecadienals was achieved using GC methods with standard capillary columns. [^{13}C]NMR spectroscopy of the *alkene* carbon atoms clearly differentiates between the *Z,Z, E,E* and either *E,Z* or *Z,E* isomers of the precursor dienols and thus of the dienals.

Key Words—(9z,11*Z*), (9*E*,11*E*), (9*E*,11*Z*), and (9*Z*,11*E*)-9,11-Hexadecadienals, (9*E*)-hexadecen-11-yn-1-yl acetate, syntheses, aldehydes, ester, conjugated dienes, dienals.

INTRODUCTION

Although the syntheses of insect pheromones having a conjugated diene structure have been well described (Henrick, 1977; Henrick et al., 1982), it is less common (Bestmann et al., 1981; Chisholm et al., 1981) for all four isomers of a single conjugated diene to be prepared in one laboratory using an excellent published procedure regardless of the authors' favorite method. In a program designed for field testing studies on a variety of moth species of the order Lepidoptera, it was essential to quickly prepare stereochemically pure, gram quantities of each of the four isomers of the (9*Z*,11*Z*), (9*E*,11*E*), (9*E*,11*Z*), and (9*Z*,11*E*)-9,11-hexadecadienals (I–IV). In fact, compound IV was only recently characterized as a pheromone for *Diatreae saccharatis*

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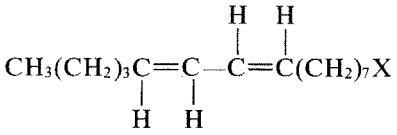


I X = CHO

Ia X = CH_2OH

FIG. 1.

(Carney and Liu, 1983) and III identified, but not fully characterized as a pheromone in *Acrolepiopsis assectella* (Renou et al., 1981).

We (Svirskaya et al., 1980) and Coffelt et al. (1979) had previously shown that pure conjugated *Z,Z* pheromone compounds can be readily obtained by a Cadiot-Chodkiewicz reaction followed by a borane reduction and hence compounds I and Ia (Figure 1) were conveniently prepared in gram quantities. A longer, but possibly commercially more applicable procedure has recently been described for the synthesis of *Z,Z* pheromones (Bishop and Morrow, 1983). The stereoisomers of I (II–IV), however, can be prepared by a wide variety of published procedures. Since we wished to prepare II–IV under the criteria stated above, we selected two literature methods each for the preparation of II–IV.[1]

The subsequent availability of all four isomers (I–IV) enabled us to find a simple method of gas–liquid chromatographic (GLC) analysis of the isomers and a simple ^{13}C method of structural identification.

METHODS AND MATERIALS

Nuclear magnetic resonance (NMR) spectra for protons were recorded on a Varian EM 360 spectrometer; NMR spectra for carbon were recorded on a Varian FT-80A spectrometer at 20 MHz using deuteriochloroform ($CDCl_3$) as solvent and tetramethylsilane as the internal standard. Samples used for ^{13}C measurements were over 99% pure by GLC analyses.

The analyses of these conjugated diene alcohols and aldehydes by gas chromatography–mass spectrometry were performed on a Pye 204 chromatograph interfaced directly to a VG Micromass 16F single focusing sector mass spectrometer. Fused silica capillary columns were employed. The column

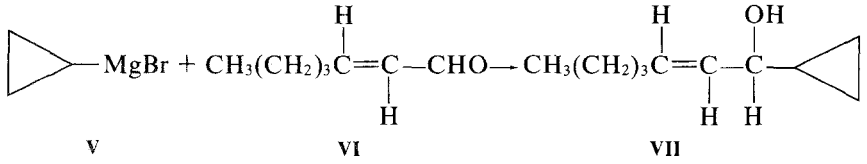
[1]We have not attempted the palladium catalyzed cross-coupling reaction between an alkenylborane and an alkenyl halide (Miyaura et al., 1983, and references therein).

used for the separation of the isomeric alcohols was 60 m × 0.25 mm *ID* coated with DBI701 having a film thickness of 0.25 μm, while that for the isomeric aldehydes was 60 m × 0.25 mm *ID* coated with SE30 with a film thickness of 0.25 μm. The temperature programing conditions were 140–200°C at 6°C/min for the aldehydes. For the diene alcohols the temperature was held at 120°C for 4 min and programed to 200°C at 8°/min. On both columns the order of elution of the diene alcohols and aldehydes were *Z,E, E,Z, Z,Z,* and *E,E.* Computer-acquired mass spectral data were recorded, through a VG2025 data system interfaced to the GC-MS system, at an ionizing energy of 70 eV in the EI mode at an ion source temperature of 200°C and a scan rate of 1.5 sec/decade. Infrared (IR) spectra were recorded on a Unicam SP 1000 IR spectrophotometer as neat films between NaCl disks. Silica gel was used for all thin- and preparative layer chromatography (TLC) and column chromatography unless otherwise specified. All glassware for reactions involving organometallics was oven-dried, cooled, and kept under argon during reaction. The reagents and reaction mixtures, containing organoboron, organolithium, and Grignard reagents were transferred via syringe and carried out under an argon atmosphere, using dry and pure solvents. Solvents were removed on a rotary evaporator. An aliquot (1 ml) of the prepared dialkylborane was hydrolyzed and analyzed for hydride concentration (Brown et al., 1977). All melting and boiling points are uncorrected. Microanalyses were performed by Guelph Chemical Laboratories Ltd., Guelph, Ontario, and by Organic Microanalyses, Montreal, Quebec. The reaction schemes are shown in Schemes 1–3.

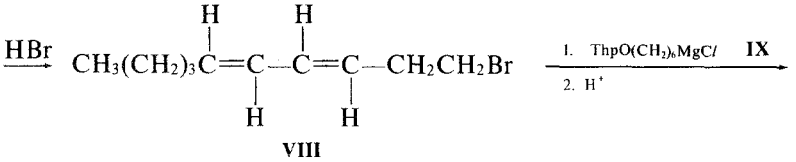
(E)-*7-Cyclopropyl-5-hepten-7-ol* (*7*). Compound VII was prepared from (*E*)-2-heptenal (6) (Svirskaya et al., 1980) according to the reaction sequence of Descoins and Henrick (1972) and Mori (1974) using a procedure originally devised by Julia et al. (1960).

Thus 7.0 g of VI reacted with cyclopropylmagnesium bromide (prepared from 9.8 g of cyclopropylbromide) to give 6.0 g of VII (bp 55–62°C/0.1 mm) in 63% yield. NMR: δ 5.75–5.4 (m, 2H), 4.01 (s, 1H), 3.6–3.3 (m, 1H), 1.78–1.1 (m, 4H), 0.88 (t, 3H, $J = 6$ Hz), 0.6–0.1 (m, 5H).

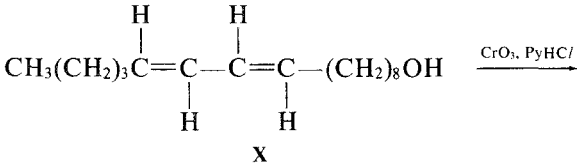
(*3*E,*5*E)-*3,5-Decadienyl bromide* (*VIII*). To (*E*)-7-cyclopropyl-5-hepten-7-ol (VII) (6.0 g) at 0°C was added 15 ml of 48% HBr in one portion (Julia et al., 1960). The reaction mixture was stirred at 0° for 15 min and extracted with hexane. The extract was washed, dried, and the solvent was evaporated. The crude product VIII (7.5 g) was chromatographed on a silica gel column using hexane as eluant to give 5.4 g of pure bromide VIII in 65% yield. IR: 980 cm^{-1}. NMR: δ 6.3–5.32 (m, 4H), 3.61 (t, 2H, $J = 8$ Hz), 2.71 (t, 2H, $J = 9$ Hz), 2.32–1.95 (m, 2H), 1.58–1.18 (m, 4H), 0.9 (t, 3H, $J = 5$ Hz). Anal: calcd. for $C_{10}H_{17}Br$: C, 55.31; H, 7.89; Br, 36.80; found: C, 55.00; H, 7.94; Br, 36.81.



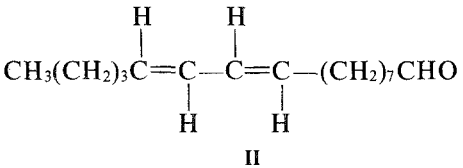
V VI VII



VIII



X



II

SCHEME 1.

(*9E,11E*)-*9,11-Hexadecadien-1-ol* (*X*). The solution of cooled (5° C) 6-tetrahydropyranyloxy-1-hexyl magnesium chloride (IX) (prepared from 8.8 g of 1-chloro-6-tetrahydropyranyloxyhexane) was added dropwise to 5.0 g of cooled (−10° C) bromide VIII using dilithium tetrachlorocuprate as a catalyst (Tamura and Kochi, 1971; Descoins and Henrick, 1972). The reaction was stirred for 3 hr at −5 to 5° C and after work-up and column chromatography on silica yielded 4.0 g of (9*E*,11*E*)-9,11-hexadecadien-1-ol (X). Recrystallization from ether–hexane gave 3.5 g of pure X, mp 34–35° C in 65% yield. Product X is >99.5% pure by GC analysis (column 1) and by [^{13}C]NMR (Table 2). IR (Nujol): 3360, 980 cm^{-1}. NMR: δ 6.24–5.18 (m, 4H), 3.62 (t, 2H, J = 8 Hz), 2.34–1.9 (m, 4H), 1.84 (s, 1H), 2.72–1.08 (m, 16H), 0.89 (t, 3H,

$J = 5$ Hz). Anal: calcd. for $C_{16}H_{30}O$: C, 80.60, H, 12.68; found C, 80.54, H, 12.64.

(*9E,11E*)-*9,11-Hexadecadienal* (*II*). Oxidation of 3.0 g of X as previously described (Svirskaya et al., 1980) yielded 2.4 g of (*9E,11E*)-9,11-hexadecadienal (II) in 80–85% yield. Even though alcohol X was >99% stereochemically pure, product II was contaminated with 5–10% of the isomeric *E,Z* and *Z,E* isomers (Henrick, 1977) as shown by GC analysis. Chromatography on silica and elution with ether–benzene (1:19) (fume hood) gave pure II, (bp 120–125°C/0.01 mm, Kugelrohr distillation), in the middle fraction in 80–85% yield. IR: 1750, 980 cm^{-1}. NMR: δ 9.85 (t, 1H, $J = 2$ Hz), 6.35–5.35 (m, 4H), 2.62–1.8 (m, 4H), 1.8–1.1 (m, 16H), 0.85 (t, 3H, $J = 7$ Hz). Anal: calcd. for $C_{16}H_{28}O$: C, 81.29, H, 11.94; found C, 80.84, H, 12.24.

(E)-*9-Hexadecen-11-yn-1-ol* (*XV*). Compound XV was prepared by some minor but important modifications of the method of Negishi et al. (1973). and hence the procedure is reproduced in full below.

A dry 500-ml flask, equipped with a thermometer, a condenser, a magnetic stirrer, and a septum inlet was flushed with argon. In the flask was placed 14.7 g (75 mmol) of 9-decynyl acetate (XI) in 20 ml of tetrahydrofuran (THF). Disiamylborane, 75 mmol at −50°C, prepared by the method of Brown et al. (1977), was added dropwise by a syringe. The stirring was continued for 3 hr (−50°C for 1 hr, −30°C for 1 hr, and then 0°C for 1 hr), and the solution of disiamyl[(*E*)-9-decenylacetate]borane (XII) was recooled to −78°C. To this solution was added, by a syringe, cooled (−50°C) lithium hexyne (XIII), freshly prepared from 6.15 g (75 mmol) of 1-hexyne in 15 ml THF and 63.75 ml (75 mmol) of *n*-BuLi. The resulting light yellow solution was stirred for 2.5 hr (−78°C for 1 hr, −50°C for 1 hr, and 0°C for 0.5 hr) and recooled again to −78°C. Iodine (20.64 g, 80 mmol) in 50 ml THF was added dropwise at −78°C, and the resulting brown solution containing a white suspension was stirred for 1 hr at −78° to −50°C and allowed to warm to room temperature. To this solution 60 ml of 3 M sodium hydroxide was added, and the stirring was continued for 0.5 hr. The mixture was extracted with ether and washed with a saturated solution of sodium thiosulfate, water, and dried over $MgSO_4$. The solvent was evaporated, and the product was taken up in 50 ml of a methanol–THF (1:1) solution. The reaction mixture was oxidized and hydrolyzed by H_2O_2 at 30–40°C. The mixture was stirred for 1 hr at 30–40°C and for 0.5–1 hr at room temperature. The usual work-up gave 22 g of crude product, which was distilled through a short-path distillation column. The fraction with bp 120–180°C/0.01 mm (14.2 g) was separated on a silica gel column using hexane, followed by a hexane–benzene (1:1) mixture as eluants. Further elution with benzene, followed by ether–benzene (1:19) yielded the product XV which contained 9% of a

saturated alcohol. A second column chromatography and distillation gave XV (>98% pure) in 61% yield (10.8 g) bp 120–122°C/0.01 mm. IR: 3350, 1060, 950, 720 cm^{-1}. NMR: δ 6.35–5.61 (m, 1H), 5.55–5.14 (m, 1H), 3.64 (t, 2H, $J = 7$ Hz), 2.28 (s, 1H), 2.5–1.8 (m, 4H), 1.8–1.1 (m, 16H), 0.86 (t, 3H, $J = 7$ Hz). Anal: calcd. for $C_{16}H_{28}O$: C, 81.29, H, 11.94; found C, 80.91, H, 12.06.

(*9E,11Z*)-9,11-Hexadecadien-1-ol XVI. Reduction of enyne XV (5.0 g) with disiamylborane as described (Negishi et al. 1973) gave, upon column chromatography and distillation, 4.4 g (87% yield) of (*9E,11Z*)-9,11-hexadecadien-1-ol XVI, bp 118–120°C/0.01 mm Lit. (Bestmann et al., 1977), bp 115–116°C/0.01 mm. GC analysis (column 1) showed that XVI was 97.3% pure and contained 2.5% of the *Z,E* and 0.2% of the *Z,Z* isomers respectively.

(*9E,11Z*)-*9,11-Hexadecadienal* (*III*). Compound III, prepared by oxidation of XVI as for II, was obtained in 80% yield (bp 135–142°C/0.02 mm, Kugelrohr distillation). IR: 1730, 985, 950, 735 cm^{-1}. NMR: δ 9.85 (t, 1H, $J = 2$ Hz), 6.6–5.1 (m, 4H), 2.6–1.9 (m, 4H), 1.9–1.1 (m, 16H), 0.9 (t, 3H, $J = 6$ Hz). Anal: calcd. for $C_{16}H_{28}O$: C, 81.29, H, 11.94; found C, 80.80, H, 12.06.

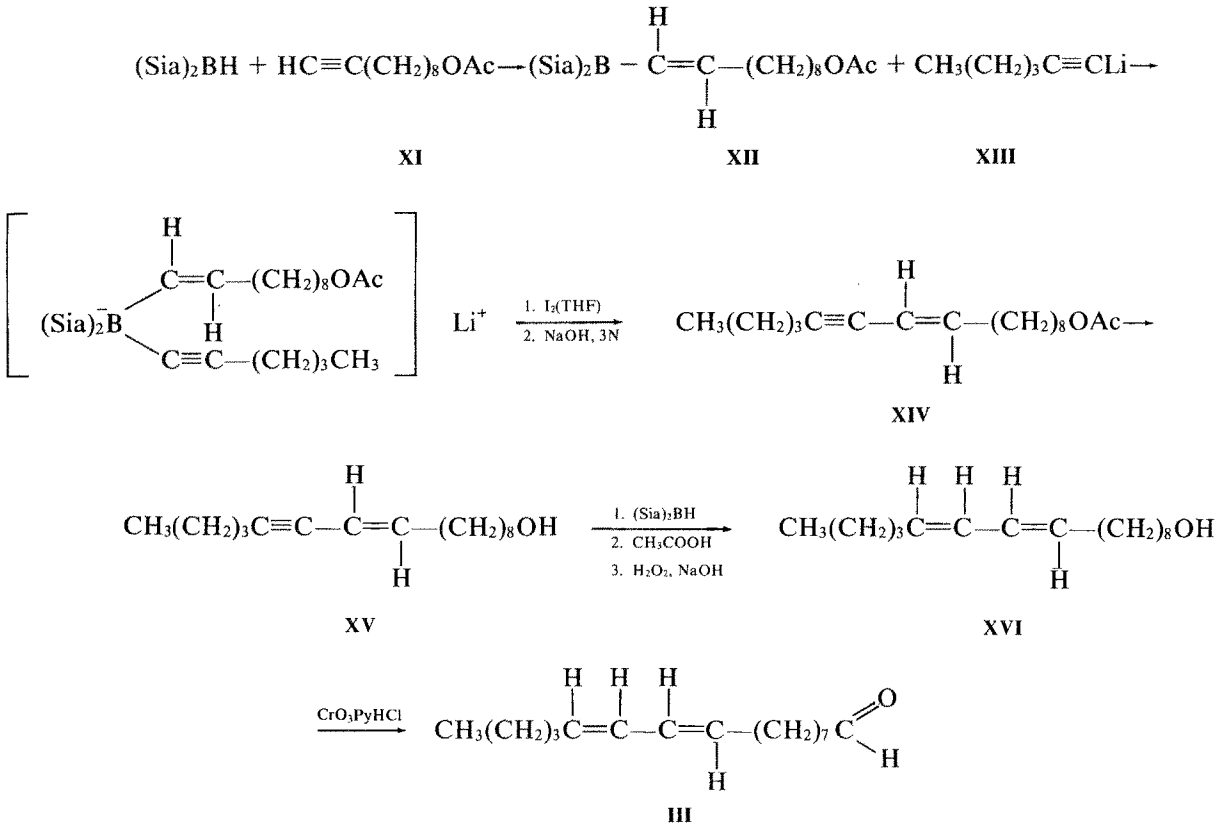
(*9Z,11E*)-*9,11-Hexadecadienal* (*IV*). Compound IV was prepared by the method of Zweifel and Backlund (1977) as shown in Scheme 3 and by the method of Bestmann (1976) for the precursor XXI. The Wittig procedure of Bestmann (1976) gave IV in low yield but high purity, and the data recorded for IV were those prepared by the Wittig procedure.

Thus, oxidation (Svirskaya et al., 1980) of XXI (3.7 g) via Bestmann et al. (1976) gave, after column chromatography and distillation, 2.0 g of pure (*9Z,11E*)-9,11-hexadecadienal (IV) (bp 130–140°C/0.01 mm, Kugelrohr distillation) as a colorless liquid in 77% yield. IR: 2720, 1730, 985, 950, 735 cm^{-1}. MS: 236 (M^+), 95, 81, 67 (100). NMR: δ 9.82–9.70 (m, 1H), 6.60–6.05 (m, 4H), 2.60–1.10 (m, 20H), 0.90 (t, 3H, $J = 6$ Hz). Anal: calcd. for $C_{16}H_{28}O$: C, 81.35, H, 11.86; found C, 81.38, H, 11.97.

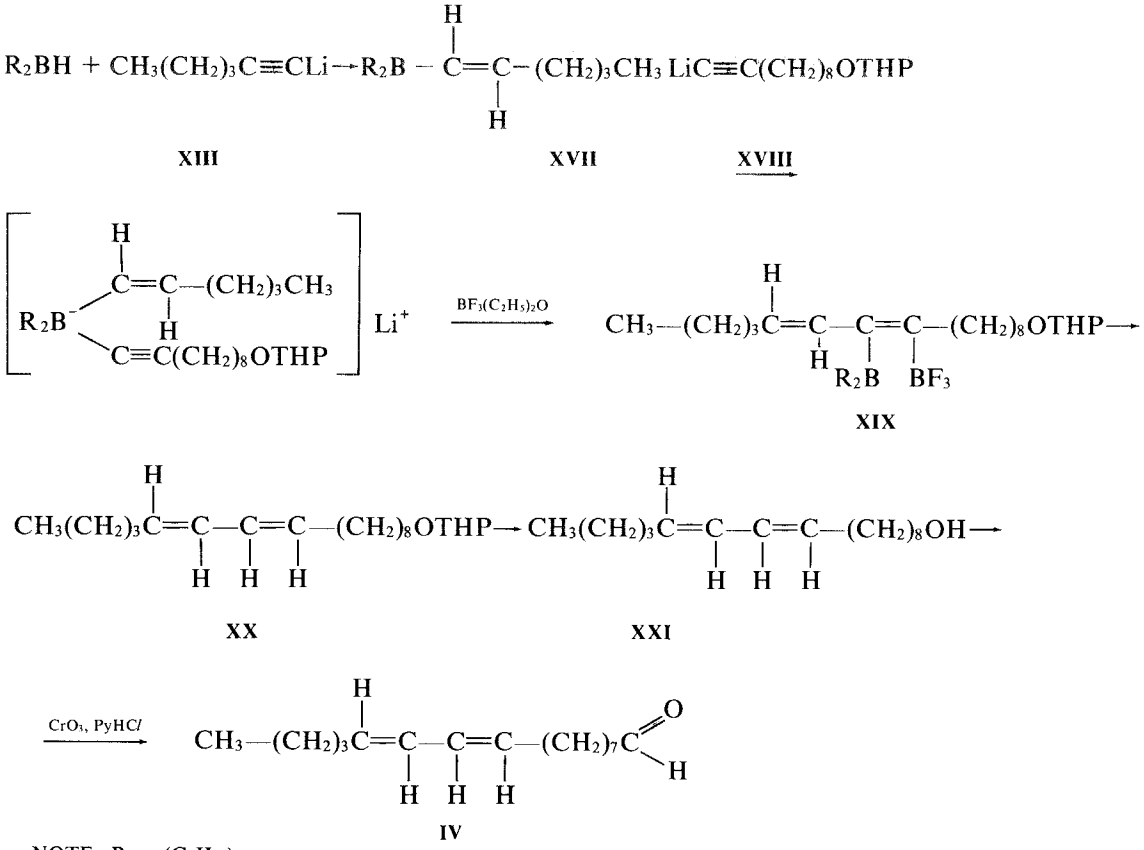
RESULTS AND DISCUSSION

(*9Z,11Z*)-*9,11-Hexadecadienal* (*I*). Compound I had been previously prepared (Svirskaya et al., 1980). In this study larger quantities of high stereochemical purity were easily prepared in good yield (Table 1).

(*9E,11E*)-*9,11-Hexadecadienal* (*II*). Compound II, previously unknown, was prepared via the cyclopropyl derivative VII by methods previously described (Descoins and Henrick, 1972; Mori, 1974). We found these methods mostly satisfactory for the preparation of the *E,E* isomer II, both with regard to overall yield and stereochemical purity (Table 1). We did find,


(Sia)2BH + HC≡C(CH2)8OAc → (Sia)2B − C(H)=C(H)−(CH2)8OAc + CH3(CH2)3C≡CLi →
XI
XII
XIII
Li+
1. I2(THF)
2. NaOH, 3N
CH3(CH2)3C≡C−C(H)=C(H)−(CH2)8OAc →
XIV
CH3(CH2)3C≡C−C(H)=C(H)−(CH2)8OH
1. (Sia)2BH
2. CH3COOH
3. H2O2, NaOH
CH3(CH2)3C(H)=C(H)−C(H)=C(H)−(CH2)8OH
XV
XVI
CrO3PyHCl
CH3(CH2)3C(H)=C(H)−C(H)=C(H)−(CH2)7C(=O)H
III

SCHEME 2.



NOTE: $R_2 = (C_6H_{11})_2$

SCHEME 3.

TABLE 1. SYNTHETIC YIELDS AND STEREOCHEMICAL PURITY OF GEOMETRICAL ISOMERS OF 9,11-HEXADECADIENOL

Method of preparation	Isomer (%)				Overall yield (%)[a]
	1a (Z,Z)	*10* (E,E)	*16* (E,Z)	*21* (Z,E)	
1. Svirskaya et al. (1980)	>99		0.2	0.2	33 (from XI, Ac = H)
2. Descoins and Henrick (1972)		>99		—	20 (from VI)
3. Negishi and Yoshida (1973)		99		—	3[b]
4. Negishi et al. (1973)	1.8		98.2	—	7 (from XI)
5. Modified Negishi and Abramovitch (1977) (this work)	0.1	0.3	99	0.3	42 (from XI)
6. Zweifel and Backlund (1978)	3.1	15.5	81.5	—	28 (from XI)
7. Modified Zweifel and Backlund (1978) (this work)	0.2	2.5	97.3	—	16 (from XI)
8. Zweifel and Backlund (1978)		3.9	[c]	77.3	28 (from XVIII)
9. Bestmann et al. (1976, 1981)	0.2		0.1	99	14[d]

[a]The yields quoted are those of the purest products obtained after all chromatographic and distillation procedures were performed. Chromatographic and distillation fractions containing less pure compounds are not included in this calculation. Pure aldehydes I–IV were obtained in isolated yields of 80–85% and having >99% purity by oxidation and chromatography of Ia, X, XVI, and XXI, respectively, and the yields quoted are the overall yields of I–IV.

[b]From 1-bromo-1-hexyne.

[c]An impurity accounts for 18.8% of this mixture and may be the *E,Z* isomer.

[d]From 9-bromononan-1-yl acetate.

however, that in the coupling reaction of VIII with IX to produce X both VIII and IX should be kept cool and the temperature of the reaction mixture should not be allowed to rise during the course of the reaction. On the other hand attempts to prepare II via thexylborane, 1-bromohexyne, and 9-decyn-1-yl tetrahydropyranyl ether (Negishi and Yoshida, 1973) gave very low yields (Table 1). Although *E,E* isomers such as II can be prepared by thiophenol-catalyzed isomerization (Henrick et al., 1975), we have found this procedure tedious and malodorous and prefer the definitive synthesis described above.

(9E,11Z)-9,11-Hexadecadienal (III). Compound III was prepared by our modified method of Negishi, et al. (1973) as well as by the published routes. Our modified version gave slightly higher yields and a significantly purer product (Table 1). As noted by Henrick et al. (1982) the original procedure of Negishi was difficult to follow, but we found it to be an excellent route as modified herein. In addition, III was prepared via the one-step procedure described (Zweifel and Backlund, 1978) in Scheme 3, and this latter procedure is definitely poorer in terms of yield and stereochemical purity of the product (Table 1). Henrick et al. (1982) note, however, that good yields are obtained in this latter procedure by using tri-*n*-butyltin chloride instead of boron trifluoride etherate as used herein.

(9Z,11E)-9,11-Hexadecadienal (IV). Compound IV, previously unknown, was prepared by the one-step procedure of Zweifel and Backlund (1978) in modest yield and barely acceptable stereochemical purity (Table 1). In addition, IV was prepared via a Wittig route previously described by Bestmann et al. (1981). This latter route in our hands afforded IV in high stereochemical purity but low chemical yield in the key Wittig reaction (Table 1). We would recommend that IV be prepared by our modified version of the method of Negishi et al. (1973) and Negishi and Abramovitch (1977).

Purification of I–IV. It is known that oxidation of conjugated dienols with chromate results in some isomerization (Henrick, 1977). In this study, however, we found that simple chromatography on silica of the aldehydes I–IV removes the 5–10% impurities of minor isomers resulting from the oxidation.[2] In all cases the minor isomers eluted first, along with some of the bulk isomer, and the major isomer was then collected with elution of only 50 ml of solvent. We feel that the major isomer can aggregate, thus ensuring that the conformationally more mobile minor impurities elute first in every case. Thus simple chromatography of these aldehydes can result in very pure final products, albeit with a 15–20% reduction in yield.

GC Analysis of I–IV. Very recently Chisholm et al. (1981) have shown that all four geometrical isomers of 5,7-dodecadienal can be separated on a

[2]A referee informs us that oxidation of olefinic alcohols using *N*-chlorosuccinimide, dimethyl sulfide, and triethylamine gives aldehydes in good yield without any appreciable isomerization.

0.25 mm ID × 23 m Supelco SP-2100 glass capillary column. We have achieved a very similar separation of I–IV using the common SE-30 column (column 2) described in Methods and Materials, and the elution order of the isomers is identical to that of Chisholm et al. (1981). The relative retention times (to pentadecane at 11.3 min) of I–IV are 1.82, 1.85, 1.91, and 1.95, respectively. The relative retention times of the corresponding alcohols of I–IV, i.e. Ia, X, XVI, and XXI on column 1 (to pentadecane at 5.3 min.) are 4.24, 4.31, 4.50, and 4.63, and it is from these alcohols that isomer ratios were determined in Table 1.

[^{13}C]*NMR Analysis of Ia, X, XVI, and XXI.* Using common correlation principles based on related unsaturated fatty acids (Batchelor et al., 1974; Gunstone et al., 1977) and some pheromones (Rossi et al., 1982; Barabas et al., 1978), it was possible to assign most of the [^{13}C]NMR absorption peaks for Ia, X, XVI, and XXI (Table 2). Although Rossi had pointed out that the allylic carbons are characteristic of the geometry about the double bonds, the olefinic carbons can also be characteristic as well. The *Z,Z, E,E,* and *E,Z* or *Z,E* isomers can be readily distinguished from each other by examining the olefin resonances. A note of caution should be added. The hexadienols Ia, X,

TABLE 2. ^{13}C CHEMICAL SHIFTS OF FOUR GEOMETRICAL ISOMERS OF 9,11-HEXADECADIENOL I, X, XVI, AND XXI

Carbon No.	δ ^{13}C (ppm)			
	Ia (*Z,Z*)	X (*E,E*)	XVI (*E,Z*)	XXI (*Z,E*)
1.	63.04	63.23	63.05	63.08
2.	32.83	32.99[a]	32.82	32.90
3.	25.77	25.90	25.90	25.82
4.	29.48	29.59	29.65	29.81
5.	29.48	29.59	29.65	29.53
6.	(29.48)[b]	29.59	29.65	29.53
7.	29.25	29.33	29.28	29.25
8.	27.48	32.74	32.82	27.75
9.	131.99	132.52	(130.07)	(134.66)
10.	123.66	130.56	125.73	128.78
11.	123.66	130.56	(128.68)	125.75
12.	132.06	132.52	(134.57)	(130.01)
13.	27.20	32.43	27.00	32.62
14.	31.88	31.96	31.88	31.68
15.	22.37	22.43	22.35	22.32
16.	13.96	14.05	13.95	13.97

[a]Assigned by intensity considerations.
[b]Assignments in parentheses are tentative.

XVI, and XXI are compounds in which the olefinic groups are surrounded by aliphatic carbons. In other closely related compounds, the functional groups may be closer to the diene moiety and will affect the clear pattern of shifts described for Ia, X, XVI, and XXI.

The preparation of all four geometrical isomers (I–IV) of a candidate insect pheromone by a total of six different methods has resulted in some improvements in the published methods and has allowed us to recommend preferred methods of synthesis at least within the framework of the methods attempted. Analysis of the products by capillary GC and [^{13}C]NMR spectroscopy allows one to readily confirm their identification and monitor their purity.

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PROPHEROMONES THAT RELEASE PHEROMONAL CARBONYL COMPOUNDS IN LIGHT

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Abstract—Pheromonal carbonyl compounds; (*Z*)-11-hexadecanal, (*E*)-citral, and 2-heptanone were treated with six alcohols to give acetals or ketals, some of which acted as propheromones by releasing the pheromonal carbonyl compounds in ultraviolet or simulated sunlight. Highest yields of pheromone were obtained from adducts prepared with *o*-nitrobenzyl alcohol and *o*-nitrophenylethane-1,2-diol. Adducts from (*Z*)-11-hexadecenal and these two alcohols were employed in lures to catch diamondback moths, *Plutella xylostella* (L.).

Key Words—Pheromone, propheromone, photolysis, carbonyl compound, slow release, diamondback moth, *Plutella xylostella,* Lepeidoptera, Plutellidae.

INTRODUCTION

Use of pheromones in managing pests and beneficial organisms is often made difficult by their rapid oxidation in air or high volatility. For example, sex attractant pheromones of many lepidopteran pests include long chain aliphatic aldehydes, such as (*Z*)-11-hexadecenal (Ia, Figure 1) (Inscoe, 1982), which suffer rapid aerial oxidation (Shaver and Ivie, 1982). Similarly, (*E*)-citral (IIa), an important component of the honeybee Nasonov pheromone (Pickett et al., 1980) that shows promise for manipulating the behavior of bees (Free et al., 1981; Williams and Pickett, 1981), is too volatile for straightforward use as well as being easily oxidized (Pickett et al., 1981). The ketone 2-heptanone (IIIa), another honeybee pheromone component with potential agricultural uses (Free and Ferguson, 1980), although less readily oxidized, is very volatile.

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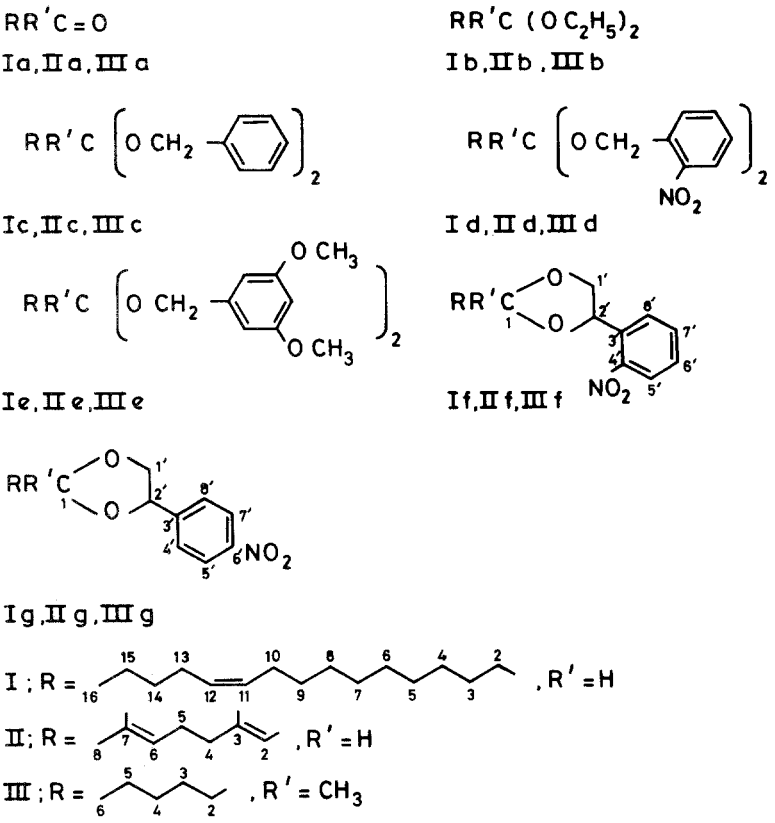

FIG. 1. Chemical structures of adducts of pheromonal carbonyl compounds.

Many pheromones, in addition to those cited, comprise carbonyl compounds (Shorey, 1977). Such compounds react readily with alcohols to form adducts (acetals or ketals) that are less volatile and less readily oxidized. Photolabile adducts have been used to provide protection for carbonyl groups in organic synthesis (Pillai, 1980). If such adducts could act as propheromones by photorelease of the carbonyl compounds under field conditions they might provide a convenient approach to use of pheromonal carbonyl compounds. The preparation of compounds that could act as the pheromonal carbonyl compounds Ia, IIa, and IIIa under sunlight are herein described.

METHODS AND MATERIALS

[^{1}H]- and [^{13}C]nuclear magnetic resonance (NMR) spectra were obtained in carbon tetrachloride and deuterochloroform on JEOL PMX-60

[^{1}H] and PFT-100 [^{13}C] spectrometers, respectively, with tetramethylsilane as internal standard. Mass spectra (MS) were obtained on a Micromass 7070F spectrometer by electron impact at 70 eV, 200°C.

Gas chromatography (GC) was on a Pye-104 chromatograph under the following conditions: (A) 1.5 m × 6 mm glass column, 10% OV-17 on Chromosorb W, 170°C isothermal, N_2 20 ml/min. (B) 2 m × 6 mm glass column, 10% Carbowax on Chromosorb W, 200°C (compound Ia), 190°C (IIa) and 140°C (IIIa) isothermal. Thin layer chromatography (TLC) was on silica gel (60 F^{254}, Merck) developed with hexane–ether. Measurements of refractive index (n_D) were at 20°C.

Starting Materials

Compounds were obtained commercially unless preparation is described; (*E*)-Citral (IIa) (Koch-Light) contained ca. 40% (*Z*)-citral. (*Z*)-11-Hexadecenal (Ia) was prepared from 1,10-decandiol by the method of Liu et al. (1984). *o*- or *p*-Nitrostyrene oxide was prepared from *o* or *p*-nitroacetophenone through bromination, reduction, and dehydrobromination (Guss and Mautner, 1951; Guss, 1952). *o*-Nitrostyrene oxide: δ 2.68 (dd, 1H, 3, 6 Hz), 3.32 (dd, 1H, 4, 6 Hz, OCH_2), 4.50 (dd, 1H, 3, 4 Hz, OCH), 7.5-8.4 (m, 4H, aromatic Hs). *p*-Nitrostyrene oxide: δ 2.78 (dd, 1H, 2.5, 6 Hz), 3.25 (dd, 1H, 4, 6 Hz, OCH_2), 3.96 (dd, 1H, 2.5, 4 Hz OCH), 7.5–7.8 (m, 2H, aromatic Hs, meta to NO_2), 8.2–8.5 (m, 2H, aromatic Hs, ortho to NO_2).

o- and p-Nitrophenylethylene Glycols. A solution of *o*- or *p*-nitrophenylethylene oxide (2.9 g, 17.6 mmol) and potassium carbonate (1.7 g) in 50% aqueous 1,4-dioxane (130 ml) was stirred and refluxed at 110°C for 24 hr, and monitored with TLC. After cooling, the reaction mixture was neutralized with 2 N hydrochloric acid, concentrated under vacuum, extracted with ether, and dried over magnesium sulfate ($MgSO_4$). After removing the ether, the residue was crystallized from benzene–hexane to give the products as light brown crystals: *o*-nitrophenylethylene glycol, 69.4% yield, mp 91–92°C (95–96°C, Hebert and Gravel, 1974), NMR: δ 3.20 (s, 2H, 2 × OH), 3.7–3.9 (m, 2H, CH_2), 5.3–5.6 (m, 1H, CH), 7.5–8.2 (m, 4H, aromatic Hs); *p*-nitrophenylethylene glycol, 51.3% yield, mp 80–82°C, NMR: δ 2.95 (s, 2H, 2 × OH), 3.7–4.0 (m, 2H, CH_2), 4.9–5.2 (m, 1H, CH), 7.8–8.1 (m, 2H, aromatic Hs, meta to NO_2), 8.2–8.5 (m, 2H, aromatic Hs, ortho to NO_2).

Acetals and Ketals

For each alcohol a general method is given followed by the particular catalysts employed and descriptions of the two acetals (from Ia and IIa) and the ketal (from IIIa) prepared; yield bp, n_D, NMR spectrum, MS.

Diethyl Acetals (Ketals)

For the general procedure, a mixture of the aldehyde or ketone (100 mmol), triethyl orthoformate (125 mmol), absolute ethanol (40 ml), and catalyst (200 mg) (added last) was stirred at room temperature under nitrogen for 24 hr. The reaction was monitored with GC (condition A). The ethanol was removed under vacuum, hexane (200 ml) was added and partitioned with saturated sodium bicarbonate solution and the hexane layer removed and dried over sodium carbonate (Na_2CO_3). After removing the solvent, the residual oil was vacuum distilled.

(Z)-*1,1-Diethoxyhexadec-11-ene* (*Ib*). Ammonium chloride (NH_4Cl), 61.5%, 118–120°C/0.05 mm, 1.4475, δ 1.05 (t, 3H, 7 Hz, CH_3), 1.14 (t, 6H, 7 Hz, 2 × OCH_2CH_3), 1.2–1.8 (m, 20H, 10 × CH_2), 1.8–2.3 (m, 4H, 2 × CH_2—C=), 3.3–3.8 (m, 4H, 2 × OCH_2), 4.45 (t, 1H, 4.5 Hz, HC(O)O), 5.3–5.5 (m, 2H, 2 × HC=).

(E)- *and* (Z)-*1,1-Diethoxy-3,7 dimethylocta-2,6-diene* (*IIb*). NH_4Cl, 83.6%, 68–70°C/0.2 mm, 1.4511 [84–85°C/2.5 mm, 1.4508 (20°C), Mikhailov and Povalov, 1959], δ 1.20 (t, 6H, 7 Hz, 2 × $\underline{CH_3}CH_2$), 1.65 (s, 3H) and 1.75 (s, 6H) (3 × CH_3C=), 2.0–2.4 (4H, 2 × CH_2C=), 3.3–3.9 (4H, 2 × $CH_3\underline{CH_2}$), 5.0–5.5 (m, 3H, 2 × HC=+ HC(O)O).

2,2-Diethoxyheptane (*IIIb*). *p*-Toluenesulfonic acid (TsOH), 71%, 88–92°C/18 mm, 1.4150 [80–82°C/15 mm, 1.4165, Utyusheva, 1941], δ 0.94 (t, 3H, 6 Hz, $\underline{CH_3}CH_2$), 1.12 (t, 6H, 7 Hz, 2 × $\underline{CH_3}CH_2O$), 1.22 (s, 3H, CH_3C), 1.3–1.6 (m, 8H, 4 × CH_2), 3.50 (q, 4H, 7 Hz, 2 × OCH_2).

Dibenzyl or Substituted Dibenzyl Acetals or Ketals

For the general procedure, diethyl acetal or ketal (10 mmol) and benzyl alcohol or substituted benzyl alcohol (30 mmol) in benzene (100 ml) were heated slowly with or without catalyst and the benzene–ethanol azeotropic mixture distilled out until the temperature reached the boiling point of benzene. The reaction mixture was basified with anhydrous potassium carbonate (K_2CO_3) and concentrated. The residue was vacuum distilled to remove the excess alcohol (in the case of benzyl alcohol) or extracted with hexane several times (in the case of *o*-nitrobenzyl alcohol or 3,5-dimethoxybenzyl alcohol). The residue from vacuum distillation or the hexane extraction was purified on a column of alumina eluting with a gradient of ether in hexane.

(Z)-*1,1-Dibenzoxyhexadec-11-ene* (*Ic*). TsOH, 98.3%, 1.5088 δ 0.92

(t, 3H, 5 Hz, CH_3), 1.1–1.8 (s, 20H, $10 \times CH_2$), 1.8–2.3 (m, 4H, $2 \times CH_2C=$), 4.64 (s, 4H, $2 \times OCH_2$), 4.74 (t, 1H, 6 Hz, HC ), 5.3–5.5 (t, 2H, 4.6 Hz $2 \times HC=$), 7.3–7.5 (m, 10H, aromatic Hs).

(Z),-*1,1-di-o-Nitrobenzoxyhexadec-11-ene* (*Id*). TsOH, 95.1%, 1.5271, δ 0.92 (t, 3H, 6 Hz, CH_3), 1.2–1.8 (m, 20H, $10 \times CH_2$), 1.9–2.2 (m, 4H, $2 \times CH_2C=$), 4.95 (t, 1H, 5 Hz, HC), 5.04 (s, 4H, $2 \times OCH_2$), 5.3–5.6 (m, 2H, 5 Hz $2 \times HC=$), 7.3–8.3 (m, 8H, aromatic Hs).

(Z)-*1,1-Bis(3,5-dimethoxybenzoxy)hexadec-11-ene* (*Ie*). TsOH, 82.8%, 1.5122, δ 0.94 (t, 3H, 6 Hz, CH_3), 1.1–1.9 (m, 20H, $10 \times CH_2$), 1.9–2.3 (m, 4H, $2 \times CH_2C=$), 3.80 (s, 12H, $4 \times OCH_3$), 4.56 (s, 4H, $2 \times OCH_2$), over 4.6 (m, 1H, HC ), 5.3–5.5 (m, 2H, 5 Hz $2 \times HC=$), 6.3–6.7 (m, 6H, aromatic Hs).

(E)- *and* (Z)-*1,1-Dibenzoxy-3,6-dimethylocta-2,6-diene* (*IIc*). NH_4Cl, 88.6%, 1.5281, δ 1.5–1.9 (m, 9H, $3 \times CH_3$), 2.0–2.3 (m, 4H, $2 \times CH_2$), 4.68 (s, 4H, $2 \times CH_2O$), 5.2 (broad, 1H, HC ), 5.4–5.7 (m, $2 \times CH=$), 7.2–7.6 (s, 10H, aromatic Hs).

(E)-*and* (Z)-*1,1-Di*-o-*nitrobenzoxy-3,6-dimethylocta-2,6-diene* (*IId*). NH_4Cl, 83.1% 1.5406, δ 1.5–1.9 (m, 9H, $3 \times CH_3$), 2.0–2.5 (m, 4H, $2 \times CH_2$), 5.02 (s, 4H, $2 \times CH_2O$), over 4.9–5.2 (m, 1H, HC ), 5.4–5.6 (m, 2H, $2 \times CH=$), 7.2–8.1 (m, 8H, aromatic Hs).

(E)- *and* (Z)-*1,1-Bis(3',5'-dimethoxybenzoxy)-3,6-dimethylocta-2,6-diene* (*IIIe*). Oxalic acid, 82.6%, 1.4528, δ 1.5–1.9 (m, 9H, $3 \times CH_3$), 2.1–2.3 (m, 4H, $2 \times CH_2$), 3.95 (s, 12H, $4 \times OCH_3$), 4.75 (s, 4H, $2 \times OCH_2$), over 4.6–4.9 (m, 1H, HC ), 5.5–5.6 (m, 2H, $2 \times HC=$), 6.5–6.8 (m, 6H, aromatic Hs).

2,2-Dibenzoxyheptane (*IIIc*). TsOH, 78.8%, 1.5224, δ 0.95 (t, 3H, 4 Hz, CH_3), 1.2–2.0 (m, 8H, $4 \times CH_2$), 1.40 (s, 3H, CH_3), 4.58 (s, 4H, $2 \times CH_2O$), 7.3–7.5 (s, 10H, aromatic Hs), *m/z* 297 (M^+-R', 0.8%), 241 (M^+-R, 3.8).

2,2-Di-o-*nitrobenzoxyheptane* (*IIId*). TsOH, 74.6%, 1.5429, δ 0.94

(t, 3H, 5 Hz, CH_3), 1.2–2.1 (m, 8H, 4 × CH_2), 1.6 (s, 3H, CH_3), 4.95 (s, 4H, 2 × CH_2O), 7.4–8.2 (m, 8H, aromatic Hs).

2,2-Bis-(3', 5'-dimethoxybenzoxy) heptane (IIIe). TsOH, 29.2%, 1.5256, δ 0.92 (t, 3H, 6 Hz, CH_3), 1.46 (s, 3H, CH_3), 1.2–2.0 (m, 8H, 4 × CH_2), 3.80 (s, 12H, 4 × CH_3O), 4.56 (s, 4H, 2 × CH_2O), 6.4–6.7 (m, 6H, aromatic Hs).

*2-Substituted 4-(*o-*or* p-*nitrophenyl)-1,3-dioxolanes.* For method A, the aldehyde (2 mmol), *o*- or *p*-nitrophenylethylene glycol (4 mmol), and *p*-toluenesulfonic acid (20 mg) were dissolved in benzene (50 ml) and refluxed under nitrogen (N_2) in a Soxhlet apparatus in which the extraction thimble was filled with $MgSO_4$. The reaction was monitored with TLC. After 6 hr, the reaction mixture was cooled and the precipitate filtered off. The filtrate was washed with saturated aqueous Na_2CO_3, then with saturated aqueous sodium chloride, and dried over $MgSO_4$. After removing solvent, the residue was chromatographed on alumina (neutral), eluting with a gradient of ether in hexane.

For method B, diethyl acetal (ketal) (10 mmol) and *o*-or *p*-nitrophenylethylene glycol (15 mmol) in benzene (100 ml) were heated slowly with or without catalyst and the benzene–ethanol azeotropic mixture distilled out until the temperature reached the boiling point of benzene. The reaction mixture was basified with anhydrous K_2CO_3 and concentrated. The residue was extracted with hexane several times. The hexane extract was applied to a Al_2O_3 column and eluted with a gradient of ether in hexane.

2-(10'-Pentadecenyl)-4-(o-*nitrophenyl)-1,3-dioxolane (If).* A, 73.3%, 1.5061, δ 0.90 (t, 3H, 6 Hz, CH_3), 1.31 (m, 20H, 10 × CH_2), 2.0–2.1 (m, 4H, 2 × CH_2C=), 3.85 (dd, 1H, 5, 8 Hz), 4.47 (dd, 1H, 4, 5 Hz, CH_2O), 5.05 (t, 1H, 5 Hz, HC<O, O>), 5.35 (t, 2H, 5 Hz, 2HC=), 5.56 (dd, 1H, 5, 8 Hz, HCO), 7.3–8.1 (m, 4H, aromatic Hs), *m/z* 194 (M^+—R, 32.9%), chemical ionization (isobutane) *m/z* 404 (M^++1).

2-(10'-Pentadecenyl)-4-(p-*nitrophenyl)-1,3-dioxolane (Ig).* A, 99.3% 1.5107, δ 0.92 (t, 3H, 6 Hz, CH_3), 1.1–1.8 (m, 20, 10×CH_2), 1.8–2.4 (m, 4H, 2×CH_2—C=), 3.4–3.8 (m, 1H), 4.1–4.5 (m, 1H) (CH_2O), 5.0–5.6 (m, 4H, HC<O, O>, 2×CH=, ArCHO), 7.5–7.8 (m, 2H, aromatic Hs meta to NO_2), 8.2–8.5 (m, 2H, aromatic Hs ortho to NO_2).

*2-[(*E*)-and (*Z*)-2',6'-dimethylhepta-1',5'-dienyl]-5-*(o-*nitrophenyl)-1,3-dioxolane (IIf).* B, NH_4Cl, 72.9%, 1.5358, δ 1.70, 1.75 (s, s, 6H, 2 × CH_3), 1.90 (s, 3H, CH_3), 2.2–2.4 (m, 4H, 2 × CH_2), 3.8–4.1 (m, 1H), 4.4–4.8 (m, 1H), (CH_2O), 5.24 (broad, 1H, HC), 5.5–6.1 (m, 3H, 2 × CH=, ArCHO), 7.4–8.4 (m, 4H, aromatic Hs), *m/z* 317 (M^+, 0.6%), 194 (M^+—R, 42.0).

2-[(E)- and (Z)-2',6'-dimethylhepta-1',5'-dienyl]-5-(p-nitrophenyl)-1,3-dioxolane (IIg). B, NH_4Cl, 85.8%, 1.5370, δ 1.70, 1.78 (s, s, 6H, 2 × CH_3 terminal), 1.90 (s, 3H, CH_3), 2.1–2.4 (m, 4H, 2 × CH_2), 3.7–4.0 (m, 1H), 4.2–4.5 (m, 1H) (CH_2O), 5.1–6.1 ((m, 4H, 2 × CH=, HC(O)O, ArCHO), 7.71 (m, 2H, aromatic Hs meta to NO_2), 8.40 (m, 2H, aromatic Hs ortho to NO_2).

2-Methyl-2-pentyl-4-(o-nitrophenyl)-1,3-dioxolane (IIIf). B (none), 79.9%, 1.5150, δ 0.96 (t, 3H, 4 Hz, CH_3), 1.48* (see below) (s, CH_3), 1.60* (s, CH_3), 1.2–2.0 (m, 8H, 4 × CH_2), 3.6–4.0 (m, 1H,), 4.6–5.0 (m, 1H), (CH_2O), 5.6–5.9 (m, 1H, ArCHO), 7.4–8.3 (m, 4H, aromtic Hs).

2-Methyl-2-pentyl-4-(p-nitrophenyl)-1,3-dioxolane (IIIg). B (none), 93%, 1.5181, δ 0.94 (t, 3H, 4 Hz, CH_3), 1.48* (s, CH_3), 1.52* (s, CH_3), 1.2–2.0 (m, 8H, 4 × CH_2), 3.6–4.0 (m, 1H), 4.3–4.7 (m, 1H), (CH_2O), 5.1–5.5 (m, 1H, ArCHO), 7.5–7.8 (m, 2H, aromatic Hs meta to NO_2), 8.2–8.5 (m, 2H, aromatic Hs ortho to NO_2).

Stereoisomers of adducts If, IIf, IIIf, Ig, IIg, and IIIg, the 2-substituted 4-(*o*- or *p*-nitrophenyl)-1,3-dioxolanes were defined by [^{13}C]NMR spectroscopy (Table 1). The dioxolanes derived from (*Z*)-11-hexadecenal and 2-heptanone were a mixture of two diastereomeric pairs and those derived from (*E*)-citral contained also the (*Z*) isomers. Peak multiplicity due to differences between *E* and *Z* isomers is indicated by *E* and *Z* designations, and those due to diastereomer differences by asterisks.

General Procedure for Irradiation

Irradiations were carried out under a CAMAG universal ultraviolet (UV) lamp emitting at 350 nm or a 20-W daylight lamp. The compound to be irradiated was dissolved in either hexane or 80% aqueous dioxane and diluted to 1 mg/ml. The sample was sealed in a borosilicate glass tube under nitrogen. The carbonyl compounds recovered after 24 hr of UV irradiation were identified by coinjection with authentic samples and quantitatively analyzed by gas–liquid chromatography (condition B). In all cases studied, no carbonyl compounds were recovered from the control which was wrapped with aluminum foil. Samples were sensitive to UV were examined in hexane under the daylight lamp over different periods.

Persistence of (*Z*)-11-hexadecenal (Ia) release from underivatized material and adduct If on glass was compared using a method similar to that of Baker et al. (1980). Either (*Z*)-11-hexadecenal (Ia) (0.10 mg, 0.42 μmol) or adduct If (0.17 mg, 0.42 μmol) dissolved in hexane (100 μl) was placed on a glass disk (16 mm) in duplicate. After irradiation (20-W daylight lamp) for 17 hr, each disk was suspended in a closed 250-ml round-bottomed flask for a further 3 hr irradiation. (*Z*)-11-Hexadecenal released from the disks onto the

TABLE 1. [^{13}C]NMR SPECTROSCOPY OF ADDUCTS If, IIf, IIIf, Ig, IIg, and IIIg, THE 2-SUBSTITUTED 4-(*o*- OR *p*-NITROPHENYL)-1,3-DIOXOLANES CHEMICAL SHIFT [ppm from $(CH_3)_4Si$]

Adduct/carbon	C-1	C-1Me	C-2	C-3	C-3Me	C-4
If	105.6*, 106.1*		33.6*, 34.2*	24.4*, 24.2*		29.3
Ig	106.4*, 106.6*		33.9*, 34.3*	24.3*, 24.1*		29.6
IIf	101.5*, 101.2*, 101.6*, 101.3* (*E,Z*)		120.2 (*E*), 121.1 (*Z*)	146	17.2 (*E*), 23.7 (*Z*)	39.6 (*E*), 32.8 (*Z*)
IIg	101.7* (*E*), 101.4* (*Z*)		120.2 (*E*), 120.7 (*Z*)	145.6 (*E*), 145.9 (*Z*)	17.0 (*E*), 23.6 (*Z*)	39.5 (*E*), 32.6 (*Z*)
IIIf	111.6*, 112.0*	22.7*, 24.0*	39.7*, 38.4*	24.0		32.2
IIIg	112.2*, 112.3*	23.8*, 24.5*	39.8*, 39.3*	23.8		32.1

Adduct/carbon	C-5	C-6	C-7	C-7Me	C-8	C-9
If	29.3	29.3	29.3		29.3	29.3
Ig	29.6	29.6	29.6		29.6	29.6
IIf	26.3 (*E*), 27.0 (*Z*)	123.6	132.0 (*E*), 132.2 (*Z*)	17.7 (*E*), 17.7 (*Z*)	25.7 (*E*), 25.7 (*Z*)	
IIg	26.1 (*E*), 26.9 (*Z*)	123.6	131.9 (*E*), 132.1 (*Z*)	17.7 (*E*), 17.7 (*Z*)	25.7 (*E*), 25.7 (*Z*)	
IIIf	22.7	14.1				
IIIg	22.6	14.0				

Adduct/carbon	C-10	C-11	C-12	C-13	C-14	C-15
If	27.2[a]	129.9	129.9	26.9[a]	32.0	22.4
Ig	27.2	129.9	129.9	26.9[a]	32.0	22.4

Adduct/carbon	C-16	C-1′	C-2′	C-3′	C-4′
If	14.0	72.3	74.0*, 74.7*	138.1	147.1*, 147.3*
Ig	14.1	77.0*, 76.6*	71.9*, 72.4*	147.7	126.9*, 126.5*
IIf		72.4	74.0*, 74.4*	138.2*, 137.8*	147.0
IIg		71.8 (*E*), 72.4 (*Z*)	76.7*, 76.0*	147.5	126.9*, 126.4*
IIIf		71.3	73.8*, 74.6*	137.2*, 137.4*	147.3
IIIg		71.5*, 71.4*	76.6*, 77.3*	146.7*, 146.9*	−26.7

TABLE 1. Continued

Adduct/carbon	C-5′	C-6′	C-7′	C-8′
If	124.6*, 124.9*	128.3	133.9	127.8*, 137.4*
Ig	123.8	147.7		
IIf	124.6	128.3	134.0	128.1*, 127.3*
IIg	123.6	148.0		
IIIf	124.6	128.3[a]	134.0	127.6[a]
IIIg	123.8	147.7		

[a]Assignments may be transposed; numbering scheme, see Figure 1.

inner walls of the flasks was quantified by washing the flasks with portions (10 ml) of a hexane solution of octadecane (0.01 mg/ml) as internal standard and then analyzing the concentrated washings by GC (condition A).

Field Studies

Lures prepared from polyethylene closures (WP/5, Fisons) containing (1) (*Z*)-11-hexadecenyl acetate (10 μg), (2) (*Z*)-11-hexadecenyl acetate (10 μg) + adduct Id (33 μg equivalent to 15 μg of aldehyde Ia), and (3) (*Z*)-11-hexadecenyl (10 μg) + adduct If (25 μg equivalent to 15 μg of aldehyde Ia) were hung in brown cardboard traps (Oecos), triangular in cross-section (side 100 mm, length 176 mm) with a removable sticky card base. Traps were placed horizontally in five groups of the three randomly arranged lure types in a north–south line with 15 m spacing and the traps aligned east–west in Brussels sprouts at crop height, on June 10, 1982, Bedfordshire, U.K. Diamondback moths, *Plutella xylostella* (L.) were counted each week and the sticky card changed, for a period of four weeks.

RESULTS AND DISCUSSION

Structures of acetal and ketal adducts of pheromonal carbonyl compounds Ia, IIa, and IIIa prepared in this study are shown in Figure 1. Adducts with *o*-nitro groups (Id, IId, IIId, If, IIf, and IIIf) were prepared because similar adducts afford photoremovable protection of carbonyl groups during synthesis (Barltrop, 1966, Hebert and Gravel, 1974). Photolysis of these adducts is facilitated by the *o*-nitro group which photochemically abstracts hydrogen from the group on the adjacent position in the aromatic ring as the first step leading to reduction of the nitro to the nitroso group and release of the carbonyl compound (Figure 2) (Pillai, 1980). The other adducts were

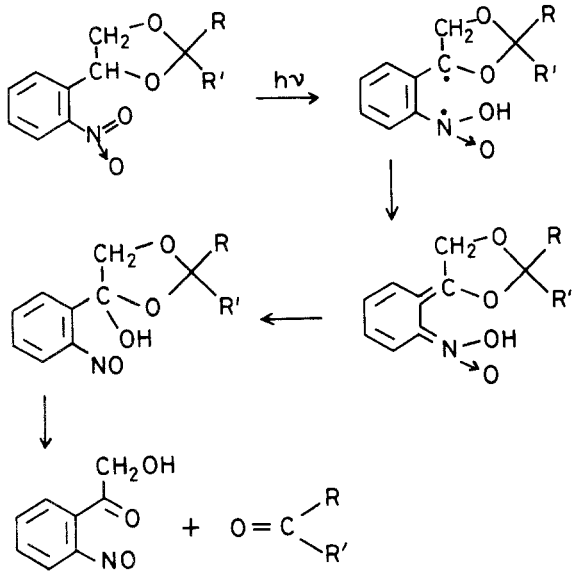

FIG. 2. Mechanism for photochemical release of carbonyl compound from adducts with *o*-nitro substituent (Pillai, 1980).

prepared in an attempt to provide propheromones with a range of release rates.

(*Z*)-11-Hexadecenal reacted with benzyl alcohol or substituted benzyl alcohols in the presence of TsOH to give the acetals in low yield (<3%); (*E*)-citral gave the acetals with many by-products; 2-heptanone did not react. However, the transacetalization of the readily formed diethyl acetals and ketal with the benzyl alcohols proceeded smoothly, mostly with yields above 70%, in the presence of a small amount of acid catalyst. The diethyl acetal of (*Z*)-11-hexadecenal and the diethyl ketal of *2*-heptanone reacted readily with the benzyl alcohols in the presence of TsOH, but in the case of *2*-heptanone the *o*-nitrobenzyl vinyl ether [MS: *m*/*z* 249 (M^+)] was formed if the reaction time was too long. The transacetalization of the diethyl acetal of (*E*)-citral and the benzyl alcohols employing the weaker acid catalyst NH_4Cl instead of TsOH avoided cyclization of the terpenoid structure. (*Z*)-11-Hexadecenal was acetalized in good yield (73.3% and 99.3%) with *o*- or *p*-nitrophenylethylene glycol in the presence of TsOH. (*E*)-Citral with the catalyst oxalic acid and 2-heptanone with TsOH reacted with *o*-nitrophenyl glycol to give the dioxolanes in low yield (<5%). However, the diethyl acetal of (*E*)-citral and the diethyl ketal of 2-heptanone were both readily transacetalized with the *o*- and *p*-nitrophenylethylene glycols, employing NH_4Cl or no catalyst, respectively.

TABLE 2. IRRADIATION OF ACETAL AND KETAL ADDUCTS OF PHEROMONAL CARBONYL COMPOUNDS UNDER UV (350 nm)

Adduct	Recovery of carbonyl compound (24 hr) (%)	
	In hexane	In 80% aqueous dioxan
Ic	0	0
IIc	0	0
IIIc	0	0
Id	82	62
IId	74	41
IIId	91	89
Ie	19	12
IIe	0	0
IIIe	0	0
If	97	90
IIf	69	71
IIIf	87	82
Ig	0	0
IIg	0	0
IIIg	0	0

Results from UV irradiation of adducts Ic to IIIg (Table 2) show that only the adducts with *o*-nitro groups (Id, IId, IIId, If, IIf, and IIIf) gave good yields (69 to 97%) of pheromonal carbonyl compounds whereas adducts containing the unsubstituted benzyl group (Ic, IIc, and IIIc) did not give detectable amounts of carbonyl compound under these conditions. Introduction of methoxyl groups (Ie, IIe, IIIe) did not lead to improved release except for adduct Ie, although in different systems such substituents assist photoremoval of groups such as benzyloxycarbonyl (Barltrop and Schofield, 1965; Chamberlin, 1966). The presence of water did not cause release by hydrolysis under these conditions. Adducts with the *p*-nitro group (Ig, IIg, IIIg) could not release the carbonyl compounds by intramolecular photochemical hydrogen abstraction and intermolecular interactions failed to give a detectable yield of carbonyl compound. Presumably subsequent photolysis of products accounted for the failure to achieve the full theoretical yield for released carbonyl compounds. Where carbonyl compounds were not released, starting material was recovered almost quantitatively except for adducts from (*E*)-citral where there had been some internal cyclization reactions involving double bonds of the terpenoid structure.

Adducts with the *o*-nitro group gave the pheromonal carbonyl compounds more slowly under simulated sunlight than under UV irradiation

TABLE 3. IRRADIATION OF ACETAL AND KETAL ADDUCTS OF PHEROMONAL CARBONYL COMPOUNDS UNDER DAYLIGHT LAMP

Adduct	Recovery of carbonyl compounds (%)			
	3 hr	6 hr	12 hr	24 hr
Id	14	20	39	53
If	11	21	44	64
IId	8	10	12	15
IIf	5	9	15	22
IIId	7	11	25	35
IIIf	11	18	33	43

(Table 3). Release rates depended more on the type of carbonyl compound than on the alcohol component of the adduct. This suggested that some pheromonal carbonyl compounds could be slowly released from these adducts in the field by sunlight. Adduct If had the additional advantage that on glass (method of Baker et al., 1980) under simulated sunlight it continued to release the pheromonal compound Ia (0.9 μg, 9% in 3 hr) after an initial irradiation (17 hr), whereas no (*Z*)-11-hexadecenal (Ia) was obtained from the underivatized sample on glass after this period.

The sex attractant pheromone of the diamondback moth, *Plutella xylostella* (L), comprises mainly (*Z*)-11-hexadecenal and (*Z*)-11-hexadecenyl acetate (Tamaki et al., 1977). Table 4 shows catches of this moth in sticky traps with lures containing (*Z*)-11-hexadecenyl acetate and the adducts Id and If. The acetate alone caught few moths. However, when the adducts were employed with the acetate there were good catches. The numbers of moths caught for each of the four weeks of the test were similar to those from nearby tests using fresh lures containing corresponding amounts of underivatized aldehyde (Ia) and acetate, but whose level of attractancy did not persist for longer than two weeks (Liu et al., 1984). The acetate, being much less

TABLE 4. CATCHES OF DIAMONDBACK MOTHS USING PROPHEROMONE LURE

Lure composition	Weekly catch (total 5 traps)			
	Week 1	Week 2	Week 3	Week 4
(Z)-11-hexadecenyl acetate (HDA)	0	1	5	3
HDA + Id	87	379	332	197
HDA + If	171	369	287	126

susceptible to aerial oxidation than the aldehyde, persists well in the polyethylene lures. The small differences between catches for lures with adduct Id and those for adduct If are not significant.

More extensive trials, particularly field tests of biological activity, are necessary to determine if these adducts have general advantages over underivatized pheromonal carbonyl compounds. Adducts giving no detectable amounts of pheromonal carbonyl compounds in the laboratory tests employed here should also be investigated further as they might act as propheromones under the more rigorous photolytic conditions obtaining when applied to crops. However, the principle of using light-sensitive propheromones has been demonstrated and may provide a new approach to exploiting pheromonally mediated behavior of agricultural pests and beneficial organisms.

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INHIBITION AND INDUCTION OF BARNACLE SETTLEMENT BY NATURAL PRODUCTS PRESENT IN OCTOCORALS

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Abstract—Barnacle settlement inhibitors and inducers are present in the gorgonian *Leptogorgia virgulata* and the pennatulacean *Renilla reniformis*. The inhibitors are low-molecular-weight compounds (<20,000 daltons) that were detected in soft tissue homogenates and dialysates of homogenate and in ambient "gorgonian water." Settlement was almost completely inhibited at a dialysate concentration of 1.0 g wet weight equivalents/liter. The inhibitors probably function in chemical defense against predation and fouling, and could prove useful in ship fouling control. The settlement inducers are high-molecular-weight substances (>20,000 daltons) that adsorb to surfaces.

Key Words—Inhibition, induction, chemical defense, larval settlement, fouling, barnacle, *Balanus amphitrite amphitrite*, octocorals, *Leptogorgia virgulata, Renilla reniformis.*

INTRODUCTION

Chemical interactions between organisms are relatively well known in terrestrial communities (Sondheimer and Simeone, 1970; Whittaker and Feeny, 1971). It is now clear that marine communities are also organized around a variety of chemical messages affecting the behavior and distribution of organisms (Kittredge et al., 1974). Particularly important to community development, and to the fouling of surfaces, is the chemical information involved in larval settlement (Crisp, 1974, 1976).

Larval settlement can be affected by two major kinds of allelochemics:

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inducers and inhibitors. Inducers are substances that encourage settlement and metamorphosis. Barnacle cyprids, for example, settle gregariously near barnacle spat and adults (Knight-Jones and Stephenson, 1950; Knight-Jones, 1953), and this response is probably due to cyprid contact with adsorbed proteins (Crisp and Meadows, 1962, 1963; Larman and Gabbott, 1975). Inducers are also implicated when the larvae of predators or symbionts settle specifically on their prey or host organisms (Scheltema, 1974; Lewis, 1978). Only a few studies have succeeded in identifying settlement inducers (Kato et al., 1975; Morse et al., 1979).

Allelochemics that discourage settlement and metamorphosis—settlement inhibitors—have received less study. Barnacle settlement may be suppressed by specific microbes or microbial films (D'Agostino and Sheridan, 1969) and by the tannins present in certain brown algae (Sieburth and Conover, 1965), but no data are given in these reports. In fact, settlement inhibition by natural products is poorly understood in any animal, and convincing evidence has yet to be reported in barnacles, probably because it is difficult to show a settlement difference between an inhibition treatment that approaches zero and a chemically neutral control that may not be much higher.

We report here that settlement of the barnacle *Balanus amphitrite amphitrite* Darwin, 1854 is inhibited by substances present in the gorgonian *Leptogorgia virgulata* (Lamarck, 1815) and the pennatulacean *Renilla reniformis* (Pallas, 1766). Curiously, these same octocorals have inducers that promote barnacle settlement.

METHODS AND MATERIALS

Octocoral Preparation. Colonies of *Leptogorgia virgulata* were collected by free-diving on the jetty at Radio Island, near Beaufort, North Carolina. *Renilla reniformis* were obtained by trawling in the adjacent Newport River estuary. The animals were maintained in the laboratory in running seawater for no more than two days before being used. Most of the work was accomplished in the summer and fall.

Soft tissues of the octocorals were weighed and homogenized in full strength ($34^0/_{00}$) seawater with a tissue grinder, followed by centrifugation (12,000 *g*) and fiber filtration (Whatman No. 1). The resulting "homogenate" was diluted with seawater to concentrations of 1.0 or 2.0 g soft tissue wet weight equivalents/liter and used as test water. In most of the experiments, however, the homogenate was dialyzed for 24 hr at 4° C in 100 times its volume of stirred seawater. This treatment separated the homogenate into dialysate and retentate fractions, both of which were used in tests after further dilution. In two experiments most of the bacteria were removed from undialyzed

gorgonian homogenate. This procedure was accomplished in three test treatments by the addition of antibiotics (15 mg sodium penicillin and 25 mg streptomycin sulfate/liter), by ultrafiltration (0.22-μm membrane filter), and by ultraviolet irradiation (close exposure to UV lamp for 14 min). In another experiment, "gorgonian water" was tested instead of homogenate or dialysate. This water was obtained by immersing healthy, living *Leptogorgia* in gently aerated seawater ($34^0/_{00}$) for 18 hr at 15° C. The water surrounding the colonies was then fiber-filtered and used in tests at 17.0 and 3.4 g soft tissue wet weight equivalents/liter.

Barnacle Rearing. Cyprid larvae of the intertidal barnacle *Balanus amphitrite amphitrite* were the assay organisms. These were obtained by rearing first stage nauplius larvae to the cyprid stage in the laboratory. The nauplii were obtained either by dissecting field-collected adults for ripe embryo masses or by collecting naturally spawned nauplii from adults maintained in breeding condition in the laboratory. These latter adults were fed on diatoms (*Skeletonema costatum*) and brine shrimp (*Artemia salina*) nauplii. All of the adult barnacles were collected from Pivers Island, near Beaufort.

Once obtained, the barnacle nauplii were reared in gently aerated polypropylene carboys at densities of about 800 nauplii/liter of glass fiber-filtered seawater ($34^0/_{00}$). The carboys were kept in environmental chambers at a constant temperature of 25° C and a 15-hr light:9-hr dark photoperiod. Nauplii were fed daily on *Skeletonema costatum* at a density of 30,000 cells/ml. The diatom was cultured separately in autoclaved seawater enriched with f/2 nutrients (Guillard, 1975). Under these conditions, first-stage nauplii were reared to cyprids in about five days, and survival was about 40%. Cyprids were then filtered from the cultures and kept in beakers of clean seawater at 15° C for one day before testing. The beakers were coated with a thin film of paraffin to minimize settlement on the glass.

Experimental Design. For each experiment cyprids were mixed by magnetic stirring, and approximately equal numbers were aliquotted into 200-ml polystyrene drinking cups that served as replicate test chambers. The cyprids in each cup were then filtered from the aliquot water and rinsed back into the cup with test water or control seawater to a final volume of 200 ml. A single settling substrate was then added to the bottom of each cup. For most of the experiments, the substrates were slate rectangles ($0.4 \times 2 \times 5$ cm) bearing ten drilled pits on their upper surfaces to promote settlement. These were identical to those used by Crisp and Meadows (1963) and were cleaned in the same way they suggest before being reused. In several experiments screened hardboard paneling was used for settlement substrates. These substrates were the same size as the slates but were soaked in running tap water for 24 hr before the tests to make them negatively buoyant. Cyprids settled almost

entirely in the tiny rough gouges covering the upper surfaces. The hardboard substrates elicited higher settlement in controls than the slates and were disposable.

Two experiments designed to test adsorption of gorgonian retentate to slate substrates involved some different procedures. In these experiments slates were first soaked in seawater or retentate without cyprids for 3 h. They were then dipped in seawater three times to remove most of the unadsorbed organic materials and finally placed in cups containing either seawater or retentate for testing with cyprids.

The cups in any single experiment were placed around the periphery of a large turntable with the long axes of the substrates arranged circumferentially. The turntable rotated at 0.7 rpm, serving to minimize lighting differences. Cups were arranged in replicate groups around the circumference; each group contained all of the experimental treatments, and treatments were assigned randomly within each group. The apparatus was located in an environmental chamber having constant temperature (25° C) and light intensity (7.0×10^{15} quanta/cm^2/sec).

Experiments ran for 12–48 hr, depending on settlement rate. When settlement ended, the replicates were preserved in 70% alcohol. Permanently attached cyprids and spat on the upper surface of each substrate were then counted and summed to give the total number of settled barnacles. The number of unattached cyprids was also counted, permitting the calculation of mean percentage settlement.

Experiments were analyzed statistically with single-classification ANOVAs (F). Percentage settlement data underwent an angular transformation prior to testing. The Student-Newman-Keuls test was used to compare treatment means.

RESULTS

Leptogorgia *Inhibitor.* In three experiments gorgonian homogenates elicited significantly lower settlement than seawater controls ($P < 0.001$; Table 1). For example, experiment 1 had eight replicates and an average of 322 cyprids available for settlement in each replicate. In this experiment nearly 31% of control cyprids settled, while only 6.5% of those exposed to homogenate settled. Homogenate concentrations of about 10^{-1} g wet weight equivalents/liter appeared to inhibit settlement.

It seemed possible, however, that the active material was produced by associated microbes rather than by the gorgonians. In two experiments (Table 2) settlement was significantly higher in seawater controls than in both unaltered homogenates and homogenates treated with antibiotics, ultrafiltration, and UV irradiation ($F = 5.1$, $0.01 < P < 0.025$ experiment 1;

TABLE 1. EFFECTS OF *Leptogorgia virgulata* HOMOGENATES, DIALYSATES, AND RETENTATES ON BARNACLE SETTLEMENT

Experiments	Mean percentage settlement				Number of replicates	Mean number of cyprids available for settlement	Concentrations (g wet weight) equivalents/liter)
	Seawater controls	Homogenates	Dialysates	Retentates			
1	30.9	6.5			8	322	1.0
2	26.5	1.0	0		4	108	2.0
3	14.7		5.0		10	229	1.0
4	10.5	2.3	0	45.1	5	381	1.0

TABLE 2. EFFECTS OF "LOW-BACTERIA" HOMOGENATES OF *Leptogorgia* ON BARNACLE SETTLEMENT

Experiments	Seawater controls	Unaltered homogenates	"Low-bacteria" homogenates			Number of replicates	Concentrations of homogenate (g wet weight equivalents/liter)
			Antibiotics	Ultrafiltration	Ultraviolet irradiation		
1	4.6[a]	1.6	1.4	2.3		8	2.0
2	128 (30.9)[b]	25 (6.5)	22 (6.4)	40 (10.9)	25 (7.1)	4	1.0

[a]Numbers not enclosed in parentheses indicate mean numbers of settled barnacles.
[b]Numbers in parentheses refer to mean percentage settlement.

$F = 65.7$, $P < 0.001$ in experiment 2). None of the homogenate treatments were significantly different from each other, except that the ultrafiltrate treatment was different from the others in experiment 2. The settlement inhibition effect was apparent whether or not bacteria were present in appreciable numbers.

To begin isolation of the active inhibitor from crude homogenate extracts, we dialyzed these extracts. This technique separates complex, high-molecular-weight retentate molecules from small dialysate molecules, with separation at about 20,000 daltons. Table 1 shows an inhibition effect in the low-molecular-weight dialysate fraction. Dialysate treatments had significantly lower settlement than seawater controls ($P < 0.001$), as was the case with the homogenates. However, dialysates were more active than undialyzed homogenates, sometimes inhibiting all settlement at 1.0 g wet weight equivalents/liter.

Other work on *Leptogorgia* dialysates has concerned concentration levels that inhibit settlement (Figure 1). In four experiments, settlement was inversely proportional to concentration. The two lowest concentrations were not significantly different from controls, but all other concentrations were

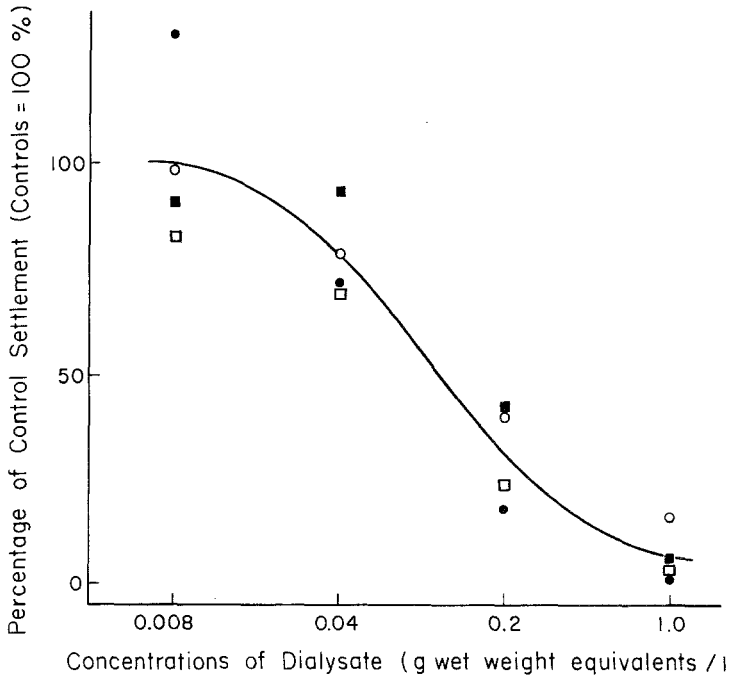

FIG. 1. Effects of concentration of *Leptogorgia* dialysate on barnacle settlement. Dose-response curve represents mean of four experiments, each with four concentrations; each point indicates mean of four replicates.

significantly different from each other ($F = 14.8$; $P < 0.001$). Settlement was almost completely inhibited at 10^{-1} g wet weight equivalents/liter. Cyprids exposed to dialysate appeared less active than those in control seawater but were not killed at the concentrations used. High concentrations of inhibitor also suppressed metamorphosis from attached cyprid to spat.

We have also shown a settlement inhibition effect in "*Leptogorgia* water" obtained in the laboratory. Seawater controls were compared with "gorgonian water" concentrations of 3.4 and 17.0 g wet weight equivalents/liter. Mean settlement percentages in these three treatments were 65.2, 50.3, and 12.6, respectively, and all of them were significantly different from each other ($F = 51.7$; $P < 0.001$). Each treatment consisted of six replicates, and the mean number of cyprids available for settlement in each replicate was 284. As expected, the inhibitor present in the "gorgonian water" was not as concentrated as that present in dialysate or homogenate.

Leptogorgia *Inducer.* In the retentate treatment of experiment 4 (Table 1) settlement was 34.6% higher than in seawater controls, and the difference was highly significant ($F = 169.5$; $P < 0.001$). These data suggest the existence of a high-molecular-weight settlement inducer in the retentate. Thus, the soft tissue of *Leptogorgia* has both a low-molecular-weight inhibitor and a high-molecular-weight inducer. The inhibition effect predominates in undialyzed homogenate, but when the homogenate is dialyzed, induction is more easily demonstrated than inhibition. Both effects were demonstrated repeatedly in experiments designed mainly for other purposes (e.g., Figure 1 and Table 3).

Important to experimental methodology, perception mechanisms, and biofouling control is the extent to which these natural products are active when adsorbed to surfaces. In two experiments involving all combinations of substrate soaking and testing in seawater and retentate (Table 3), the control treatment that was both soaked and tested in seawater had significantly lower settlement than the three retentate treatments ($F = 6.7$, $0.001 < P < 0.005$ in experiment 1; $F = 14.7$, $P < 0.001$ in experiment 2). The retentate treatments were not significantly different from each other. In experiment 4 (Table 1) only 36% of the cyprids in the retentate treatment settled in the pits, which are physically favorable for settlement; the remaining cyprids (64%) settled on the less-favorable smooth surfaces between the pits, presumably because the inducer had adsorbed onto all surfaces and was a more powerful stimulus than the pit contours. These experiments suggest that the inducer adsorbs to slate substrates and is not easily rinsed off. Similar experiments with the inhibitor did not provide clear results.

Renilla *Inhibitor and Inducer.* In addition to the *Leptogorgia* work, we have discovered a second inhibitor and another inducer in the pennatulacean octocoral *Renilla reniformis* (Table 4). As with the gorgonian, the settlement inhibition effect is present in both the undialyzed homogenate and the

TABLE 3. ADSORPTION OF *Leptogorgia* RETENTATES ON SLATE SETTLEMENT SUBSTRATES

	Slates: soaked/tested				
	Controls[a]	Retentate treatments[a,b]			Mean number of cyprids available for settlement
Experiments	Seawater/seawater	Seawater/retentate	Retentate/seawater	Retentate/retentate	
1	0.3[c]	18.5	14.4	9.4	175
2	9.2	25.8	38.5	35.4	302

[a] Each treatment consisted of three replicates.
[b] Concentrations were all 1.0 g wet weight equivalents/liter.
[c] Numbers refer to mean percentage barnacle settlement.

TABLE 4. EFFECTS OF *Renilla reniformis* HOMOGENATES, DIALYSATES, AND RETENTATES ON BARNACLE SETTLEMENT

	Mean percentage settlement				Number of replicates	Mean number of cyprids available for settlement	Concentrations (g wet weight equivalents/liter)
Experiments	Seawater controls	Homogenates	Dialysates	Retentates			
1	30.9	0			4	370	1.0
2	15.1	2.5	0.2	61.7	5	200	1.0

dialysate, with a slightly greater (although not significantly different) effect in the dialysate than in the homogenate. All other differences were significant ($F = 402.5$, $P < 0.001$ in experiment 1; $F = 68.9$, $P < 0.001$ in experiment 2). *Renilla* is also similar to *Leptogorgia* in that a settlement inducer was present in the retentate fraction (Table 4, experiment 2).

DISCUSSION

Our work began with the observation that healthy colonies of *Leptogorgia virgulata* were remarkably free of free-living barnacles and other fouling organisms. Although planktonic cyprids of at least eight free-living barnacle species occur in the immediate vicinity of the gorgonians, the adults of only two of these were to be found on the colonies, and then only rarely. In contrast, dead gorgonians became heavily fouled in a few weeks (Burkholder, 1973; J.D.S., personal observations). Settlement inhibition seemed a likely explanation for this apparent anomaly.

Inhibitors. The present work demonstrates that barnacle settlement inhibitors are present in the soft tissue of *Leptogorgia virgulata* and *Renilla reniformis* (Tables 1 and 4, Figure 1). The inhibitors are low-molecular-weight molecules that can be collected from the water surrounding gorgonians, at least in the laboratory. They effectively inhibit settlement at low concentrations (10^{-2} to 10^{-1} wet weight equivalents/liter; Figure 1) by suppressing cyprid activity, but higher concentrations could be quite toxic. In nature they probably function to chemically defend the octocorals against predation and fouling.

Although no previous studies have dealt with *L. virgulata* and *R. reniformis,* there is a substantial literature on octocoral chemistry. Much of this work concerns the terpenoids that are so ubiquitous in these animals (Ciereszko and Karns, 1973; Fenical, 1978; Tursch et al., 1978). Like our inhibitors, these low-molecular-weight compounds are toxic in low concentrations (Burkholder and Burkholder, 1958; Perkins and Ciereszko, 1973). For example, a cembranolide terpenoid that is a potent neuromuscular toxin occurs in *Lophogorgia* spp. (Fenical et al., 1981), a genus closely related to *Leptogorgia.* Particularly relevant to the octocorals may be the antimicrobial and icthyotoxic effects that have been studied (Burkholder, 1973; Bakus, 1974; Tursch, 1976). Octocoral defense against larval settlement was first proposed by Ciereszko (1962), and Hadfield and Ciereszko (1978) showed that several cembranolides isolated from gorgonians were very toxic to larvae of the nudibranch mollusc *Phestilla sibogae.* No previous studies have focused on larval barnacles, but D.J. Faulkner found juvenile barnacles to be highly susceptible to crassin acetate from *Pseudoplexaura* spp. (footnoted in Wein-

heimer and Matson, 1975). Terpenoids would seem to be likely candidates for the settlement inhibitors described herein.

The present work suggests the possible importance of natural settlement inhibitors to biofouling control. Fouling is controlled nowadays with paints containing inorganic copper or organic tin compounds, or with halogens. Unfortunately, these toxins may pose long-term pollution hazards in inland waters (Hoare and Hiscock, 1974; Good et al., 1979). Natural products, however, may be more biodegradable than heavy metals, thus restricting their toxicity both spatially and temporally. Further evaluation of their use as antifoulants would require isolation studies, toxicity analyses, and development of binding and release technologies.

Inducers. Work on the inhibitors in *Leptogorgia* and *Renilla* led us to the discovery of settlement inducers in the soft tissue retentate fractions of these same octocorals (Tables 1, 3, and 4). Unlike the inhibitors, the inducers are high-molecular-weight substances that adsorb to surfaces. In these respects they appear similar to the adsorbed proteins responsible for the gregarious settlement of barnacles (Crisp and Meadows, 1962, 1963; Larman and Gabbott, 1975). Although *Leptogorgia* colonies are generally free of free-living barnacles and other fouling organisms, they do harbor several symbiotic animals (Patton, 1972). One of these is the interesting barnacle *Conopea galeata* (=*Balanus galeatus*), which occurs exclusively on gorgonians. Settlement of this barnacle could be dependent on gorgonian inducers.

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ISOLATION, IDENTIFICATION, AND BIOLOGICAL ACTIVITY OF TRAIL-FOLLOWING PHEROMONE OF TERMITE *Trinervitermes bettonianus* (SJÖSTEDT) (TERMITIDAE:NASUTITERMITINAE)

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Abstract—The principal compound which posseses trail-following activity at low concentration has been isolated from workers and from female alates of the grass feeding termite *Trinervitermes bettonianus*. Male alates also produce the pheromone but in much smaller quantities than the female. The structure of the pheromone was determined as the diterpene hydrocarbon cembrene-A from spectra and microscale reaction data.

Key Words—Trail pheromone, termite, *Trinervitermes bettonianus*, Isoptera, Termitidae, diterpene, cembrene-A.

INTRODUCTION

The presence and utilization of trail pheromones in termites for orientation during foraging has been known for many years (Lüscher and Müller, 1960; Stuart, 1961, 1963a,b). Despite much research on the behavioral aspects of trail following, relatively few trail pheromones have actually been identified chemically (Hummel and Karlson, 1968; Hummel, 1968, Tai et al., 1969; Birch et al., 1972). Several pheromones have been partially characterized (Verron and Barbier, 1962; Moore, 1966, 1974; Tschinkel and Close, 1973; Larue and Barbier, 1976; Roomi et al., 1981). Howard et al. (1976) have indicated that major problems in chemical identification are in establishing an adequate bioassay and in distinguishing an authentic trail pheromone from a potent nonpheromonal attractant.

Trail-laying and trail-following behavior in *Trinervitermes bettonianus* (Sjostedt) and related species has been the subject of investigation in these

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laboratories for several years (Leuthold and Lüscher, 1974; Leuthold, 1977; Oloo and Leuthold, 1979, 1980; Oloo, 1981a,b). In the present study we have analyzed the chemical nature of the *T. bettonianus* trail pheromone and report the isolation and identification of the diterpene cembrene-A (1) as the principal component of worker foraging trails. The same compound also occurs in male and female alates in larger quantities than the workers, female alates containing much larger quantities than male alates.

METHODS AND MATERIALS

Whole *T. bettonianus* nests were collected from Machakos, 60 km southeast of Nairobi and from Narok, 160 km northwest of Nairobi, Kenya, and maintained in the laboratory as previously described (Oloo and Leuthold, 1979). Extracts of natural trails were obtained by allowing workers to forage across a bridge, the floor of which was carpeted with a paper substrate (filter paper, Whatman No. 1) which was replaced every 0.5 hr for 1.5–2 hr or as long as foraging lasted. The paper strips with fresh trails were immediately cut into pieces directly into a vial; sufficient redistilled hexane was added to just cover the pieces and extracted for 24 hr at 4° C. Cóntrols were obtained by extracting similar quantities of clean filter paper strips in a similar manner. Gland extracts were prepared by dissecting 200 sternal glands of major workers, or 50 sternal glands of swarming allates which were extracted with 500 μl of redistilled hexane for 24 hr at 4° C. Whole-body extracts were obtained by extracting alates with a minimum of redistilled hexane (just sufficient to cover the bodies) for 24 hr at 4° C. Extracts were concentrated to 100 μl before gas chromatographic (GC) analysis. Trail extracts were assayed for trail-following activity using the standard technique of Leuthold and Luscher (1974). A soldier extract was also obtained for comparative purposes from 50 major soldiers (whole bodies) in 1 ml of redistilled hexane for 24 hr at 4° C, followed by concentration to 100 μl. In the case of larger extracts of female alate whole bodies, greater than 100 individuals, the extracts were passed through a short column of silica gel (silica gel 60, 70–270 mesh, Machery-Nagel, Duren, W. Germany) eluting with hexane to remove polar lipid materials which interfered with the reduction in volume of these extracts.

Gas chromatography (GC) was performed on a Packard model 428 chromatograph equipped with flame ionization detectors (Packard-Becker B.V. Delft, Netherlands). Columns were all glass with dimensions 3 m × 2 mm ID × 6 mm OD. The following stationary phases were employed: column A, 5% OV-101; column B, 5% OV-17; column C, 5% Carbowax 20 M; and column D, 5% Silar 5CP. All phases were coated on Chromosorb W-HP, 80–100 mesh (Johns-Manville, Denver, Colorado, USA). A 23-m OV-17 glass WCOT capillary column ID 0.5 mm) was also used with a splitless injection system.

For GC of whole, crude extracts, a standard temperature program was employed; Ti = 60° C (3 min), T_7 = 5° C/min, Tf = 260° C. Preparative GC was carried out using an all-glass splitter as described by Baker et al. (1976), modified in shape to fit the Packard model 428 configuration. GC peak area integration was achieved by the use of a Packard model 603 computing integrater.

Combined gas chromatography–mass spectrometry (GC-MS) was performed on a Finnigan Instrument 1015D GC-MS fitted with a 15-m OV-101 glass WCOT capillary column (ID 0.5 mm) directly coupled to the ion source via a platinum capillary interface. A splitless injection system was employed. The ion source was operated at 70 eV energy.

Fourier transform nuclear magnetic resonance (FT-NMR) spectra were obtained on either a Bruker 200-MHz instrument or a Nicolet 300-MHz instrument (NMC-300).

Microscale chemical reactions were performed in the following manner. Silylation was carried out either directly by on-column injection of the sample into the GC (1 μl) followed by injection of the silylating agent [bistrimethyl silylacetamide (BSA), or trimethylsilylimidazole (TSIM, 0.1 μl)] 15 sec later, or by the addition of the reagent (1 μl) to the sample (10 μl) in a Kontes microvial. Bromination was carried out in a 10-μl syringe by drawing the sample (2 μl) into the syringe followed by a 1% aqueous bromine solution (2 μl). The contents were mixed by barrel action and allowed to react (1–10 min in the dark) when the aqueous layer was expelled together with some of the sample to leave 1 μl in the syringe which was injected into the GC in the normal fashion. Sodium borohydride reductions were performed in a similar manner in a 10-μl syringe using an aqueous, ethanolic solution of sodium borohydride. Hydrogenations were performed on 100 μl of ethanolic sample solutions stirred in a microvial with an atmosphere of hydrogen maintained in the vial. Platinum dioxide was used as the catalyst. Microozonolysis was carried out on 100-μl samples (in carbon disulfide solution) at Dry Ice temperatures. Ozone was generated in a 10-ml chamber with an inlet and outlet consisting of $\frac{1}{16}$-in. stainless steel tubing to allow a slow stream of oxygen to pass through the chamber. A discharge between two electrodes (4 kV) situated in the chamber was employed to produce the ozone which was detected with starch–potassium iodide indicator paper moistened with dilute hydrochloric acid. Work-up was achieved by addition of a trace of triphenylphosphine.

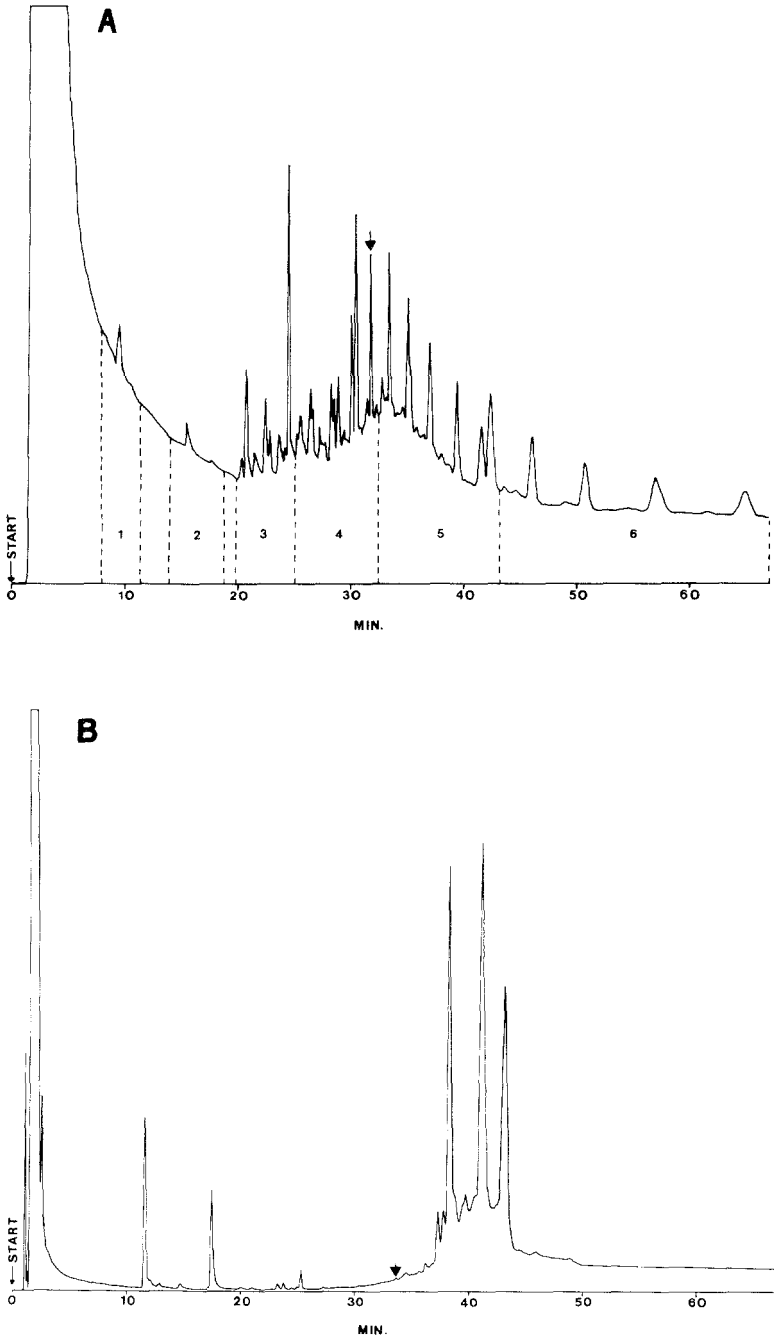
RESULTS

Figure 1A shows the temperature programed gas chromatogram of *T. bettonianus* worker trails collected on filter paper substrate and extracted with hexane. These crude extracts were active in the bioassay (Table 1). Six

gas chromatographic fractions were obtained (indicated by the numbered sections on chromatogram 1A) and subjected to the bioassay. Fraction 4 and to a lesser extent fractions 3 and 5 showed significant activity at high dilution (Table 1). Fraction 4, when further investigated on column B, contained seven significant components (Figure 2). Components 2–5 appeared to have the retention times of the normal hydrocarbons heptadecane through to eicosane (C-17 to C-20). Component 5, however, was removed from the chromatogram by reaction with bromine water. This reaction also resulted in the loss of activity of the fraction in the bioassay. Components 6 and 7 appeared to contain carbonyl functions, since they reacted with sodium borohydride. The activity of the fraction was little affected, however (components 6 and 7 had shorter retention times than component 5 on column A). These results indicated that component 5 was of importance in eliciting trail-following responses from workers. However, further fractionation of this fraction (4) was not practical without extremely time-consuming and laborious collection of natural trails.

Freshly swarmed female alates, on the other hand, are known to produce substantial quantities of trail-active compounds in the sternal gland (Oloo and Leuthold, 1980). A GC comparison was made between male and female alates with both whole-body extracts and with dissected sternal gland extracts. The chromatograms in Figures 1C and 1D show sternal gland extracts of the female and male, respectively, while 1E and 1F show whole-body extracts of female and male, respectively. Only in the female extracts does the single component indicated by the arrow appear in large quantities. This component is much reduced in the male. Again, six preparative GC fractions were obtained from the female alate extracts (sections 1–6, Figure 1C). Bioassay indicated fraction 4 to be highly active, while fraction 5 also elicited some activity (Table 2). Chromatographically, this single component in the alate fraction 4 was found to be identical to component 5 in worker trail extract fraction 4. Alate fraction 4 was further investigated by spectroscopic

FIG. 1. Temperature programed gas chromatograms of *Trinervitermes bettonianus* castes: 5% OV-101, 60° C (isothermal for 3 min) programed to 260° C at 5° C/min. (A) Natural trails of major workers laid on filter paper substrate; (B) major soldiers; (C) dissected sternal glands of female alates; (D) dissected sternal glands of male alates; (E) whole bodies of female alates; (F) whole bodies of male alates (the arrows indicate the position of cembrene-A). The position of cembrene-A in the chromatograms was always checked by coinjection of the isolated material. While the whole body extract of female appears to contain a greater proportion of active compound than does the gland extract, the former was filtered through silica gel (the gland extract was not). It is possible that some of the components were sufficiently polar as to be incompletely eluted from the silica with hexane.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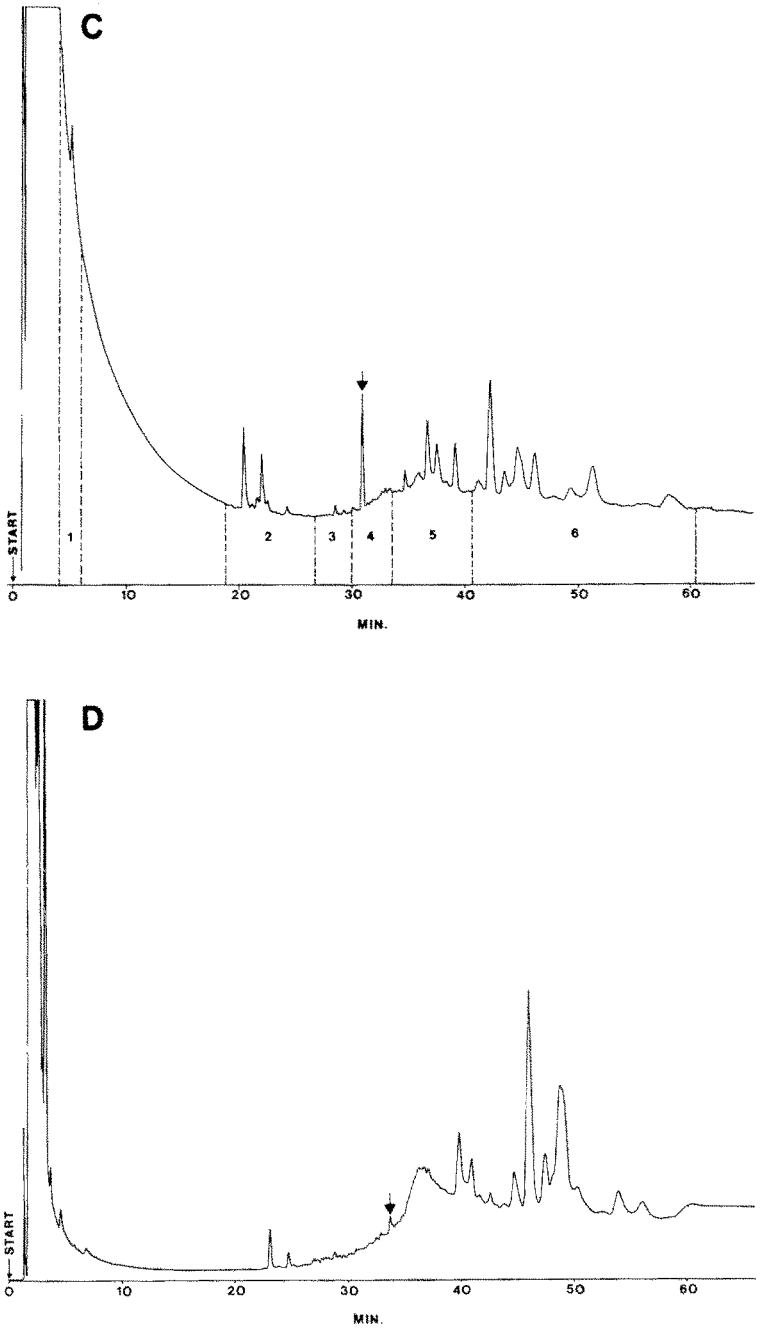

FIG. 1. Continued

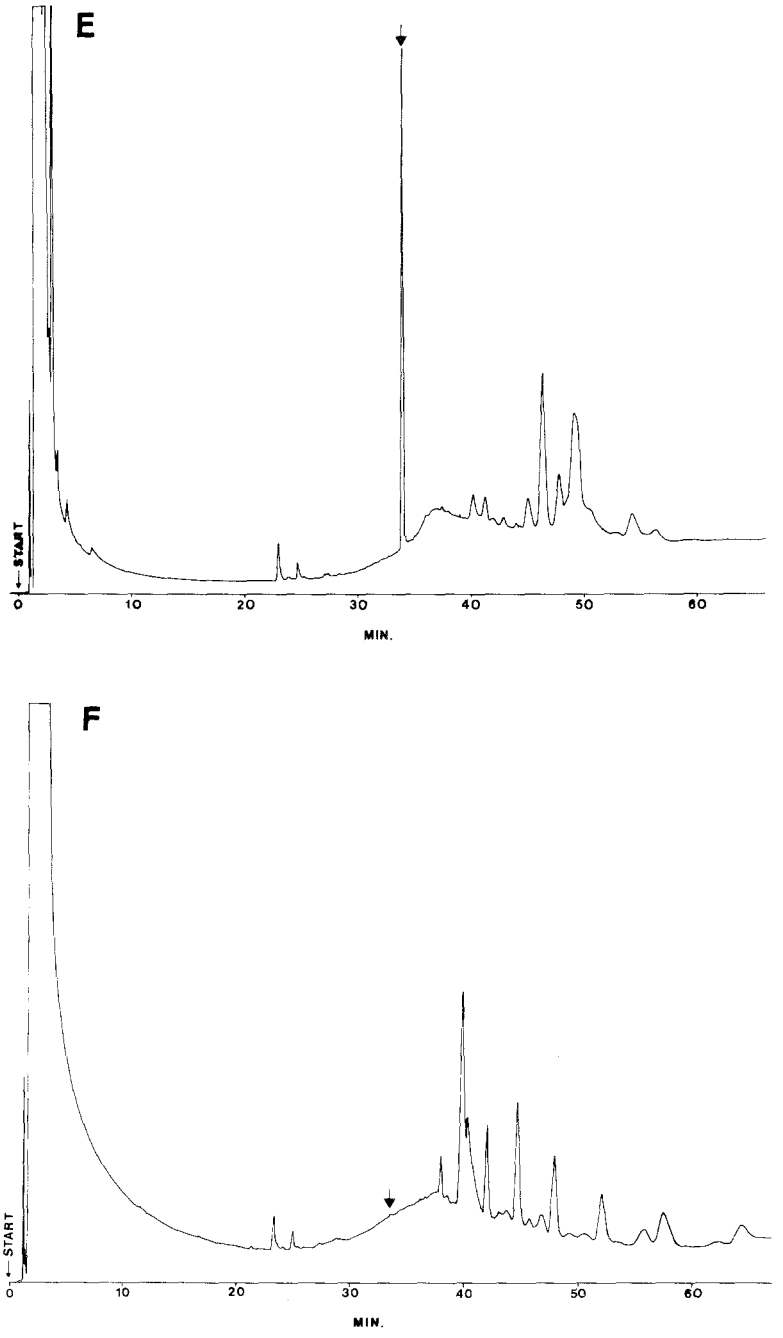

FIG. 1. Continued

Table 1. Worker Foraging Trails of *Trinervitermes bettonianus*: Trail-Following Activity of Whole Extracts and Fractions in a "Figure 8" Bioassay

Test material	Bioassay score[a]	Activity[b]
Extract	19/20	+ !
	20/20	+ !
	18/20	+ !
Fraction 1	2/20	—
	10/20	—
Fraction 2	6/20	—
	12/20	—
Fraction 3	11/20	—
	16/20	+ $
Fraction 4	16/20	+ $
	20/20	+ !
	20/20	+ !
Fraction 5	10/20	—
	16/20	+ $
Fraction 6	4/20	—
	14/20	—

[a] In the "figure 8" bioassay 2.5 μl of test solution are applied along one 5-cm S-shaped line on a paper substrate while solvent only is applied on a second 5-cm S-shaped line such that the two lines form a figure 8. A worker following the figure must make two choices between test material and solvent. Ten workers are used with 10 fresh trails for each concentration which results in 20 choices; 15 or more choices out of 20 for the test material indicates a significant preference ($P < 0.05$) (Oloo and Leuthold, 1979; Leuthold and Lüscher, 1974).

[b] + indicates active; − indicates inactive; !, $P < 0.01$; $, $P < 0.05$.

and microchemical techniques (ca. 2 mg were available from whole-body female alate extracts).

The GC retention times of the alate fraction 4 component on columns A and B were identical to that of *n*-eicosane ($C_{20}H_{42}$), while on column D the component had a longer retention time than *n*-eicosane. Indeed, analysis on column D indicated that the fraction contained *n*-eicosane to the extent of 5%. The eicosane was confirmed by its retention time and its mass spectrum which was identical to that of an authentic standard. Evidence that the major component contained double bonds arose from microscale bromination which caused the removal of the component from the GC analysis while the 5% eicosane peak was unaffected. The mass spectrum of the trail compound (capillary GC-MS) possessed a molecular ion at m/z 272 (7%), consistent with the hydrocarbon formula $C_{20}H_{32}$ having five units of unsaturation. Major fragment ions occurred at m/z 257 (10%, M − 15), 229 (3%), 189 (7%), 175 (6%), 161 (10%), 147 (13%), 134 (12%), 133 (17%), 121 (37%), 107 (41%), 93 (68%), 81 (57%), 79 (38%), 68 (100%), 67 (63%), 55 (27%), indicative of a terpenoid structure.

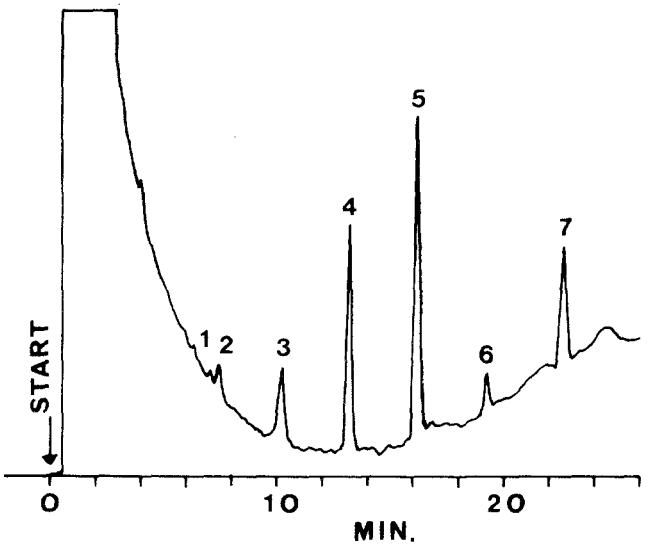

FIG. 2. Gas chromatogram of preparative fraction 4 from worker trail extract: Column 5% OV-17, 3 m × 2 mm ID. Temperature program: 180°C (isothermal for 5 min) to 220°C at 5°C/min.

TABLE 2. ALATE FEMALE EXTRACTS OF *Trinervitermes bettonianus:* TRAIL-FOLLOWING ACTIVITY OF WHOLE FEMALE EXTRACTS AND FRACTIONS 4 AND 5 IN A "FIGURE 8" BIOASSAY

Test material	Bioassay score[a]	Activity[b]
Whole female extract		
Undiluted[c]	16/20	+ $
Diluted extract	19/20	+ !
(1/1000)	18/20	+ !
Diluted extract	16/20	+ $
(1/2000)		
Diluted extract	15/20	+ $
(1/5000)	13/20	—
Fraction 4	18/20	+ !
	19/20	+ !
Diluted	16/20	+ $
(1/2000)	15/20	+ $
Fraction 5	15/20	+ $
	14/20	—

[a] As for Table 1.
[b] As for Table 1.
[c] Some undiluted extracts were actually repellent to workers.

The number of double bonds in the structure was determined by microscale hydrogenation in ethanol over platinum dioxide which produced a mixture of epimers (presumably) not separable under the GC conditions employed. The mass spectrum of the products possessed a molecular ion at m/z 280 (1%), confirming the presence of four double bonds and one ring in the structure. Other significant fragment ions were at m/z 237 (14%, M − 43), 236 (25%), and 55 (100%). The ions at m/z 237 and 236 suggested a macrocyclic ring which was confirmed by comparing the hydrogenation products with those of hydrogenated cembrene (II). The mass spectrum of the hydrogenated cembrene closely resembled that of the hydrogenated trail compound (m/z M + 280 (1%), 237 (16%), 236 (27%), 55 (100%)). The two hydrogenation products also possessed the same GC retention times (columns A and B).

Double bond positions were determined by microozonolysis which led to the detection of 2 mol of 4-oxopentanal (III) and 1 mol of the diketoaldehyde (IV) per mole of trail compound. These were identified by comparison with standards produced by the ozonolysis of 6-methyl-5-hepten-2-one (V) and limonene (VI), respectively. GC analysis of the ozonolysis products was performed on column C (110° C held isothermally for 2 min, then programed to 190° C at 4° C/min). *n*-Dodecane was used as an internal standard for the quantitative measurements. Formaldehyde was detected separately in a qualitative fashion by spotting the ozonolysis solution on a thin-layer plate (silica gel) followed immediately by spotting dilute 2,4-dinitrophenylhydrazine reagent. The hydrazone formed in situ was eluted in toluene–ethyl acetate (95:5). A hydrazone spot from the ozonolysis solution was observed with the same R_f value as that of the hydrazone of an authentic formaldehyde sample.

The above data suggest that the trail compound was cembrene-A (I). The 200-MHz proton NMR spectrum further confirmed this and is shown in Figure 3 (the two major regions of the spectrum are shown on an expanded scale (×5) in Figure 3B). The isopropenyl unit was confirmed by the presence of a vinyl methyl resonance at δ 1.66 (3H) as a broad singlet together with the corresponding vinyl protons at δ 4.69 (2H) constituting an AB pair. Irradiation of the vinyl protons at δ 4.69 caused the methyl resonance at δ 1.66 to collapse to a sharp singlet. Three other vinyl protons were observed in the region δ 4.90–5.25, each coupled to adjacent methylene groups (J ca. 6.5 Hz) and to respective vinyl methyl groups which were observed at δ 1.56 (ca. 9H, broad singlet). The methylene region δ 1.70–2.30 accounted for approximately 11 protons while the broad resonance at δ 1.59 accounted for three protons which may combine the C-14 methylene and the C-1 methine protons. The spectrum compares well with published NMR data on cembrene-A (Birch et al., 1972; Patil et al., 1973; Kodama et al., 1975; Wiemer et al., 1979).

Cembrene-A has been isolated from several natural sources, including

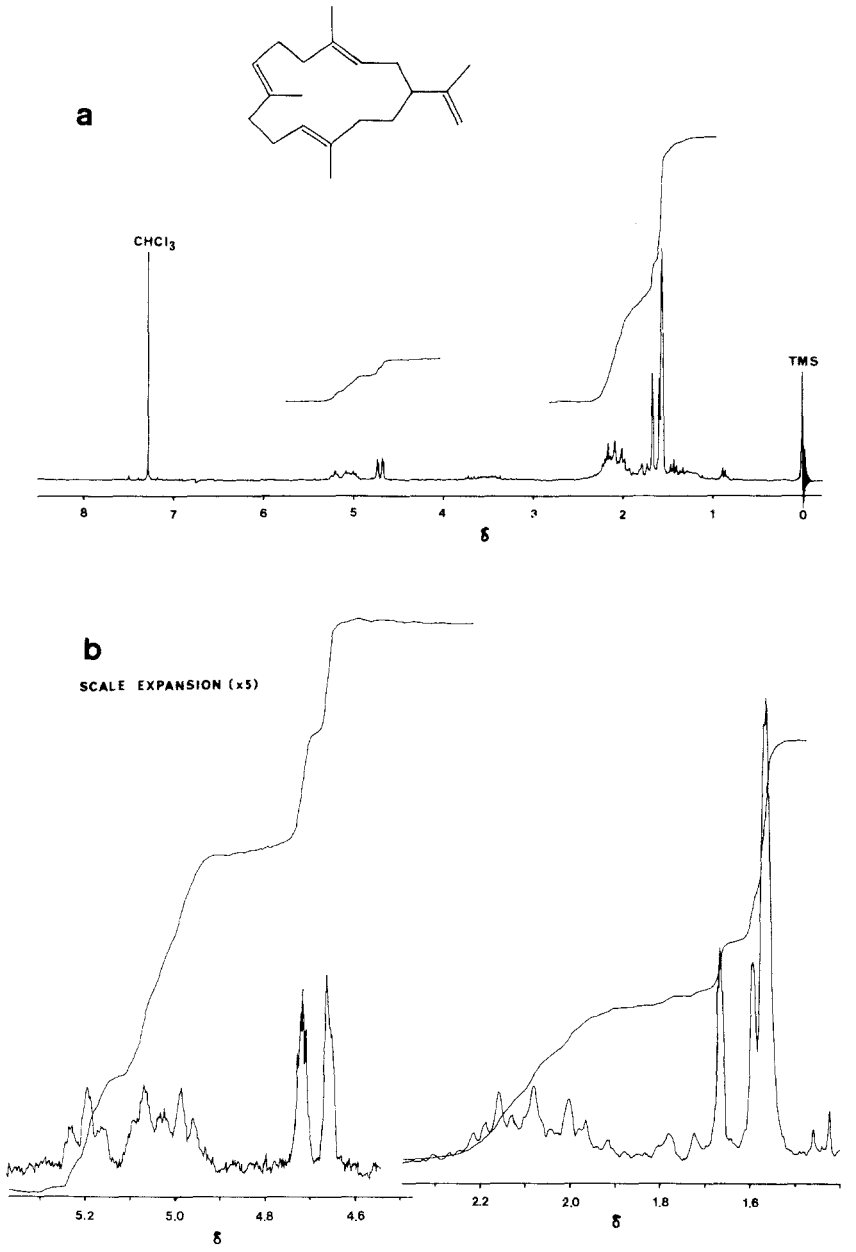

FIG. 3. 200-MHz proton NMR spectrum of *Trinervitermes bettonianus* trail pheromone (cembrene-A). (a) Full spectrum, in $CDCl_3$ using tetramethylsilane as the reference; (b) expanded (×5) regions of interest: δ 1.4–2.3 and 4.6–5.3.

the defense secretion of soldiers of the East African termite *Cubitermes umbratus*. The major component (50%) of the defense secretion is cembrene-A (Prestwich et al., 1978; Wiemer et al., 1979). A sample was isolated from the soldiers of *C. umbratus* by preparative GC and compared to the trail compound isolated from *T. bettonianus*. The two compounds from the different sources were identical by GC on four packed columns and one capillary column. Mass and NMR spectra were also identical, and the sample isolated from *C. umbratus* was active in the bioassay.

Quantitation of cembrene-A in workers and alates was achieved by extracting dissected sternal glands and comparing GC peak areas with known concentrations of *n*-eicosane (initially the FID response of *n*-eicosane was compared to that of cembrene-A, the latter response being ca. 8% less than that of *n*-eicosane; calculations of the gland quantities were adjusted accordingly). The results are summarized in Table 3.

The ratio of the gland content in the three castes was: worker–male–female, 1:62:1090, which is of the same order of magnitude as the ratio of trail activity of the three castes, 1:70:1200, as reported by Leuthold and Lüscher (1974). Table 4 shows the activity of various concentrations of cembrene-A in the "figure 8" bioassay and the "figure Y" bioassay (Oloo and Leuthold, 1979, Leuthold and Lüscher, 1974). In the figure 8 bioassay (where the test solution is applied at the rate of 2.5 μl/5 cm of trail), the 1 ng/μl (0.5 ng/cm) test solution showed activity while the 100 pg/μl (50 pg/cm) test solution did not (the threshold probably lies between the two). In the "figure Y" bioassay (where the test solution is applied at the rate of 2.5 μl/2.5 cm of trail), activity has been observed with 1 pg/μl (1 pg/cm) test solutions.

Three minor components from fractions of the worker natural trail extracts have also been identified from fractions 1 and 2 (Figure 1A). Fraction 1 consisted of two components in the ratio 9:1. The major component was

Table 3. Approximate Quantities of Cembrene-A in Three Castes of *Trinervitermes bettonianus*: Major Workers, Male Alates, and Female Alates

	Major workers	Male alates	Female alates
Cembrene-A per termite (ng)	11	680	12,000
(number of determinations)	(13)	(8)	(6)
Approximate ratio of gland content	1	62	1090

TABLE 4. ACTIVITY OF CEMBRENE-A AT DECREASING CONCENTRATIONS: ACTIVITY OF SIX CONCENTRATIONS IN "FIGURE 8" BIOASSAY AND THREE CONCENTRATIONS IN "FIGURE Y" BIOASSAY

	Figure 8 assay		Figure "Y" assay[c]	
Concentration	Score[a]	Activity[b]	Score[a]	Activity[b]
100 ng/μl	16/20	+ $		
	18/20	+ !		
10 ng/μl	15/20	+ $		
1 ng/μl	16/20	+ $		
	18/20	+ !		
100 pg/μl	11/20	—	18/20	+ !
			17/20	+ !
10 pg/μl	5/20	—	18/20	+ !
			16/20	+ $
1 pg/μl	3/20	—	15/20	+ $
			16/20	+ $

[a] As for Table 1.
[b] As for table 1.
[c] In the "Y" bioassay, the test solutions were applied at the rate of 2.5 μl/2.5 cm of trail as opposed to 2.5 μl/5 cm of trail in the figure 8 bioassay.

identified as the monoterpene myrcene from its retention data and its mass spectrum, which were identical to the data from an authentic sample. The minor component was considered to be limonene from its retention data, but it was insufficient for a useful mass spectrum.

The single component occurring in fraction 2 was identified as 2-decanol by its retention data in comparison to an authentic sample. The component was easily silylated by both BSA and TSIM and the silyl derivative compared with the silyl derivative of the authentic sample. The occurrence of these three compounds is in keeping with their presence in the soldier defense secretion of *T. bettonianus* (Prestwich and Chen, 1981).

As indicated earlier, fraction 5 from both the worker trail extract and the female alate extract elicit a degree of trail activity. However, structures of the components of these fractions have not been elucidated, although preliminary GC-MS data indicated the presence of terpenoid molecules.

T. gratiosus trails were briefly examined by collection of major worker natural trails on filter paper. The presence of cembrene-A was indicated by chromatographic coinjection with the material isolated from *T. bettonianus* (Oloo and McDowell, 1982). Other closely related terpenoids are probably present.

DISCUSSION

Our evidence indicates that the principal component responsible for trail following in *T. bettonianus* is the diterpene hydrocarbon cembrene-A (Figure 4, I), the same compound, also called nasutene, reported by Birch et al. (1972) to be the trail pheromone of the Australian nasute termite *Nasutitermes exitiosus* (Hill). Whereas Birch and colleagues extracted the compound from workers only, we have isolated the compound from freshly swarmed female imagoes and established its presence in the workers by chromatographic comparisons. The compound is active at high dilution, and as little as 0.5 ng/cm of trail is effective as determined in the "figure 8" bioassay and, in some tests with the "figure Y" bioassay, as little as 1 pg/cm elicited a response. Birch et al. (1972) indicated that the activity of cembrene-A in trail bioassays with *N. exitiosus* fell within the range 10^{-5} to 10^{-8} g/ml (10 ng/μl to 10 pg/μl). These investigators do not give a figure for the amount of solution applied per unit distance.

Leuthold and Lüscher (1974) indicated that the female imago sternal glands possess much greater trail activity than the worker sternal glands (in the ratio 1200:1) which was suggested to be due either to (1) the dispropor-

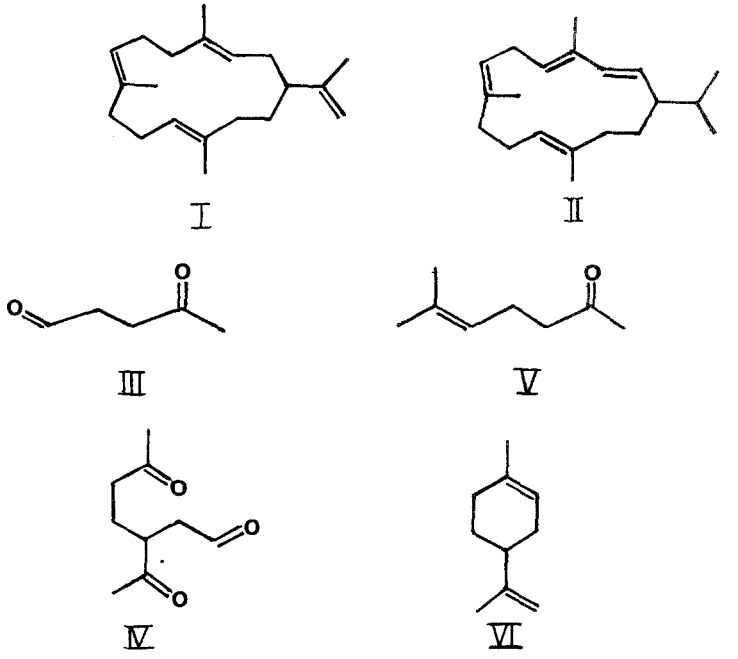

FIG. 4.

tionate growth of the functional parts of the alate gland (in comparison to the worker gland) therefore producing larger quantities of the pheromone, or (2) the presence of a different, more potent compound. Comparative fine structural studies of the sternal glands of various castes of *T. bettonianus* by Quennedy and Leuthold (1978) indicated no notable differences in the sternal gland structure (other than size). Furthermore, when worker extract was applied in sufficient quantity, its attractiveness to male imagoes was similar to that of the female imago extract, suggesting no real difference in the secretion between the two castes. Our discovery of cembrene-A in both the workers and in the female alates with much larger quantities in the alates, approximately 12 μg per alate, would seem to bear out this hypothesis. Furthermore, the relative quantity of cembrene-A in female alates and major workers approximates to 12 μg and 11 ng, respectively (ratio approximately 1100:1) and parallels the relative potential trail strength of the two castes (1200:1) (Leuthold and Lüscher, 1974).

Leuthold (1977) suggested three distinct phases of postflight courtship behavior in *T. bettonianus:* (1) rambling with dragging of the abdomen and laying a strong, continuous pheromone trail; (2) slow walking with trail-laying interrupted regularly for seconds by calling, the abdomen raised exposing the sternal and tergal glands; and (3) continuous calling for several minutes at a time. It seems likely that cembrene-A is involved in the first two stages of behavior described above, but it is not known if this compound is significant in the calling behavior, although Leuthold indicated that sternal gland volatiles were important at a distance of 3 cm or less, while at greater distances (up to 20 cm) tergal gland volatiles were implicated. We have not yet extended this work to study the behavioral responses of alates to cembrene-A. Leuthold and Lüscher (1974) also found that male imagoes were capable of laying trails (70:1 relative strength to worker trails). Our analyses showed much smaller quantities of cembrene-A in the male imagoes, approximately 680 ng per male, which correlates with their potential trail strength.

Preparative GC fraction 5 from both the worker trail extracts and the female alate extracts elicited some activity in the trail bioassay. Preliminary GC-MS data of the components of these fractions indicated the presence of terpenoid components. However, much more work is required to identify the active compounds. It has been suggested that secondary components play a role in trail following. Moore (1974) indicated that nasutene (cembrene-A) elicited trail following in several Australian species of nasute termite and suggested that secondary components played a part in species specificity. We have also found that the E. African species *T. gratiosus* possesses cembrene-A in the worker trail extracts (by GC comparisons). Although it is not known if cembrene-A is the principal pheromone of this species, its presence is in keeping with the fact that *T. bettonianus* and *T. gratiosus* follow each other's

trails with little or no discrimination in the laboratory situation (Oloo and McDowell, 1982). However, it may require much more refined bioassay techniques to detect subtle differences in the trails of closely related species.

Soldiers of *T. bettonianus* do not take part in active trail laying (Leuthold and Lüscher, 1974), unlike the soldiers of *Nasutitermes costalis* which actively lays trails and are responsible for the organization of foraging in this species (Traniello, 1981). We were unable to detect significant quantities of cembrene-A in the soldiers of *T. bettonianus,* which is in keeping with their not taking part in trail laying.

Kato et al. (1980) have shown that for *N. exitiosus,* optical isomerism in cembrene-A is of much less importance to trail activity in the latter species than stereoisomerism in the molecule. The optical activity of cembrene-A from *T. bettonianus* has not been determined, and it is unknown whether or not this is important in *T. bettonianus.*

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HOUSE FLY OVIPOSITION INHIBITION BY LARVAE OF *Hermetia illucens,* THE BLACK SOLDIER FLY[1,2,3]

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Abstract—Wild populations of house flies were inhibited from ovipositing into poultry manure containing larvae of the black soldier fly, *Hermetia illucens* (L.). A laboratory strain of house fly responded differently, readily ovipositing into manure with lower densities of soldier fly larvae, but avoiding the higher densities tested. The amount of time *H. illucens* larvae occupy the manure prior to an oviposition test influences ovipositional responses of house flies. Manure conditioned by *H. illucens* larvae for 4–5 days did not significantly inhibit house fly oviposition. We suggest that some type of interspecific chemical communication (allomone) is present.

Key Words—Diptera, Stratiomyidae, *Hermetia illucens,* black soldier fly, Muscidae, *Musca domestica,* house fly, oviposition inhibition, oviposition-deterring allomone, larval inhibition.

INTRODUCTION

Hermetia illucens (L.) (Diptera: Stratiomyidae), the black soldier fly, is recognized as significantly controlling populations of the house fly, *Musca domestica* L., in caged layer poultry operations (Furman et al., 1959; Gonzalez et al., 1963; Tingle et al., 1975; Axtell and Edwards, 1970; Sheppard, 1983). The exact mechanisms involved in this interaction between soldier fly and house fly populations are not known. Interspecific competition for food, space, or other requirements has been suggested (Furman et al., 1959; Gonzalez et al., 1963; Tingle et al., 1975). Kilpatrick and Schoof (1959)

[1]Diptera: Stratiomyidae.
[2]Portion of an MS thesis by S.W.B.
[3]Research partially supported by State and Hatch funds.

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theorized that soldier fly larvae modify the breeding medium, rendering it unsuitable for house fly development and survival.

We suggest that, in addition to the competition and modification theories, the possibility of oviposition inhibition be considered. Oviposition-deterring pheromones are recognized as an intraspecific population regulation mechansim in tephritid flies, the Azuki bean weevil, *Callosobruchus chinensis* (L.), the cabbage looper, *Trichoplusia ni* (Hubner), and the European apple sawfly, *Hoplocampa testudinea* (Klug) (Prokopy, 1972; Oshima et al., 1973; Renwick and Radke, 1980; Prokopy, 1981). Under crowded conditions, several species of mosquitoes apparently produce chemical factors that are highly toxic to larvae of both their own and other species (Ikeshoji and Mulla, 1970).

Allomonal effects on competing species have also been demonstrated in scolytids. *Dendroctonus brevicomis* LeConte adults secrete an epideictic pheromone, verbenone, which inhibits tree colonization of the competing bark beetle, *Ips paraconfusus* Lanier (Byers and Wood, 1980). Also, pheromone emitted by *D. frontalis* Zimmerman adults inhibits nearby landing of *Ips grandicollis* Eichhoff adults (Birch et al., 1980).

This study was conducted to determine if *H. illucens* larvae inhibit house fly oviposition. We propose that an oviposition-deterring allomone in soldier fly larvae may exist as an interspecific population regulating mechanism of house fly populations in poultry manure.

METHODS AND MATERIALS

All studies were conducted in a laboratory under ambient temperature and humidity conditions of 25 ± 3°C and 60 ± 10% relative humidity at the Coastal Plain Experiment Station (CPES), Tifton, Georgia, during the summers of 1981 and 1982. Two strains of house flies were utilized. One colony, designated as "wild" was established from adults collected at a caged layer house in Cook County, Georgia. The "lab" colony was started from pupae obtained from Carolina Biological Supply and reared over several generations on CSMA larval medium (D. Fendley, personal communication).[4] Soldier fly larvae were collected from a naturally occurring field population at a 100-bird experimental caged layer house maintained at the CPES.

In 1981, oviposition preference tests were conducted in a 0.75 m^3 screened cage with ca. 1500–2000 wild adults. Female house flies were given a choice of ovipositing into three 113-g capacity styrofoam cups containing 75 g insect-free layer manure of ca. 81% moisture, and 0, 10, or 100 third–fifth

[4]Conversation with Daniel M. Fendley, April 15, 1981, University of Georgia, Athens, Georgia.

instar soldier fly larvae (average weight = 0.096 g, length = 15–20 mm), which had been placed in the cups 30 min prior to the test. These and higher levels of soldier fly larvae are commonly seen in the field. Cups containing soldier fly larvae were exposed to ovipositing house flies for 24 hr after which all soldier fly larvae were carefully removed. Cups were held for 4–5 days in the laboratory, and total number of house fly pupae and developing larvae was recorded. Six trials were conducted, with cups being rotated such that each treatment occupied each location twice.

In 1982 both lab and wild flies were utilized in separate oviposition preference studies. About 500–1000 adults of each fly strain were maintained in separate 33 cm^3 cages. Female flies were given a choice of ovipositing in 137.8-g capacity cups with 75 g of insect-free poultry manure and either 0, 10, or 100 fifth instar (length = 18–20 mm) soldier fly larvae. Soldier fly larvae were placed in the cups 0.5, 2.5, or 24 hr before being placed into house fly cages. Cups were left exposed to house fly oviposition for 5 hr. Eggs laid in each cup were then counted using a fine, moistened 000 artist's brush and a dissecting scope. This technique enabled us to (1) obtain immediate results, (2) eliminate removal of soldier fly larvae, thus disturbing newly laid house fly eggs, and (3) eliminate the possibility of oviposition by adult flies that may have been loose in the laboratory.

A difference in response between the two fly strains in the above experiments led us to conduct a comparison test. The tests were set up as described above, with cages of wild flies and laboratory flies being tested together on the same day. Soldier fly larvae were placed in the cups 1 and 24 hr prior to offering them to ovipositing female house flies. Six trials for each time period with both strains were conducted.

We also tested ovipositional response of laboratory and wild house flies to manure which had been occupied by 0, 10, or 100 soldier fly larvae for 4–5 days. The larvae were removed from the cup and the manure was homogenized prior to offering it to females for a 3-hr trial.

The data were transformed to $\log_{10}(x + 1)$ and subjected to analysis of variance ($P = 0.05$) with mean separation by Duncan's multiple-range test.

RESULTS

Experiments conducted in 1981 demonstrated that significantly fewer house fly eggs were laid in cups with 10 and 100 soldier fly larvae (average 8 and 11 eggs, respectively) than cups without soldier fly larvae (average = 184 eggs, $P < 0.05$). We repeated these experiments in 1982, initially using a laboratory strain of flies and letting the soldier fly larvae occupy the manure for 30 min prior to the test. Results (Table 1) indicated that there was no significant difference in the number of eggs laid between the various density

TABLE 1. EFFECT OF THREE DENSITY LEVELS AND THREE TIME INTERVALS OF *H. illucens* LARVAE ON OVIPOSITION OF TWO STRAINS OF HOUSE FLIES IN THE LABORATORY (TIFTON, GA, 1982)

No. *H. illucens* larvae/cup	Hours *H. illucens* in manure					
	0.5		2.5		24	
	Lab	Wild	Lab	Wild	Lab	Wild
	$N = 7$		$N = 11$		$N = 10$	
0	722.1a[a,b]		570.7ab	375.4a	421.0a	73.4a
10	233.7a		417.0a	169.3b	146.9b	2.0b
100	153.4a		142.6b	0 c	0 c	0 c

[a]Values are mean number of house fly eggs laid/cup.
[b]Means within columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test.

levels of soldier fly larvae even though 67% and 79% fewer eggs were laid in cups with 10 and 100 larvae, respectively, than cups without larvae. In separate tests we then utilized lab and field flies and times of 2.5 and 24 hr to see if our results were being influenced by the strain of fly and/or the amount of time soldier fly larvae were allowed to occupy the manure prior to a test. These studies resulted in significant interaction between the strain of house fly, the time the soldier fly larvae were left in the manure prior to placement in the cage, and the number of soldier fly larvae ($P < 0.0187$). Number of larvae had the greatest influence on house fly oviposition ($P < 0.001$). Strain of fly and time also affected house fly oviposition significantly ($P < 0.0160$ and $P < 0.002$, respectively).

Results (Table 1) indicated a difference in response between laboratory and wild flies. At 2.5 hr lab flies and wild flies laid 26% and 55% fewer eggs, respectively, in cups with 10 larvae than cups without larvae. Wild flies were strongly inhibited from laying in cups with 100 larvae, whereas lab flies laid an average of 142.6 eggs/cup. At 24 hr, both lab and wild flies were inhibited from ovipositing into cups with 100 larvae. Although both lab and wild strains laid significantly more eggs in cups without larvae than cups with 10 larvae, the lab flies laid 65% fewer eggs in cups with 10 larvae than cups without larvae, while wild flies laid 97% fewer eggs.

These results between wild and lab flies led us to conduct a comparison test between the two strains. Table 2 summarizes these results. They concur with our other results (Table 1), indicating a difference in response to soldier fly larvae due to both strain of fly and time soldier fly larvae were allowed to

TABLE 2. EFFECT OF THREE DENISTY LEVELS AND TWO TIME PERIODS OF *H. illucens* LARVAE ON AVERAGE NUMBER OF EGGS LAID/CUP OF TWO STRAINS OF HOUSE FLIES IN THE LABORATORY (TIFTON, GA, 1982)

No. *H. illucens* larvae/cup	Hours *H. illucens* in manure			
	1		24	
	Lab	Wild	Lab	Wild
0	74.2a[a,b]	345a	434.8a	27.8a
10	139.3a	32b	95.3ab	.3b
100	2.3a	0b	0 b	0 b

[a] $N = 6$.
[b] Means within columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple-range test.

occupy the manure. One hundred soldier fly larvae per cup strongly inhibited house fly oviposition regardless of strain of fly or time. Wild flies were strongly inhibited from ovipositing into cups with 10 soldier fly larvae, but lab flies were not.

Medium Conditioning by Soldier Fly Larvae. There was no significant difference among treatments ($P > 0.3676$) or strain of fly ($P > 0.6687$) in tests examining the behavior of wild and lab flies to manure conditioned by soldier fly larvae for 4–5 days. An average of 616 house fly eggs (wild and lab) were laid in cups without soldier fly larvae, compared with 347 and 205 eggs in cups which had held 10 and 100 soldier fly larvae, respectively.

DISCUSSION

Results of this study indicate that wild populations of house flies strongly avoided ovipositing into manure containing soldier fly larvae. Laboratory flies behaved differently, readily ovipositing into manure with lower densities of soldier fly larvae. A possible explanation may be that selection has given rise to a population of laboratory flies with different behavioral traits than wild fly populations, perhaps less sensitive to chemical or physical communication with an interspecific competitor such as *H. illucens*.

We also found that manure conditioned by soldier fly larvae for 4–5 days did not significantly inhibit house fly oviposition. Apparently soldier fly larvae are not releasing long-lasting inhibitory chemical factors into the manure. The fact that we observed strong oviposition inhibition by wild populations of house flies to both low and high numbers of soldier fly larvae leads us to

believe that some type of interspecific chemical communication (allomone) is present. However, physical factors such as the activity of soldier fly larvae on the surface of the manure, repelling house flies, should not be overlooked. It is possible that bóth chemical and physical factors are acting together to repel ovipositing house flies. Intraspecific communication is recognized as a population regulation mechanism allowing for an optimal density range of individuals on a given resource (Prokopy, 1972, 1981; Corbet, 1971), and it is suggested that other cases of chemical communication between interacting species competing for a similar resource in the same habitat may exist (Prokopy, 1981).

Many muscoid Diptera exhibit contagious oviposition behavior as a mechanism for optimizing larval density (Bryant and Hall, 1975). Larval condition of the medium then acts to deter female house flies from further oviposition (Bryant and Hall, 1975). It is possible the differences in house fly oviposition in cups with and without soldier fly larvae was influenced by this intraspecific oviposition behavior. However, in several of our tests there was no significant difference between any of the treatments. Therefore, we believe our studies do demonstrate interspecific interactions.

House fly adults are known to communicate chemically with their own larvae, being attracted to general metabolites or specific pheromones of excretion products of the larvae in the medium (Bryant and Hall, 1975). Therefore, it is possible that they can also communicate chemically with a competing species to avoid habitats that are already optimally dense and selectively disadvantageous. This may explain why low densities of soldier fly larvae do not deter house flies from ovipositing as strongly as high densities of soldier fly larvae. Perhaps adult house flies are not receiving the signal that an optimal larval density in the medium exists.

Soldier fly larvae may be inhibiting colonization by other decomposers. Dunn (1916) reports that a cadaver recovered from a jungle in the Canal Zone was found covered with *H. illucens* larvae. No other dipterous species were present. He notes this to be "remarkable," considering the numbers of other carrion feeding fly species found in the region.

Intraspecific communication in *H. illucens* has been demonstrated with the presence of an adult oviposition-stimulating pheromone (Booth and Sheppard, unpublished data). Also, field observations indicate that soldier fly larvae or possibly their excretion products are attractive to ovipositing female soldier flies, which exhibit contagious egg laying behavior.

Further studies are necessary to confirm the existence of an oviposition inhibition chemical utilized by *H. illucens* as an interspecific population regulation mechanism and to examine the behavioral reactions of other Diptera to *H. illucens*. Studies on intraspecific communication between *H. illucens* larvae and adults and their role in regulating larval densities are also needed.

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ATTRACTIVE AND INHIBITORY PHEROMONES PRODUCED IN THE BARK BEETLE, *Dendroctonus brevicomis*,[1] DURING HOST COLONIZATION: Regulation of Inter- and Intraspecific Competition

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Abstract—Quantities of attractive (*exo*-brevicomin and frontalin) and inhibitory (*trans*-verbenol, verbenone, and ipsdienol) pheromones were monitored in both sexes of *Dendroctonus brevicomis* during their colonization of a ponderosa pine. Verbenone was found in males in the greatest amounts at the time of landing, and it declined more rapidly than the other pheromones in either sex. The amounts of frontalin and *exo*-brevicomin in males and females, respectively, increased after initial boring within the host but began to decline after mating. The quantity of *trans*-verbenol in both sexes (females had significantly more) declined more gradually than *exo*-brevicomin, frontalin, and verbenone. Ipsdienol was found only in males during the initial stages of attack when encountering the resin. It is suggested that along with a general decline in all pheromonal components, a sufficient change in the ratio of the attractive pheromones to an inhibitory pheromone, *trans*-verbenol, may play a role in termination of aggregation. *trans*-Verbenol may also function along with verbenone and ipsdienol in limiting the density of attack and thus intraspecific competition. These inhibitory pheromones also appear to cause several competing species of bark beetle to avoid landing in areas infested with *D. brevicomis*, even when their own pheromone is present.

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Key Words—Coleoptera, Scolytidae, Cleridae, *Dendroctonus brevicomis, Enoclerus lecontei, Pinus ponderosa,* bark beetle, *exo*-brevicomin, frontalin, verbenone, *trans*-verbenol, ipsdienol, aggregation, pheromone, competition.

INTRODUCTION

Intraspecific competition is assumed to occur in most species which utilize a limited food resource (Pianka, 1976), such as a "weakened" and beetle-infested host tree. Furthermore, it is always advantageous for an individual to avoid competition (both intra- and interspecific) whenever possible (Pianka, 1976). As expected from ecological theory, reports of intraspecific competition in several bark beetle species in the genera *Ips, Dendroctonus, Scolytus,* and *Tomicus* have shown that brood output per female decreases at higher densities on the bark (Miller and Keen, 1960; Cole, 1962; Reid, 1963; Eidmann and Nuorteva, 1968; Ogibin, 1972; Svihra, 1972; Berryman, 1974; Mayyasi et al., 1976).

The western pine beetle, *Dendroctonus brevicomis* LeConte, begins the attack of its host, ponderosa pine (*Pinus ponderosa* Doug. ex. Laws.), when a female penetrates the bark and excavates a gallery in the phloem. The attractive pheromone component, *exo*-brevicomin, is produced in females and released with the frass, a mixture of fecal pellets and host material (Silverstein et al., 1968). A male attracted to the entrance tunnel soon releases frontalin (Kinzer et al., 1969; Pitman et al., 1969; Browne et al., 1979) which, together with *exo*-brevicomin and a major component of host resin, myrcene, significantly enhances the attraction of both sexes (Wood et al., 1976; Bedard et al., 1980b) to initiate the mass attack (concentration phase). Several compounds in the beetles that inhibit the attraction response have been suggested to play a role in regulating attack density and intraspecific competition (Byers and Wood, 1980; Byers, 1982, 1983a). Verbenone alone (Renwick and Vité, 1970; Bedard et al., 1980a), verbenone plus *trans*-verbenol (Bedard et al., 1980a), and *trans*-verbenol alone (Bedard et al., 1980a; Byers, 1983a) have been shown to inhibit the response of *D. brevicomis* to the attractive components in the field. In a recent report, Byers (1982) found that only males could synthesize (+)-ipsdienol from myrcene vapors and that ipsdienol inhibited the attraction of both sexes to their pheromone components in the field. Emergent females contain *trans*-verbenol and emergent males *trans*-verbenol and verbenone (Renwick, 1967; Vité and Renwick, 1970; Byers, 1983c), but the amounts in beetles during the period of host colonization have not been determined.

D. brevicomis appears to limit intraspecific competition for food and space by regulating the density of attack. Miller and Keen (1960) have

summarized several early reports on attack densities and found them to range from 5.9 to 23.2 per 0.1 m^2 or "always within certain limits." Production and release of attractants by an individual must be synchronized at the population level with a mechanism of regulating denisty (possibly olfactory/pheromonal) to prevent overcrowding and to terminate aggregation. One theory proposed by Renwick and Vité (1970) and McNew (1970) suggested that males may release verbenone during the latter stages of attack which would reduce response to attractive components in order to terminate the aggregation of beetles. However, Byers and Wood (1980) questioned this theory because they found that males contained the largest quantities of verbenone at the beginning of colonization, and as the attractiveness of the infested log decreased over a 5-day period, the amount of verbenone declined along with *exo*-brevicomin and frontalin. They suggested that verbenone does not terminate the mass attack but may regulate the density of attack at close range, while a reduction in *exo*-brevicomin and frontalin caused termination of long-range attraction (Byers and Wood, 1981).

Some of the behavioral chemicals produced by *D. brevicomis* may be used to avoid competition by other bark beetle species that also inhabit ponderosa pine phloem and frequently occur together in the same pine tree (Miller and Keen, 1960). For instance, the attraction of *Ips paraconfusus* Lanier to natural pheromone was shown by Byers and Wood (1980, 1981) to be inhibited by volatiles from *D. brevicomis*-infested logs and also by verbenone. (+)-Ipsdienol produced by males of *D. brevicomis* (Byers, 1982) was shown to inhibit the response of *I. pini* (Say) to natural pheromone (Birch et al., 1980). Therefore, in order to better understand the mechanisms that may regulate attack density, terminate aggregation, and reduce interspecific competition, we wanted to determine the quantitative relationships between the above behavioral chemicals found in both sexes of *D. brevicomis* during their colonization of a pine tree and compare them to landing rates of the beetle on the tree.

METHODS AND MATERIALS

A mass attack of *D. brevicomis* was induced on an apparently healthy ponderosa pine to determine when certain behavioral chemicals were present within each sex during host colonization (August 25–September 27, 1978). The ponderosa pine (50.9 cm diam at 1.5-m height) was located in the Sierra National Forest near Oakhurst, California, at 1000 m elevation in a nearly pure stand of this species. A pulley with climbing rope was attached to a tree limb 13 m above ground so that by means of counterbalancing weights and a sling one could hoist oneself up the trunk to obtain ready access to any sampling area of the tree without damaging it. Flat sticky-traps (15.25 × 15.25

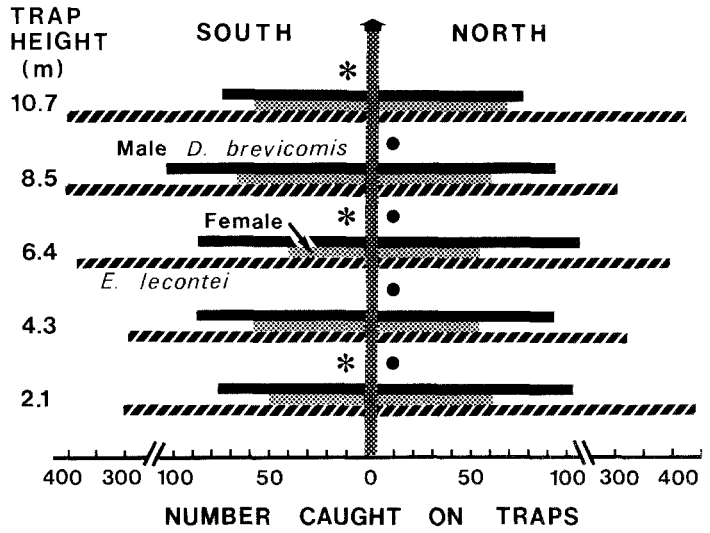

FIG. 1. Number of male and female *Dendroctonus brevicomis* and *Encolerus lecontei* caught on sticky traps at several heights and directions on a ponderosa pine tree from August 25 to September 27, 1978. The tree was baited with synthetic pheromone of *D. brevicomis* (*exo*-brevicomin, frontalin, and myrcene) for one day (August 25) at three heights indicated with an asterisk. *D. brevicomis* were obtained from bark sampled between the traps at heights indicated with darkened circles to determine pheromone content in hindguts.

cm) made from 6.3-mm wire mesh and coated with Stickem Special® were placed on nails 2 cm away from the bark of the tree in north- and south-facing directions at five heights on August 24, 1978 (Figure 1). On the next day three glass salt-shakers were placed on the tree; each one contained three glass tubes filled with neat *exo*-brevicomin, frontalin, (both racemic) and myrcene (all Chemical Samples Co., Cleveland, Ohio), each one releasing about 2 mg/day (Byers and Wood, 1980). The following morning these baits were removed. *D. brevicomis* and *Enoclerus lecontei* Wolc. (Coleoptera:Cleridae) were collected daily for 11 days after the initial attack induced by the baiting (Figure 1).

Chi-square tests were used to compare sex ratios of catch between various trap levels and between north- and south-facing traps. The extent and duration of male stridulation, length of gallery construction, egg maturation within females, and host resin exudation were noted during the first part of the attack sequence from August 25 to September 5 (Table 1). On September 20 the attack density was recorded in several areas between the 2.1- and 10.7-m heights.

D. brevicomis were collected live as they walked on the bark surface at two times in the afternoon on the first day of baiting, August 25, and three

TABLE 1. RELATIONSHIPS BETWEEN AGGREGATION OF *Dendroctonus brevicomis* and *Enoclerus lecontei* ON PONDEROSA PINE AND MALE STRIDULATION, EGG MATURATION, AND GALLERY LENGTH OF *D. brevicomis*, AND HOST RESIN EXUDATION (AUGUST–SEPTEMBER 1978)

	Dendroctonus brevicomis					
Days after initial attack	Trap catch ♂:♀	Male stridulation	Gravid ♀♀	Range of gallery lengths (cm)	*E. lecontei*	Resin exudation
Aug. 24	0	0	0	0	0	0
Aug. 25[a]	57:34	++[b]	0	0	5	0
Day 1	97:53	+++	0	*[d]	15	0
Day 2	151:120	+++	0	*	15	+[e]
Day 3	255:153	+++	+[c]	*	455	+++
Day 4	172:111	++	+++	0.6–2.5	614	+++
Day 5	54:27	++	+++	1.2–3.8	519	+
Day 6	25:15	+	+++	2.0–5.1	302	0
Day 7	29:18	+	+++	2.5–6.2	483	0
Day 11	rain	0	+++	< 9.0	rain	0

[a] The tree was baited with *exo*-brevicomin, frontalin, and myrcene for one day only (August 25) which elicited a mass attack of *D. brevicomis*.

[b] Relative levels (from none = 0 to maximum = +++ as judged by J.A. Byers) of male stridulation, number of eggs in dissected females, and rate of resin flow.

[c] Little or no feeding occurred in *D. brevicomis* prior to August 28 (day 3) when phloem was first observed in hindguts of most males and females. Approximate percentage levels of dissected females with eggs, from none = 0 to >95% = +++.

[d] Not measured as beetles were taken from resin tubes, outer bark area, or after just penetrating the phloem.

[e] Relative rates of resin flow from none = 0 to maximum = +++ (judged by J.A. Byers).

times one day later (results pooled and shown for August 26 in Figure 2) and from galleries (lengths in Table 1) at 1200 and 1800 hr of each day (2–6, 8, 11, 14, 20, and 27 days) after the initial attack. The beetles were obtained in about equal portions from 2-m sections of the tree (Figure 1). Within 1 hr of collections, the beetles from each date and time were separated by sex, and the hindguts (about 20–25) of each group were excised and extracted with 300 μl diethyl ether (Byers and Wood, 1980). The amounts of pheromones present in these gut extracts were analyzed by GLC (3.6 m × 2 mm ID glass column of Ultrabond II on 100/120 mesh at 60° and 110° C and N_2 flow of 30 ml/min; 1.8 × 2 mm ID glass column of 3% Apiezon L on 100/120 Gas Chrom Q at 100° C and N_2 flow of 12 ml/min). Authentic samples of verbenone (Chemical Samples Co.), frontalin, *exo*-brevicomin, myrcene, and *trans*-verbenol (Glidden Organics, Jacksonville, Florida) were each GLC purified >99% and used for comparison to the gut extracts. Exponential regressions of the amounts of

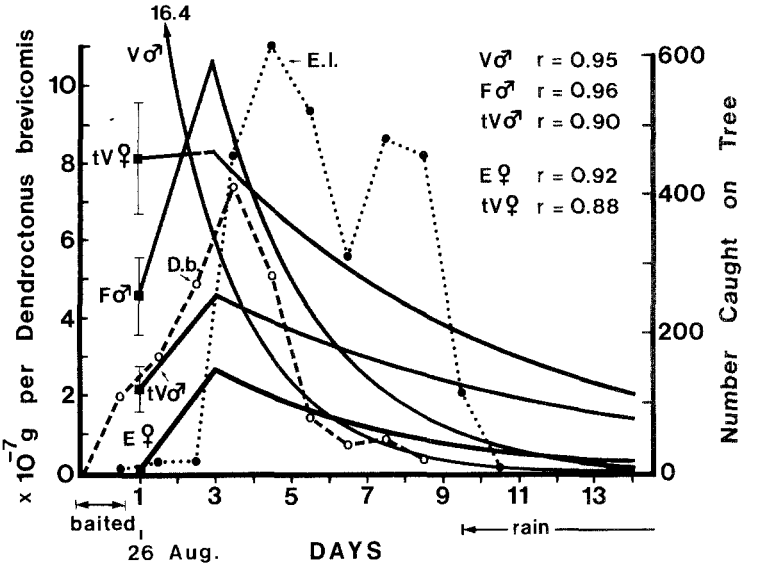

FIG. 2. Average content of the pheromones, verbenone (V), frontalin (F), *trans*-verbenol (tV), and *exo*-brevicomin (E), in guts of male and female *Dendroctonus brevicomis* feeding in a ponderosa pine tree. Daily catch of *D. brevicomis* (D.b.) and *E. lecontei* (E.l.) indicated by broken and dotted lines, respectively. Exponential regression curves are from data collected on days 3–20 except for V (1–20), V: $Y = 26.4e^{-0.48X}$, F: $Y = 31.4e^{-0.36X}$, tV♂: $Y = 6.4e^{-0.11X}$, E: $Y = 5.0e^{-0.20X}$, tV♀: $Y = 12.3e^{-0.13X}$. Brackets represent ± SEX for $N = 5$ on day 1 (August 26) for nonfeeding, recently landed *D. brevicomis*. Tree baited with E + F + myrcene for time shown to attract beetles to begin colonization.

pheromones in each sex through time were calculated from the GLC analyses (as indicated in Figure 2) since this type of function might be expected for release of volatile compounds from a substrate.

One bark beetle extract for each sex collected at 1800 hr on days 2, 8, 11, 14, and 20, and the above authentic standards as well as ipsdienol (Chemical Samples Co., GLC purified >99.5%) were further analyzed by GC-MS to confirm the identifications and relative amounts obtained with GLC. A Finnigan 4023 gas chromatograph–mass spectrometer–computer system with a 60-m Carbowax 60 N column (J & W) was used isothermally at 180° C with a helium carrier gas flow rate of 28 cm/sec using a Grob injection technique. A compound was considered identified if the mass spectrum and retention time of the unknown and the standard were similar (retention time ±5 sec). For quantitative analyses, ion characteristics of the spectra of the pheromonal standard were measured in comparison to the unknown at the appropriate retention times (min:sec):frontalin (*m/e* 142; 4:48); *exo*-

brevicomin (*m/e* 114; 5:02); ipsdienol (*m/e* 85; 6:51); *trans*-verbenol (*m/e* 109; 7:10); and verbenone (*m/e* 107; 7:59).

RESULTS

There were no significant differences ($P > 0.1$) in sex ratios of total catch in all comparisons between the five heights or between the north and south directions on the tree (Figure 1). Also, no significant differences were observed in the proportion of males to females caught each day from the initial attraction induced by the pheromone bait, day 0, to day 7 ($P > 0.1$). A total of 866 males and 557 females were caught during the entire period yielding a sex ratio of 1.55 (1.35–1.79, 99% binomial confidence limits). This ratio was significantly different from 1 ($P < 0.001$), the sex ratio of broods as they emerge from infested trees (Miller and Keen, 1960; Stephen and Dahlsten, 1976). The catch of *E. lecontei,* a predator, was substantially higher than *D. brevicomis.* The catch of *E. lecontei* also appears to be uniformly distributed in areas of the tree where traps were placed (Figure 1).

No beetles were caught until placing the pheromone baits on the tree. The aggregation was initiated, and it continued after the baits were removed for about eight days during a period of warm sunny weather (daily highs of 30–34° C). This concentration phase was essentially finished on day 8 after the initial attack, at which time only 4.7% as many beetles were caught as on the peak catch on day 3. Flight ceased the next day when rain and cooler weather intervened for over a week. The sex ratio in galleries was usually 1 : 1 (from day 4), and no males were found alone. The concentration phase was synchronized over a relatively short period of time as indicated by the ranges of gallery lengths and the large proportion of the total catch (66%) during the first four days (Table 1). Females began to produce mature eggs at about the time phloem was first observed in hindguts of both males and females. Male stridulation was heard almost as soon as females penetrated the bark, reaching a maximum during the peak aggregation three days after baiting and then diminishing as the catch decreased (Table 1). Host resin exudation began when females reached the phloem–xylem interface and apparently caused significant mortality of *D. brevicomis* since many "pitch tubes" contained from one to three or more dead beetles of either sex. However, the resin flow appeared to cease by day 6, and the death of the tree was confirmed the following year. Evidence of successful brood production and emergence holes were also observed. The final density of attack was about 15.1/0.1 m^2 (range 11.8–17.2, $N = 8$) and appeared relatively uniform in all areas sampled.

The peak aggregation of *D. brevicomis* was correlated with higher quantities of *exo*-brevicomin, frontalin, and other pheromone components in their guts (Figure 2). Males contained large quantities of verbenone (1.6

μg/male) when they landed on the tree, and the levels appeared to decline the most rapidly of all pheromone components measured (Figure 2). Frontalin levels in males that had just landed may increase upon exposure to resin or feeding, but its depletion was also rapid after 2–3 days. Females contained only trace or undetectable amounts of *exo*-brevicomin upon landing on the tree but apparently synthesize the component during feeding (Figure 2). *trans*-Verbenol was found in both sexes upon landing, although females had significantly more (Figure 2). The decline in *trans*-verbenol content in hindguts appeared to be the most gradual of all the components measured.

The identifications and relative amounts of pheromone components in gut extracts as determined by GLC (Figure 2) were confirmed by GC-MS. Females apparently did not have ipsdienol (<0.1 ng/female) during the attack, while males contained the largest amount (80 ng/male) when in the resin (day 2) but less on days 8 and 11 (5 and 7 ng, respectively) and none (<0.1 ng) was detected in the male extract on day 14.

The aggregation of *E. lecontei* on the tree appeared to immediately follow the peak aggregation of *D. brevicomis,* but the catch of this predator remained high for several days after attraction of the bark beetle had essentially ended. The attractive components released on the tree on day 0 (August 25) appeared to have resulted in very little attraction of *E. lecontei.*

DISCUSSION

Intraspecific Competition. The final attack density of *D. brevicomis* on the ponderosa pine was within normal limits (Miller and Keen, 1960), and the range of densities in various sections indicated a rather uniform distribution throughout the bole. The duration of landing and mass attack on the tree also was within the ranges observed in earlier studies in California (Miller and Keen, 1960; Byers and Wood, 1980; Bedard et al., 1983). The catch was highest when galleries were under 2.5 cm which agrees with Bedard et al. (1983), who found logs most attractive when galleries ranged from 2 to 5 cm with both sexes present.

Landing places with respect to height and direction on the tree appeared similar for *E. lecontei* and both sexes of *D. brevisomis.* However, more males were caught than females (1.55:1) and this ratio was significantly different from the brood emergence ratio (1:1). This may have been due to (1) the absolute sex ratio in the area, (2) a sexual difference in attraction as noted recently for *Ips paraconfusus* (Byers, 1983b), and (3) differential landing activity by the male when "searching" for female entrance holes—as suggested by Stephen and Dahlsten (1976) to explain the higher proportions of male *D. brevicomis* caught on baited trees. The third possibility appears more likely in view of the high numbers of clerids also caught on traps which appeared due to

their high activity with multiple landings in search of prey on the bark. Furthermore, sexual differences in response to attractive components have not been observed in the laboratory olfactometer or in the field, or to infested logs (Byers and Wood, 1980, 1981).

Based on the diameters near the base of the tree (50.9 cm) and at the 10.7 m height (41.2 cm), the ten traps covered at most 1.5% of the bark surface. If extrapolations are made from this figure and the catch, then over 27,000 beetle landings occurred in this area of the tree on day 3 and over 91,000 during the mass attack period. However, only about 4700 beetles would need to land in this part of the tree over the entire period (15.1 attacks/0.1 m^2) if every pair were successful. The discrepancy between the number of galleries and the apparently much higher number of landings, as reflected in trap catch, was probably caused by some combination of the following: (1) multiple landings by beetles, (2) mortlity caused by predators and tree resin, and (3) beetles that visited the tree and did not stay. The amounts of *exo*-brevicomin and frontalin (each 6 mg/day) released from the baits on the first day were similar to what might be expected to be released per day from the 4700 beetles as calculated from the results of Browne et al. (1979) during the early stage of attack (however, the amount of myrcene from baits was about two orders of magnitude less).

Frontalin and verbenone from males, and *trans*-verbenol from both sexes, appear to be released soon after "contact" with the host (cf. Vité et al., 1972) since they are found in large amounts upon landing. However, the production of *exo*-brevicomin and possibly frontalin may be influenced by feeding and/or hormones. *exo*-Brevicomin in females increased as feeding commenced (also found by Pitman et al., 1969; Hughes and Renwick, 1977), while its decline appeared to begin immediately after mating (eggs in females). Earlier, Hughes (1973) found that mating caused a significant decline in the content of *exo*-brevicomin in females feeding in logs of ponderosa pine. Hughes and Renwick (1977) discovered that *D. brevicomis* females produced large quantities of *exo*-brevicomin when treated with juvenile hormone (JH III). The amounts of ipsdienol found only in males during the early stages of colonization are apparently produced after exposure to myrcene precursor in the host resin (Byers, 1982). The long-term decline in *trans*-verbenol levels of beetles during the colonization appears due to its continued but declining production from the α-pinene precursor in the resin and phloem (Hughes, 1973; Byers, 1983a).

The highest quantities of *exo*-brevicomin and frontalin were found in *D. brevicomis* guts when the maximum catch occurred (Figure 2). This indicates that maximal flight attraction resulted from the release of the highest relative amounts of these pheromone components. The relative amounts of various pheromones within the beetles during the colonization presumably reflect the release rates of these compounds in nature. This assumption is probably

correct since feeding and defecation are known to occur at the beginning of colonization (Silverstein et al., 1968), and females must continue to feed to sustain egg production (e.g., 56 eggs contain over 3.4 times the beetles' weight, from Figure 2 in Miller and Keen, 1960). Furthermore, the evaporation rates of compounds of similar volatility from fecal pellets would be expected to be nearly porportional to their mole percent (Raoult's Law, cf. Byers, 1981b). However, absolute release rates are dependent on the number and density of beetles attacking the tree. Therefore, comparisons between dates of relative release rates from the infested tree (Figure 2) cannot be done accurately until the population density has stabilized, a few days after baiting (gallery lengths in Table 1 indicate maximum density was reached after only a few days). Some loss of pheromone components may have occurred during the <1-hr transport of beetles from the tree to the laboratory, although amounts are similar to those found in *D. brevicomis* feeding in logs that had been immediately dissected and extracted (Byers and Wood, 1980; Byers, 1983c).

The quantities of frontalin and *exo*-brevicomin in beetles during the first 3–4 days of colonization (Figure 2) agree with the relative release rates of these components observed by Browne et al. (1979) from beetles for only 1 day and for 3 days in the field. If the average amounts of *exo*-brevicomin and frontalin in beetles are compared to their estimates of release (Browne et al., 1979) for the same period of time (frontalin = 8.6×10^{-7} g/day and *exo*-brevicomin = 4.1×10^{-6} g/day), then males would have a turnover rate of about one gut content per day and females about 20 gut contents per day. It appears that further work is needed before we can have confidence in this type of comparison. However, it does indicate that at least *exo*-brevicomin and *trans*-verbenol were produced over an extended period, otherwise they would have been exhausted after just a few days assuming the above release rates.

Renwick (1967) and Pitman et al. (1969) reported that emergent males contain large amounts of verbenone. Byers (1983a) further showed that the appearance of verbenone in emergent beetles occurred in the absence of host material and that (+)- and (−)-α-pinene did not appear to serve as a precursor, at least at this time. We found verbenone in the largest amounts in males as they landed on the tree, and its content appears to immediately decline and more rapidly than that of any other pheromone component in the hindgut (although ipsdienol was not compared). These results indicate the verbenone is not synthesized ore released in the latter stages of the concentration phase when termination is occurring. This compound was earlier hypothesized to cause termination (Renwick and Vité, 1970; NcNew, 1970). Instead, verbenone is produced before landing and possibly shortly thereafter and may operate as a "close-range" inhibitor (Byers and Wood, 1981) to regulate density of attack, since a "long-range" inhibition at this time would be nonadaptive. Browne et al. (1979) indicated that verbenone is associated with females because they collected the compound from air

passed over females in logs inside steel barrels. Addition of males did not significantly increase the release of verbenone. However, the ratios of verbenone–*exo*-brevicomin and verbenone–frontalin were much less (1 : 106 and 1 : 11.2) than that observed in our study (days 1–4, Figure 2). This discrepancy may be due to the artificial conditions inside the barrels affecting behavior or due to problems of differential entrainment of the volatiles.

Both the increase and decrease in the landing rate, and probably the attraction, of *D. brevicomis* were positively correlated with the presence of *exo*-brevicomin and frontalin in the gut. However, the decline in landing may have been made more precipitous because of a change in the ratio of attractive to inhibitory pheromones. There is evidence that *trans*-verbenol, in combination with verbenone (Bedard et al., 1980a) or by itself (Hughes and Pitman, 1970; Bedard et al, 1980a; Byers, 1983a) inhibits the attraction of *D. brevicomis* to their attractive components. Feeding males contained about half as much *trans*-verbenol as females (Figure 2), which is in contrast to earlier reports that males contained little or none of this compound (Renwick, 1967; Renwick and Vité, 1970; Vité and Renwick, 1970; Pitman et al., 1969). *trans*-Verbenol may function with verbenone and ipsdienol during the aggregation to regulate density of attack. In addition, *trans*-verbenol may inhibit new attacks during the termination phase since it decreased in both sexes more gradually than other components and was the only component still present in significant amounts when the catch decreased to low levels. Therefore, the ratio of *trans*-verbenol to *exo*-brevicomin and frontalin within certain absolute release rates may function to terminate attack as well as regulate attack density and intraspecific competition. In Figure 3 we propose a revised version of the mechansim of attack (Renwick and Vité, 1970) based on the above discussion. However, additional work is needed to establish and further delimit the functions of the inhibitory pheromones, (+)-ipsdienol, verbenone, and (−)-*trans*-verbenol and their interactions with the attractive pheromones, (+)-*exo*-brevicomin and (−)-frontalin.

Several other bark beetles appear to utilize inhibitory pheromones to regulate density and/or termination of attack. *D. pseudotsugae* males release methylcyclohexenone that inhibits response to female-released pheromone (Rudinsky and Michael, 1972; Pitman and Vité, 1974). Male *Trypodendron lineatum* release volatiles that inhibit response to female pheromone (Nijholt, 1973). In *D. frontalis,* males release verbenone which at high release rates inhibits both sexes or males more (Renwick and Vité, 1970; Payne et al., 1978), and may be used in a similar way as in *D. brevicomis.* Another inhibitor, *endo*-brevicomin, from males reduces attraction of flying beetles (Payne et al., 1978). In *I. paraconfusus,* however, attractive pheromene components from males at high release rates appear to have a sex-specific inhibitory effect on males which may function to regulate their attack density (Byers, 1983b). In contrast, the attractive response of *D. brevicomis* does not

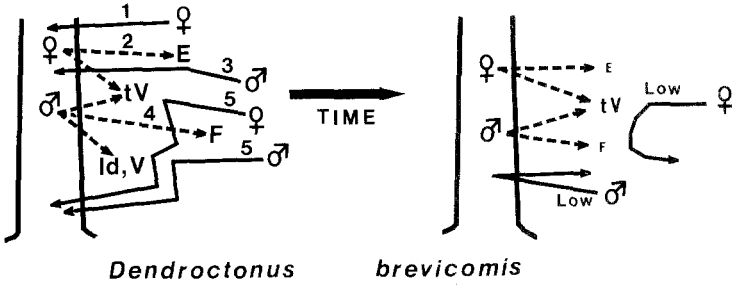

FIG. 3. Theoretical mechanism for regulation of attack density (intraspecific competition) and termination of aggregation in *Dendroctonus brevicomis* during colonization of a ponderosa pine. The female beetle arrives first (1) and bores into the trunk and after feeding produces *exo*-brevicomin, E (2), which primarily attracts males (3). Males, upon locating a female gallery, soon release frontalin, F (4), which synergizes with E to elicit a mass aggregation (5). However, at the same time females and males produce *trans*-verbenol (tV) and males produce verbenone (V) and (+)-ipsdienol (Id). At close range these compounds apparently inhibit the attraction of beetles to E and F (tV appears to primarily affect females while Id and V affect both sexes), which would regulate the attack density. After several days the production and release of E and F diminishes to unattractive levels (at long range). The few females attracted during this latter period may be inhibited from attacking by the still significant, although reduced, levels of tV. The few males would not find any unpaired females and so would continue searching elsewhere.

seem to be inhibited by similar levels of its attractive components released in the same olfactometer in which *I. paraconfusus* was inhibited (Byers and Wood, 1981; Byers, 1983b). Mating appears to cause a reduction in pheromone production in *D. brevicomis* (Hughes, 1973; and our study), *D. frontalis* (Coster and Vité, 1972), *Scolytus multistriatus* (Peacock et al., 1971; Elliott et al., 1975; Gore et al., 1977), and *I. paraconfusus* (Byers, 1981a). Thus, the reduction in the quantity of attractive pheromones is as important as the release of inhibitory compounds during termination of the concentration phase. In fact, a reduction in attractive pheromones is apparently the only olfactory mechanism of termination in some bark beetles, *S. multistriatus* and *I. paraconfusus* (Gore et al., 1977; Byers, 1981a), although these species may have as yet undiscovered short-range olfactory mechanisms.

Interspecific Competition. The inhibitory pheromone components may not only play a role in intraspecific communication but also may serve as interspecific messages (allomones) for reducing possible competition between cohabiting species (Figure 4). *D. brevicomis* and *I. paraconfusus* are sympatric in our study area near Yosemite National Park but compete for the same host tissue with a third beetle, *I. pini,* about 100 km northward where

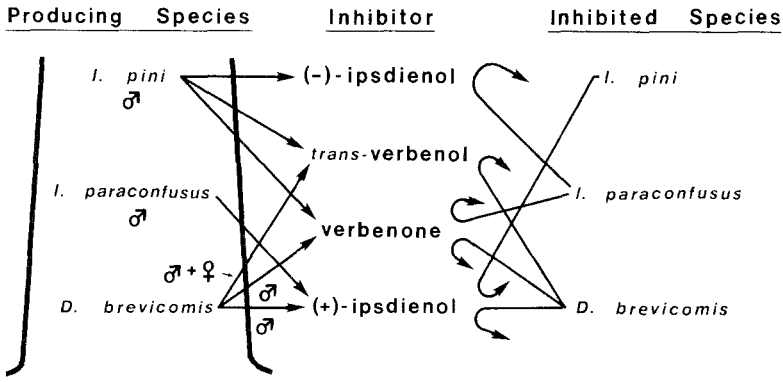

FIG. 4. Inhibition of the attraction response to conspecific pheromone by pheromones/kairo-allomones produced by three sympatric bark beetles, *Dendroctonus brevicomis, Ips paraconfusus,* and *I. pini* which may function to reduce interspecific competition for their host ponderosa pine in California. Response inhibition by *trans*-verbenol, verbenone, and (+)-ipsdienol may also reduce intraspecific competition in *D. brevicomis.*

they all occur continuously along the Sierra to the Cascades and into Oregon. In Oregon and Idaho, *D. brevicomis* and *I. pini* are predominant. Verbenone, in addition to its intraspecific effects, appears to have another function for *D. brevicomis* since its release by males inhibits the response of its competitor, *I. paraconfusus* (Byers and Wood, 1980, 1981). Lanier et al. (1980) found verbenone in *I. pini* males from Idaho that had fed in red pine (*P. resinosa* Ait.) logs, but they could not ascribe any "biological activity" to the compound. Birch and Wood (1975) showed that the responses of *I. pini* and *I. paraconfusus* were mutually inhibited by volatiles from infested logs of the opposite species, and Light and Birch (1979) determined that (−)-ipsdienol from *I. pini* inhibited the attraction of *I. paraconfusus.* However, it may be that the verbenone in *I. pini,* a major component, contributes to this inhibition of *I. paraconfusus* by (−)-ipsdienol, and thus the behavioral effect of verbenone on *I. paraconfusus* has been naturally selected because of pressures to reduce interspecific competition from both *D. brevicomis* and *I. pini* (Figure 4). Furthermore, the inhibitory effects of verbenone on *D. brevicomis* could be not only the result of intraspecific competition but partly the result of selection pressure exerted by *I. pini.* This could also be true of the inhibitory effects of *trans*-verbenol on *D. brevicomis* (Byers, 1983a) which is produced by *I. pini* (Vité et al., 1972; Lanier et al., 1980) (Figure 4). The (+)-ipsdienol produced by male *I. paraconfusus* (Silverstein et al., 1966) and by male *D. brevicomis* in the early stages of colonization may not only function to reduce intraspecific competition in both species (Byers, 1982; Byers, 1983b) but also

reduce interspecific competition from *I. pini* in both species (Birch et al., 1980; Byers, 1982).

The system of density regulation and termination of aggregation in *D. brevicomis* appears to be complex. It apparently involves several attractive and inhibitory pheromones that are produced and released at varying ratios and absolute concentrations, depending on the stage of colonization. The absolute and relative concentrations of various pheromones are influenced by many factors such as attack density and sequence of arrival (compression of mass attack) which in turn are dependent on weather, population density, and host resistance. Physiological factors such as mating and nutrition as well as presence of pheromone precursors in the host (Byers, 1981b) also may effect release rates of pheromones. Male stridulation and other behavioral processes also probably have a role in regulation of attack density. Interspecific effects also could influence the density of colonization as hypothesized by Byers and Wood (1980, 1981) for *I. paraconfusus* and *D. brevicomis*. In Figure 5, a theoretical scheme is presented for the interaction of ecological factors which appear to influence the significance of pheromones, kairomones, and

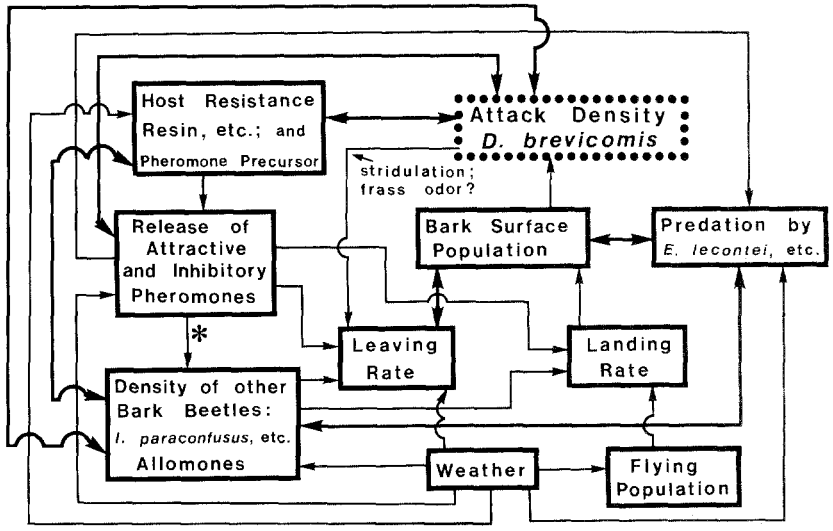

FIG. 5. Schematic of theoretical interactions between ecological components which may influence the attack densities of *Dendroctonus brevicomis* (and competing bark beetle species) on a ponderosa pine. Heavy lines indicate two-way interactions and possible direct feedback or regulatory functions, while thin lines indicate one-way effects which in some cases can have indirect feedback effects. The model assumes that pheromones/allomones are released after beetles have located sites of attack. *Pheromone components of *D. brevicomis* can also act as allomones in reducing the response of competing species to their pheromones.

allomones in regulating inter- and intraspecific competition (attack density) among *D. brevicomis* and other bark beetle species (e.g., *I. paraconfusus*).

Further work on determining the absolute concentrations of pheromones released from a tree under colonization and on the variation of production (cf. Birgersson et al., 1984) and release between individual galleries is needed. In addition, detailed studies of response to combinations of pheromones must be done before a rather complete understanding of aggregation and colonization can be achieved.

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CHEMOSENSITIVITY OF LOBSTER, *Homarus americanus*, TO SECONDARY PLANT COMPOUNDS: Unused Receptor Capabilities

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Abstract—Chemosensitivity to secondary plant substances was examined electrophysiologically and behaviorally for the lobster *Homarus americanus*. Neurophysiological experiments show that some chemoreceptor cells in the antennules (representing the sense of smell) and walking legs (representing the sense of taste) were excited by secondary compounds from plants of marine and terrestrial origin. These compounds include amygdalin, atropine sulfate, bromoform, caffeine, *p*-coumaric acid, diiodomethane, ferulic acid, heliotropin, phloroglucinol, quinine sulfate, salicin, sinigrin, tannic acid, and tomatine. The possible behavioral function of three of these compounds was tested. Phloroglucinol and ferulic acid had no observable effect on any aspect of feeding behavior at any concentration tested. Tannic acid, which is related to polyphenols found in marine algae, had no observable effect at any concentration tested on orientation to and grasping of food (activities controlled primarily by antennular and leg chemoreceptors, respectively) but did have an inhibitory effect on food ingestion (an activity controlled primarily by mouthpart chemoreceptors). These electrophysiological and behavioral results suggest that potential chemoreceptive information derived from many secondary plant compounds may not be used in feeding behavior. The receptors sensitive to these compounds may represent a "common chemical sense" as suggested by Dethier (1980). However, at least one compound, tannic acid, that is smelled and tasted by lobsters can function as a feeding inhibitor at the level of the mouthparts.

Key Words—Lobster, *Homarus americanus*, chemoreception, feeding deterrents, feeding inhibition, secondary plant compounds, common chemical sense, Crustacea.

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INTRODUCTION

Substances produced by plants, known as secondary plant compounds, inhibit the consumption of these plants by many terrestrial herbivorous invertebrates and vertebrates (Fraenkel, 1959; Whittaker and Feeny, 1971; Swain, 1977). These compounds include tannins, quinones, alkaloids, terpenoids, glucosinolates, cyanogenic glycosides, and many others.

There is ample neurophysiological evidence that primary chemosensory neurons of insects can detect certain secondary plant substances. The response can either be excitation of one set of chemoreceptors sensitive to such compounds (Morita et al., 1957; Stürckow, 1959; Ishikawa, 1966; Dethier, 1973, 1976, 1980) or inhibition of another set of chemoreceptors that are excited by feeding stimulants (Dethier, 1980). These studies, along with behavioral evidence (Waldbauer and Fraenkel, 1961; Dethier, 1980), indicate that taste is an important modality used by insects in avoiding plants containing these compounds.

Comparatively little is known concerning the effects of secondary plant compounds on aquatic invertebrates. Early workers noted that several marine species, including sponges (Parker, 1910), holothurians (Olmstead, 1917), ascidians (Hecht, 1918), chitons (Arey and Crozier, 1919), and nudibranchs (Crozier and Arey, 1919), can respond behaviorally to a variety of secondary plant compounds, including such alkaloids as strychnine, quinine, atropine sulfate, and nicotine. The crayfish *Cambarus affinis* (Bell, 1906) and the shrimp *Palaemon treillanus* (Balss, 1913) responded to 30–60 mM quinine, and the shrimp *Crangon vulgaris* responded to 30 μM quinine (Spiegel, 1927). Later studies have shown that various substances found in marine angiosperms and algae (Valiela et al., 1979; Carlton, 1980; Geiselman, 1980; Geiselman and McConnell, 1981; Targett and McConnell, 1982; McConnell et al., 1982), in ascidians (Stoecker, 1980), or in other invertebrates (Mackie and Grant, 1974; Prezant et al., 1981) can inhibit feeding by marine invertebrates.

Neurophysiological studies have demonstrated that chemoreceptors of marine crustaceans can detect a wide variety of compounds. The most stimulatory are small nitrogenous compounds such as ammonia and select amino acids, amines, and nucleotides (Case, 1964; Shepheard, 1974; Ache et al., 1978; Johnson and Ache, 1978; Derby and Atema, 1982a; Derby, unpublished data). Neurophysiological and behavioral experiments show that marine crustaceans also can detect substances that are not normally found in their environment (Luther, 1930; Atema et al., 1979; Pearson et al., 1980; Derby and Atema, 1981). This raises the question of the range of receptor types and their specificities in these crustaceans. Many secondary compounds from terrestrial plants are presently not known to occur in the

marine environment. If crustacean chemoreceptors are sensitive to such compounds, it could imply two possibilities. First, these or related compounds actually do occur in the marine environment and have an as yet unknown functional significance in plant–crustacean interactions. For example, marine bryozoans have recently been shown to contain two terpenoids—citral and geraniol—which are common to terrestrial plants but previously unknown in the marine environment and which may have antifouling or antifeeding functions (Christophersen and Carlé, 1978). A second possibility, as suggested by Dethier (1980), is that there are chemosensory cells which are broadly sensitive to a wide range of chemicals, including secondary plant compounds, and these cells may represent a "common chemical sense." Dethier (1980) offers the salt receptor of blowflies as an example; such cells can detect secondary plant substances but cannot provide enough information to allow for discrimination among potential food plants based on these substances. He further suggests that from this "primitive" type of cell, specialized cell types evolved that are sensitive to a particular compound or set of compounds (e.g., amino acid receptors of crustaceans and sugar receptors of insects).

To further explore this hypothesis, neurophysiological and behavioral experiments were performed which examine the chemosensitivity of the lobster *Homarus americanus* to marine and terrestrial secondary plant compounds, including substances which a priori are not expected to be natural stimuli for lobsters.

METHODS AND MATERIALS

Chemical Stimuli. Chemicals used in this study include representatives of classes of compounds identified as constituents of either marine or terrestrial plants. Most of these compounds have also been shown to inhibit the feeding behavior of various animals. Ferulic acid and *p*-coumaric acid are cinnamic acids present in the marine angiosperm *Spartina alterniflora* which inhibit ingestion of this plant by two marine invertebrates (Valiela et al., 1979). Diiodomethane and bromoform are halomethanes released by the marine green alga *Codium* sp. and have some inhibitory effects on the feeding behavior of the marine snail *Littorina littorea* (Geiselman, 1980). Phloroglucinol and tannic acid are related to polyphenols found in the marine brown algae *Fucus vesiculosus* and *Ascophyllum nodosum* and inhibit ingestion by *L. littorea* (Geiselman, 1980; Geiselman and McConnell, 1981). Secondary compounds from terrestrial plants (e.g., Whittaker and Feeny, 1971; Swain, 1977; Dethier, 1980) that were tested in the present experiments include amygdalin, atropine sulfate, caffeine, heliotropin, quinine sulfate, salicin,

sinigrin, and tomatine; to our knowledge, these compounds do not occur in the marine environment.

Neurophysiology. Neurophysiological experiments were performed on excised lateral antennular flagella and walking legs of locally caught lobsters. The recording procedure for only the antennular preparation will be described in some detail (see also Ache et al., 1978; Fuzessery et al., 1978; Atema et al., 1979). The procedure for legs is very similar and is described in more detail in Derby and Atema (1982a). After a lateral flagellum was excised, it was inserted into a Sylgard cork and placed in a stimulating-recording chamber. Several annuli at the proximal end were carefully removed, exposing the sensory nerve containing the axons of the primary receptor cells; this nerve was bathed in *Homarus* Ringer's solution (Derby and Atema, 1982a). The distal tip was cut and a micropipet was inserted into this end, perfusing the antennule with oxygenated Ringer's solution. A 10-ml/min flow of artificial sea water (ASW) (Derby and Atema, 1982a) continuously rinsed over the distal section of the antennule. Chemical stimuli in 50-μl aliquots were injected into this flow while the resulting multiunit neurophysiological responses were recorded with a platinum hook electrode from subdivided bundles of the sensory nerve. Conventional electrophysiological equipment was used, and the data were stored on magnetic tape for later analysis. In the leg preparation, responses were recorded only from the two most distal segments, the dactylus and propodus.

Chemical stimuli introduced into the ASW flow of the recording chamber reached a maximum concentration of 0.035 times that introduced, as measured by the change in conductance following introduction of a concentrated salt solution into flowing distilled water. This dilution factor is included in the reported concentrations of chemical stimuli. Mussel (*Mytilus edulis*) extract was prepared by homogenizing 20 g wet weight of soft tissue in 1 liter of ASW, centrifuging at 25,000 *g* for 15 min, discarding the pellet, and diluting the supernatant tenfold. Stock solutions of chemical compounds were made in ASW, and when necessary the pH was adjusted to 7.6. Compounds which were less soluble in water were initially dissolved in a small amount of ethanol and later added to ASW; the final ethanol concentration in these solutions as the stimulus reached the receptors was approximately 10^{-4} M. Equivalent concentrations of ethanol were also tested neurophysiologically as control stimuli. Previous neurophysiological experiments demonstrated that 3.5×10^{-4} M ethanol elicited no or minimal neural activity (Derby and Atema, 1982a).

In order to broadly search for bundles of nerves containing a wide variety of chemoreceptor cell types, we used a complex natural mixture (mussel extract) as a search stimulus. Multiunit responses to equimolar concentrations (10^{-5} M) of L-glutamate and 14 secondary plant compounds were then

recorded. Response magnitudes were determined from each multiunit response by computing the difference in the total number of action potentials during equal time periods before and after stimulus introduction. This period was set to include most or all of the response of the cells, usually with a maximum of 10 sec. For each nerve bundle, the responses to ASW or, when applicable, ethanol control stimuli were also measured and subtracted from the above value. To compare responses between nerve bundles, activity ratios for each of the compounds were determined for each bundle. Mean activity ratios were then calculated for each compound. Activity ratios are defined according to Johnson and Ache (1978) and Derby and Atema (1982a) as the ratio of the response to each secondary plant compound and the response to mussel extract.

The experiments were carried out in two different years, leading to a complication in data analysis. Responses of leg chemoreceptors to all plant compounds and responses of antennular receptors (antennule study I) to the majority of compounds (atropine sulfate, tomatine, quinine sulfate, amygdalin, sinigrin, salicin, heliotropin, caffeine) were recorded in the summer of 1979. The remainder of the compounds (bromoform, ferulic acid, tannic acid, diiodomethane, *p*-coumaric acid, phloroglucinol) were tested on antennules in the summer of 1982 (antennule study II). The activity ratios for L-glutamate in the leg study and in antennule study I were nearly the same (24.4 and 26.0, respectively), but the ratio was much higher in antennule study II (71.5). Similarly, the activity ratios for the secondary plant compounds were all higher in antennule study II than in the leg study or antennule study I. These results suggest that there may have been differences in the composition of the mussel extract between study I and study II, even though the concentration in terms of wet weight of tissue per volume of sea water was constant. In fact, it has been previously reported that mussels collected from different locations or at different times can have a qualitatively and/or quantitatively different chemical composition (Bayne and Widdows, 1978; Pierce, 1982).

One method of circumventing this problem during data analysis is to determine the activity ratios using L-glutamate instead of mussel extract as the standard for each bundle, since L-glutamate was the only common stimulus besides mussel extract in studies I and II. However, since L-glutamate was not tested on all cells in antennule study I, the data were instead standardized by using the mean activity ratios to L-glutamate for each study (24.4, 26.0, and 71.5 in the leg study, antennule study I, and antennule study II, respectively). This transform is defined by the following equation:

$$\frac{\text{mean activity ratio of each secondary plant compound}}{\text{mean activity ratio of L-glutamate for study in which plant compound was tested}} \times 100$$

Behavior. This study examined whether secondary plant compounds

TABLE 1. RESPONSES OF CHEMORECEPTORS TO SECONDARY PLANT COMPOUNDS

Stimulus[a]	Leg chemoreceptors[b]	N[c]	Antennular chemoreceptors[b]	N[c]
L-Glutamate	100	24	100	30
Ferulic acid	56.6	13	32.5	11
Atropine sulfate	34.0	11	34.6	9
Salicin	31.6	11	3.9	7
Sinigrin	27.1	10	4.2	10
Amygdalin	26.6	10	10.4	7
p-Coumaric acid	23.0	13	15.0	10
Tomatine	22.5	7	17.7	9
Heliotropin	22.1	7	0	8
Caffeine	20.5	11	0	7
Phloroglucinol	18.0	7	7.6	8
Bromoform	17.2	6	33.9	7
Tannic acid	14.8	7	32.0	7
Diiodomethane	3.7	6	24.9	7
Quinine sulfate	2.5	7	14.2	7

[a] All compounds tested at 10^{-5} M.

[b] Values represent: $\frac{\text{mean activity ratio of each secondary plant compound}}{\text{mean activity ratio of L-glutamate standard for relevant study}} \times 100.$

[c] N = Number of nerve bundles tested for each substance.

affected the feeding behavior of lobsters. The three compounds used in this study, selected because each elicited a different level of electrophysiological activity (Table 1) and because each has been shown to inhibit feeding behavior of other marine invertebrates (Valiela et al., 1979; Geiselman, 1980; Geiselman and McConnell, 1981), are: ferulic acid (= 4-hydroxy-3-methoxycinnamic acid, mol wt 194), one of the most highly excitatory plant compounds for both leg and antennular chemoreceptors; tannic acid (Sigma Chem. Co., mol wt 1700), highly stimulatory for the antennular but not the leg chemoreceptors; and phloroglucinol (= 1,3,5-trihydroxybenzene, mol wt 126), relatively poorly stimulatory for both the leg and antennular chemoreceptors. Six lobsters with carapace lengths of 5–7 cm were selected for inclusion in these experiments because each reliably showed the full complement of feeding behaviors, as described later, when presented with food. Each lobster was individually held in a 100-liter aquarium (measuring 0.7 m long by 0.4 m wide by 0.3 m deep), with continuous flow-through sea water and a 14:10 light–dark photoperiod regime. Observations were made during the dark phase, using illumination from dim red lights. Temperature during the experiments ranged from 19 to 21° C.

The feeding behavior of each lobster toward Whatman glass fiber disks

(type GF/A, 2.1 cm diameter) soaked in different chemical substances was observed. These substances included: (1) 10 g mussel tissue homogenized as described for the neurophysiological experiments in 1 liter of either ASW, tannic acid (10^{-3}, 10^{-4}, or 10^{-5} M), phloroglucinol (10^{-2}, 10^{-3}, or 10^{-4} M), or ferulic acid (10^{-2}, 10^{-3}, or 10^{-4} M); (2) tannic acid alone (10^{-3}, 10^{-4}, or 10^{-5} M); (3) phloroglucinol alone (10^{-2}, 10^{-3}, or 10^{-4} M); (4) ferulic acid alone (10^{-2}, 10^{-3}, or 10^{-4} M); and (5) ASW alone. Both the ASW solution and the mussel extract in ASW had ethanol added in order to control for the ethanol used to dissolve the secondary plant compounds before adding them to the sea water; the final ethanol concentration in these solutions was 10^{-2} M. Mussel extract at a concentration of 10 g tissue/liter ASW was used in this study since this was the lowest concentration that consistently evoked the full complement of behaviors (as described below) from all six lobsters.

The release rates of chemicals from the disks are unknown. A lobster that touched a disk soon after the introduction of the disk into the aquarium is likely to have experienced close to the maximum concentration, but with time the concentration released from the disk undoubtedly decreased. The short time course of each trial (a maximum of 10 min but often much less; see Table 2) partially alleviated this problem.

Lobsters display a relatively stereotyped series of searching and feeding behaviors when presented with a chemically attractive food such as a disk soaked in mussel extract. Lobsters search the aquarium while performing such behaviors as antennule flicking, exopodite fanning, antennal waving, and walking high on legs. When a leg touches the food, the food is usually immediately grabbed and transferred to the mouthparts and is then usually partially or completely ingested. A more complete description of the normal searching and feeding behavior of lobsters can be found in Derby and Atema (1982b). Based on these findings, the effects of secondary plant compounds on the feeding behavior of lobsters were evaluated in the present study by monitoring the following behaviors: (1) number of lobsters to grab the disk in the mouthparts within 10 min of introduction; (2) time required to grab the disk after its introduction; (3) time required to start eating the disk after grabbing it; (4) time required to either drop the uneaten or partially eaten disk or to eat the entire disk after grabbing it; and (5) amount of disk eaten (estimated to the nearest 10% from the portion of the disk remaining at the end of the experiment; for statistical analysis, these data were transformed from percentages using the arcsine transformation).

Each of the six lobsters was fed every type of disk at least once, with each lobster thus serving as its own control. A trial was not included in the analysis if the lobster did not touch a disk with any of its walking legs within the 10-min time period. Differences between treatment groups were statistically analyzed with randomized block analysis of variance and Dunnett's test for multiple comparisons using the 5% significance level.

TABLE 2. EFFECTS OF TANNIC ACID ON FEEDING BEHAVIOR OF LOBSTERS[a]

Disk soaked in:	Number of lobsters to grab disk ($N = 6$)	Time to grab disk after introduction (sec)	Time to start eating disk after grabbing (sec)	Time to drop or finish disk after grabbing (sec)	Amount of disk eaten (%)
Part A:					
mussel extract[b] in:					
ASW	6	18 ± 3	15 ± 2	106 ± 22	98 ± 2
10^{-5} M tannic acid	6	24 ± 8	13 ± 3	81 ± 16	97 ± 3
10^{-4} M tannic acid	6	37 ± 14	15 ± 4	110 ± 17	83 ± 6[c]
10^{-3} M tannic acid	6	26 ± 8	17 ± 2	105 ± 6	55 ± 4[c]
Part B:					
ASW	1	31	—[d]	7	0 0
10^{-5} M tannic acid	2	83 ± 39	—	6 ± 1	0 0
10^{-4} M tannic acid	1	60	—	26	0 0
10^{-3} M tannic acid	2	135 ± 61	—	19 ± 5	0 0

[a] All values represent mean ± standard error; $N = 6$. Addition of any concentration of tannic acid to mussel extract had no significant effect ($P > 0.05$, randomized block analysis of variance) on any of the measured parameters of feeding behavior in Table 2A except for "amount of disk eaten."

[b] 10 g wet weight mussel (*Mytilus edulis*) tissue in 1 liter of indicated solution.

[c] Randomized block analysis of variance with Dunnett's multiple comparison test of data in Table 2A demonstrates that 10^{-3} or 10^{-4} M tannic acid added to mussel extract caused a significant ($P < 0.05$) reduction in "amount of disk eaten;" 10^{-5} M tannic acid added to the mussel did not have this effect (using data transformed from percentages by arcsine transformation).

[d] Dashes signify that no lobsters began eating the disks within 10 min.

RESULTS

Neurophysiology. The populations of antennular and walking leg chemoreceptors were excited by almost all of the secondary plant compounds tested (Table 1). Several secondary plant compounds were 30–60% as effective as L-glutamate. Among the compounds most excitatory for both leg and antennular chemoreceptors were ferulic acid and atropine sulfate. The majority of the compounds generated relatively low-level yet discernible responses (e.g., Figure 1).

Only the ability of plant compounds to excite chemoreceptors was monitored in these experiments. The low rate of spontaneous discharge of these chemoreceptors (e.g., Figure 1, ASW trace) made it difficult to identify with extracellular techniques cells that might have been inhibited by chemicals. Experiments to test if the responses of receptors to feeding excitants could be reduced by the simultaneous presentation of plant compounds were not performed.

Behavior. Lobsters were highly excited when a disk soaked in mussel extract was introduced into the aquarium. In response to mussel extract, the lobsters performed the normal sequence of searching and feeding behaviors previously described (i.e., they always grabbed the disk once it was touched with a leg and they subsequently ate most of the disk) (Table 2A). In contrast, ASW disks were either not grabbed after being touched by the legs ("number of lobsters to grab disk") or, if they were picked up, they were always dropped after a few seconds ("time to drop disk after grabbing") without ever being eaten ("amount of disk eaten") (Table 2B). Lobsters behaved similarly toward filter paper disks impregnated with ASW or with any of the three concentrations of tannic acid alone (Table 2B). However, when tannic acid was added to

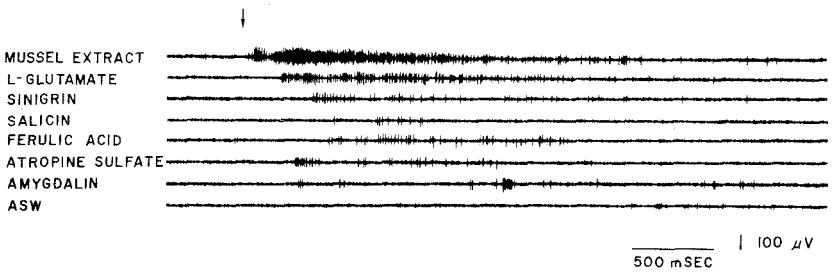

FIG. 1. Multiunit recording of primary chemosensory neurons sensitive to secondary plant compounds. Sensory cells in a nerve bundle in a second walking leg of *H. americanus* were excited by mussel extract (70 mg/l) and by equimolar concentrations (10^{-5} M) of L-glutamate and five secondary plant compounds (sinigrin, salicin, ferulic acid, atropine sulfate, amygdalin); there was little to no response to artificial sea water (ASW). Arrow indicates time of stimulus introduction.

the mussel extract at a concentration of either 10^{-3} or 10^{-4} M (1.7 and 0.17 mg/ml solution, respectively), there was a significant reduction of feeding but only in the amount of disk eaten (Table 2A). A 10^{-5} M concentration of tannic acid in mussel extract did not inhibit ingestion of the disc. Tannic acid at any of the three concentrations added to mussel extract had no effect on the number of lobsters to grab disk, time to grab disk after introduction, time to start eating disk after grabbing, or time to drop a partially eaten disk or to eat the entire disk after grabbing (Table 2A).

Lobsters attempting to eat the disks soaked in mussel extract with 10^{-3} or 10^{-4} M tannic acid performed a distinct behavior. They initially grabbed such a disk and transferred it to their mouthparts but usually quickly dropped it after briefly manipulating it. This behavior was often repeated several times in rapid succession until the partially ripped disk was finally dropped and ignored. Since the value for "time to drop or finish disk after grabbing" was measured as the time that the uneaten or partially eaten disk was dropped the final time, this repeated grabbing–dropping behavior could perhaps explain why these values were not different from disks soaked in either mussel extract in ASW or in mussel extract in tannic acid (10^{-3} or 10^{-4} M) even though the values for "amount of disk eaten" were different for these two types of disks.

Phloroglucinol and ferulic acid did not induce lobsters to either grab or eat disks which were impregnated with them at any concentration (10^{-2}, 10^{-3}, or 10^{-4} M). Disks that were soaked with mussel extract in either phloroglucinol or ferulic acid (10^{-2}, 10^{-3}, or 10^{-4} M) were not treated differently by lobsters than were disks soaked with mussel extract in ASW. Neither phloroglucinol nor ferulic acid had a significant effect (randomized block analysis of variance, $P > 0.05$) on any of the measured feeding behaviors, including "amount of disk eaten."

DISCUSSION

The neurophysiological data in this study clearly demonstrate that chemoreceptor neurons in the antennules and walking legs of lobsters are sensitive to various secondary plant compounds at a concentration of 10^{-5} M. When the mean responses were scaled relative to an equimolar concentration of L-glutamate, one of the chemicals most stimulatory to *H. americanus* chemoreceptors (Shepheard, 1974; Derby and Atema, 1982a,c), many compounds were found to be 20–35% as excitatory as glutamate, and one was 57% as effective (Table 1). What must be addressed is how lobsters use such receptors.

Curiously, the behavioral experiments suggest that lobsters do not use this antennular or leg input regarding secondary plant compounds. Neither attraction to the food odor source (mediated primarily by antennular

chemoreceptors: Reeder and Ache, 1980; Devine and Atema, 1982) nor grasping of the food (mediated by leg chemoreceptors: Derby and Atema, 1982b) was affected by tannic acid, phloroglucinol, or ferulic acid, even though all of these compounds excited leg and antennular receptors and ferulic acid gave the largest overall chemoreceptor response of all 14 secondary plant compounds. Tannic acid did reduce food ingestion and caused repeated dropping of mussel extract-soaked disks. But this behavioral change is very likely due to mouthpart chemoreceptors (Derby and Atema, 1982b) which were not studied here physiologically. Tannic acid did not affect those behaviors dependent on antennular or leg chemoreceptor input, the focus of this study. Why then are these receptors a part of the lobster's antennular and leg sensory system?

One explanation which cannot be discounted is that these receptors may be involved in evoking some other type of behavior, possibly unrelated to feeding, which was not monitored in the present behavioral experiments.

Another possibility is that the concentrations used in this study were too low to evoke food rejection and that if concentrations were increased, the neural activity carried by legs or antennules would increase to the point of eliciting food rejection. Arguing against the possibility is the fact that the concentration tested for each of the three compounds in these behavioral experiments (disks soaked in a 1–2 mg/ml solution) was 100 times greater than the concentration used in the electrophysiological experiments and was as great or greater than the effective concentrations for these same compounds on other marine invertebrates. Tannic acid caused over 50% feeding reduction in snails (*Littorina littorea*) at 0.01 mg/g medium, and phloroglucinol caused 40% reduction at 0.1 mg/g medium (Geiselman, 1980; Geiselman and McConnell, 1981). Ferulic acid reduced feeding by amphipod crustaceans (*Orchestia grillus*) and snails (*Melampus bidentatus*) at 3 mg/g medium (Valiela et al. 1979).

A third hypothesis is offered by Dethier (1980). When he analyzed receptor sensitivity of three insect species, two herbivores and one nonherbivore, he found that the nonherbivorous insect (blowfly, *Phormia regina*) had fewer types of cells that were sensitive to secondary plant substances. However, *Phormia* did resemble the herbivores in possessing a salt receptor. Although this cell type is sensitive to many secondary plant substances, it is probably relatively unimportant in the discrimination of such compounds. Dethier suggests that since salt sensitivity is found in virtually all animals (Dethier, 1977), it is possible that a salt cell represents a receptor type that has changed little from those that constitute an undifferentiated "common chemical sense" (Dethier, 1980). In the nonherbivorous lobster, such an undifferentiated type of cell may be responsible for the detection of secondary plant substances without causing an effect on food acquisition behavior. Such

an unused receptor capability may be an important substrate for future evolution of more specialized receptors.

The behavioral experiments with tannic acid (Table 2) further indicate that marine algae which contain polyphenols related to this compound (Geiselman, 1980; Geiselman and McConnell, 1981) may derive some protection against ingestion by lobsters. Factors other than chemical inhibitors (e.g., attractiveness, palatability, texture and toughness, nutritive value) undoubtedly also contribute to the low preference of lobsters for plants (Squires, 1970; Weiss, 1970; Miller et al., 1971; Ennis, 1973; personal observations).

The repeated grabbing–dropping behavior observed for lobsters eating disks soaked in a mixture of mussel extract and tannic acid is similar to that observed by Luther (1930) for *Carcinus maenas* eating pieces of blotting paper soaked with acetic acid. It also resembles the behavior of lobsters with deafferented mouthpart chemoreceptors attempting to eat a preferred food, *Mytilus edulis* (Derby and Atema, 1982b). This behavior may be the crustacean equivalent to "mouthing" behavior as described for fish feeding on distasteful items (Kinnel et al., 1979) and for fish with mouth taste lesions (Atema, 1971). Thus, lobsters are no exception to the great variety of animals, including invertebrates and vertebrates (Waldbauer and Fraenkel, 1961; Atema, 1971; Dethier, 1973, 1976, 1980), which use the mouth taste system to judge food palatability by integrating information about a presence of unacceptable chemicals or a lack of acceptable chemicals.

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FATE OF PHOTOSENSITIZING FURANOCOUMARINS IN TOLERANT AND SENSITIVE INSECTS

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Abstract—The fate of [^{14}C]xanthotoxin (8-methoxypsoralen) was studied in larvae of insect species that are tolerant (*Papilio polyxenes* Stoll) or sensitive (*Spodoptera frugiperda* J.E. Smith) to the phototoxic effects of photosensitizing psoralens. Both insects metabolize xanthotoxin by oxidative cleavage of the furan ring, but the detoxification occurs at a much more rapid rate in *P. polyxenes* in which >95% of an oral 5 μg/g xanthotoxin dose is metabolized within 1.5 hr after treatment. The detoxification of psoralens by *P. polyxenes* appears to occur primarily in the midgut tissue prior to absorption, with the result that the intact phototoxin does not reach appreciable levels in body tissues. Studies with an angular furanocoumarin indicated that isopsoralens are metabolized by *P. polyxenes* at a somewhat slower rate than observed for psoralens; however, a reduced rate of metabolic detoxification of isopsoralens probably does not explain the fact that psoralen tolerance in *P. polyxenes* does not extend to the isopsoralen series.

Key Words—Xanthotoxin, psoralen, isopsoralen, furanocoumarin, *Papilio polyxenes, Lepidoptera, Papilionidae, Spodoptera frugiperda,* Noctuidae, metabolism, detoxification, tolerance.

INTRODUCTION

Furanocoumarins occur widely in nature as constituents of hundreds of plant species, particularly among the Umbelliferae and Rutaceae (Pathak et al., 1962; Ivie, 1978b; Berenbaum, 1981b). Linear furanocoumarins (psoralens) arise biogenetically through reactions ultimately leading to a linear fusion of the furan and coumarin rings at the 6, 7 positions, whereas in the isopsoralen series the fusion is angular at the 7, 8 positions.

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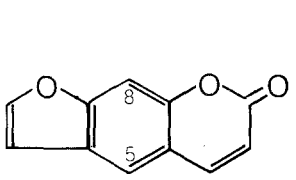
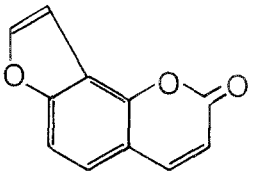


PSORALEN



ISOPSORALEN

Furanocoumarins have been well studied because of their photoactive properties when activated by long-wavelength ultraviolet light. Psoralens in particular have been used since ancient times in the treatment of certain human skin disorders, particularly skin depigmentation (vitiligo) and, more recently, psoriasis (Scott et al., 1976; Van Scott, 1976; Stern et al., 1979). Furanocoumarins form photoinduced mono- and/or diadducts with DNA, a property that accounts for certain of their medicinal and other biological effects (Scott et al., 1976), but which also leads to potentially serious phototoxicological problems in humans (Pathak et al., 1962; Ashwood-Smith et al., 1980; Ivie et al., 1981) and other organisms, including grazing livestock (Ivie, 1978b). The medicinal use of psoralens has been correlated with an increased incidence of skin cancer in man (Stern et al., 1979; Grekin and Epstein, 1981).

Furanocoumarins in nature appear to be act as phytoalexins (Beier and Oertli, 1983), as allelopathic agents (Friedman et al., 1982; Shimomura et al., 1982), and as allomones against insect, mammalian, and other herbivores. Furanocoumarins are well documented as livestock phototoxins (Ivie, 1978b) and, in recent years, the role of these compounds as insect antifeedants and insect phototoxins has received increasing attention (Berenbaum, 1978; Muckensturm et al., 1981).

Because many natural furanocoumarins (particularly psoralens) are potent nonspecific phototoxins, it would be expected that psoralen-rich plants, particularly those in habitats of high light intensity, would not constitute major forage targets for herbivorous vertebrate and invertebrate species. Some insects, however, particularly larvae of certain species of Lepidoptera in the family Papilionidae, are known to feed successfully and, in fact, preferentially on psoralen-rich plants, a phenomenon whose coevolutionary implications have been considered in detail (Berenbaum, 1981a,b,; Berenbaum and Feeny, 1981). We have initiated studies aimed at evaluating the extent to which metabolic detoxification contributes to the successful circumvention by such butterflies of the phototoxic effects of dietary psoralens (Ivie et al., 1983), and we report here extended observations on the interactions of both linear and angular furanocoumarins in psoralen-tolerant (*Papilio polyxenes* Stoll) and psoralen-sensitive (*Spodoptera frugiperda* J.E. Smith) insect species.

METHODS AND MATERIALS

Insects. The black swallowtail butterfly, *Papilio polyxenes* Stoll (Lepidoptera: Papilionidae), and the fall armyworm, *Spodoptera frugiperda* J.E. Smith (Lepidoptera: Noctuidae), were studied as representative psoralen-tolerant and -sensitive insect species. The dietary preference of *P. polyxenes* for psoralen-rich plants is well documented (Berenbaum, 1981a), and *Spodoptera* spp., althoughly highly polyphagous (or generalized) insect herbivores, are known to be unwilling or unable to thrive on plants that contain appreciable levels of psoralens (Berenbaum, 1978).

Furanocoumarins. Unlabeled samples of authentic furanocoumarins were obtained as follows: psoralen from M.A. Pathak, Harvard Medical School, Boston, Massachusetts; xanthotoxin (8-methoxypsoralen) from Biochemical Laboratories, Redondo Beach, California; and isopsoralen from HRI Associates, Emergyville, California. A radiocarbon-labeled sample of xanthotoxin was prepared by demethylation of xanthotoxin to xanthotoxol as previously described (Ivie, 1978a), followed by remethylation with [^{14}C]methyl iodide in acetone–potassium carbonate. The resulting [^{14}C]xanthotoxin (9.86 mCi/mM) was purified by thin-layer chromatography (see below) to a radiochemical purity of >99%. Xanthotoxin was chosen as the major compound of study because it is a potent photosensitizing psoralen that is a common constituent of psoralen-containing plants (Pathak et al., 1962).

In Vivo Metabolism of [^{14}C]*Xanthotoxin.* All studies were conducted with last-instar larvae of *P. polyxenes* or *S. frugiperda* that had been reared since hatched on sprigs of fresh parsley obtained from a local supermarket. Larvae were weighed individually, and the appropriate [^{14}C]xanthotoxin dosage was calculated for each larva. Weighed larvae were held without food for 2 hr prior to treatment. The appropriate amount of [^{14}C]xanthotoxin (in several μl of acetone carrier) was spread as uniformly as possible with a microsyringe over the dorsal surface of small parsley twigs, the solvent was allowed to dry for a few minutes, and then the treated parsley was offered to the individual larvae. Two treatment levels were used for both insect species—5 μg xanthotoxin/g body weight and 100 μg/g. In most cases, essentially all of the treated parsley was consumed within 5–15 min, with the exception of armyworms treated at 100 μg/g. Armyworms showed great reluctance in consuming parsley treated at the higher dosage rate; ultimately a 6-hr starvation period prior to treatment was used for armyworms treated at 100 μg/g in order to obtain good dosage consumption. With each treated insect, the actual amount of [^{14}C]xanthotoxin consumed was calculated by subtracting the radiocarbon remaining on any unconsumed parsley (as determined by liquid scintillation counting) after feeding had stopped or, at most, 15 min after the treated parsley was offered. Immediately after

treatment, insects were transferred to individual covered glass petri dishes, fed untreated parsley, and held at 32°C until sacrificed.

For each species, dosage level, and sacrifice interval, 4–8 replicates were run. In all experiments, several "extra" replicates were done to allow for the periodic circumstance encountered where a given larva refused to consume the dosage offered within 15 min—such replicates were discarded at that point. In all samples ultimately analyzed, the level of radiocarbon consumption within 15 min exceeded 95% of that offered. At the 5 μg/g treatment level the [^{14}C]xanthotoxin was used as synthesized (9.86 mCi/mM), but for the 100 μg/g treatments, the radiochemical was first diluted with unlabeled xanthotoxin to a specific activity of 0.49 mCi/mM.

At appropriate sacrifice intervals, larvae were quickly frozen and held for later analysis. All frass deposited posttreatment was collected and held frozen for later study. Subsequent washes of the petri dishes used to hold the treated larvae during the posttreatment period contained essentially no radiocarbon as determined by liquid scintillation counting.

Action of P. polyxenes *Gut Contents on* [^{14}C]*Xanthotoxin.* To evaluate the possible role that gut microflora or enzymes secreted into the gut lumen might play in the metabolism of furanocoumarins in *Papilio,* in vitro studies were conducted in which gut contents of *P. polyxenes* larvae were incubated with [^{14}C]xanthotoxin. For these studies, 5 μg of [^{14}C]xanthotoxin in acetone carrier were pipetted into each of several 20-ml-capacity glass scintillation vials and the solvent was allowed to evaporate. Phosphate buffer (0.2 M, pH 7.8, 2.0 ml) was added to each vial, then the entire gut contents from a single freshly dissected *P. polyxenes* larva were added to each vial. The vials were flushed with carbon dioxide or nitrogen gas, quickly and tightly capped, and incubated for 12 hr in a shaking water bath at 32°. After incubation, samples were immediately frozen for later analysis.

Xanthotoxin Metabolism by Midgut and Whole Body Insect Preparations. [^{14}C]Xanthotoxin (25 μg in 25 μl acetone) was pipetted into each of several 20-ml-capacity glass scintillation vials, the solvent allowed to evaporate, and phosphate buffer (2.0 ml) was added to each vial. Last instar larvae of both insect species were dissected under cold buffer and the midguts were removed, slit open lengthwise, and the gut contents washed out with cold buffer and discarded. To each of the prepared scintillation vials was added one *P. polyxenes* midgut (cut into three approximately equal pieces) or three individual armyworm midguts. Similarly, the bodies of the dissected larvae (exclusive of gut and contents) were added to prepared vials (one *P. polyxenes* body/vial cut into nine approximately equal pieces or three armyworm bodies each cut into three pieces). The total wet weight of midgut tissue/vial averaged 86 mg and 91 mg for *P. polyxenes* and *S. frugiperda,* respectively; comparable average weights for the body tissues were 480 mg

and 469 mg. Nonenzymatic controls consisted of appropriate insect midgut or body preparations that were held in buffer at 100° for 30 min prior to addition to the vials containing [^{14}C]xanthotoxin. All samples (uncapped) were incubated at 32° C in a shaking water bath for 4 hr and then capped and frozen.

Comparative Degradation of Xanthotoxin, Psoralen, and Isopsoralen by P. polyxenes *Midgut Tissue Preparations.* To evaluate the relative rates of degradation of these compounds by *P. polyxenes* midgut tissue, single midguts were incubated in buffer with 25 μg of unlabeled xanthotoxin, psoralen, or isopsoralen as described above for studies with [^{14}C]xanthotoxin. After 4 hr incubation at 32°, the samples were frozen for later analysis. Six replicates were run for each compound, and appropriate controls (heat-deactivated midgut tissue) were included.

Fate of Isopsoralen in P. polyxenes *In Vivo.* To evaluate certain aspects of the kinetics of an angular furanocoumarin in *P. polyxenes,* last-instar larvae were fed parsley twigs treated with unlabeled isopsoralen at a dosage equivalent to 5 μg/g, using procedures identical to those described above. At 1.5 or 3 hr after dosing, the larvae were quickly frozen for later analysis of levels of unmetabolized isopsoralen in body tissues. Four replicates were run for each sampling interval.

Sample Extraction and Analysis. Samples of larval excreta, gut and contents, or body tissues from the in vivo studies with [^{14}C]xanthotoxin were transferred to 50-ml-capacity glass culture tubes, distilled water (10 ml) was added, and the samples were adjusted to pH $\leq$ 2.0 using concentrated HCl. Ethyl acetate (10–15 ml) was added to the samples, which were then homogenized thoroughly (Willems Polytron, Brinkmann, Westbury, New York). After centrifugation to break emulsions, the ethyl acetate was pipetted off and the extraction step was repeated as above 2–4 times, depending on the sample. Aliquots of the organic extracts (combined) and aqueous/residue slurries were subjected to liquid scintillation counting (LSC) to quantitate the radiocarbon levels present in each fraction. The organic phases were dried over anhydrous sodium sulfate, concentrated to a small volume (~0.1 ml) by rotary evaporation and finally a gentle stream of nitrogen, then subjected to thin-layer chromatographic (TLC) analysis.

Samples from the in vitro [^{14}C]xanthotoxin studies were transferred to culture tubes, water (8 ml) was added (first rinsing the sample vial), then ethyl acetate (15 ml) was added (first rinsing the sample vial). These samples were subsequently acidified, extracted, the radiocarbon content quantitated, and the organic phases prepared for TLC as described above.

TLC resolution of [^{14}C]xanthotoxin metabolites was accomplished using precoated silica gel chromatoplates (Silplate F-254, 20 $\times$ 20 cm, 0.25 mm gel thickness, with fluorescent indicator, Brinkmann). Extracts were

spotted as short bands near one edge of the plates, the plates were developed in a mixture of ethyl acetate–methanol–glacial acetic acid (150 : 50 : 2), then exposed to X-ray film (Kodak No-Screen) for 7 days to visualize the resolved [^{14}C]components. Quantitation was effected by scraping the appropriate gel regions into scintillation vials for LSC. In all LSC determinations, appropriate corrections for quench were made.

Samples from studies that involved incubation of unlabeled xanthotoxin, psoralen, or isopsoralen with *P. polyxenes* midgut tissue were diluted with 8 ml of water, then extracted three times with 20 ml of diethyl ether. The combined ether extracts were dried over sodium sulfate, then concentrated to dryness. The residue was taken up in 1.0 ml of 5% chloroform in hexane, and this solution was passed through a silica SEP-PAK (Waters Associates, Milford, Massachusetts) that had been prewashed with ~5 ml of the same solvent mixture. After application of the sample to the SEP-PAK (discarding eluate), an additional 4.0 ml of 5% chloroform in hexane was used to rinse the sample flask and this too was passed through the SEP-PAK, discarding the eluate. Finally, the SEP-PAK was eluted with 5 ml of 7.5% ethyl acetate in chloroform to recover the appropriate psoralen or isopsoralen. The final eluate was concentrated to dryness, dissolved in 2.5 ml of chloroform, and 20-μl aliquots were analyzed directly by high-performance liquid chromatoggraphy (HPLC) (see below).

Body tissue samples (whole body less gut and contents) from *P. polyxenes* larvae treated with isopsoralen were extracted and cleaned up by silica SEP-PAK exactly as described above but were subjected further to the following cleanup procedure. The final eluates off silica SEP-PAKs were concentrated to dryness and the residue dissolved in 0.6 ml of acetonitrile. Water (0.4 ml) was added, and this solution was passed through a C18 SEP-PAK (eluate collected) that had previously been activated by flushing with 5 to 10-ml volumes of acetonitrile and then water. The sample flask was rinsed with 0.6 ml of acetonitrile, 0.4 ml of water was added, and this was flushed through the C18 SEP-PAK. This procedure was repeated three additional times, and the combined eluates were concentrated to dryness by rotary evaporation (anhydrous ethanol was added to effect azeotropic distillation of the water present). Finally, the residue was dissolved in 0.5 ml of chloroform and 20-μl aliquots were analyzed by HPLC. Recovery studies with midgut and body tissues fortified with 25 μg of the appropriate furanocoumarin standards were done to confirm the efficiency of the described extraction, cleanup, and analysis procedures.

HPLC studies were conducted with a Waters model M-6000 pump and a Tracor model 970A variable wavelength detector set at 250 nm. The column was a 25-cm Supelco 5 μm silica, and the solvent was 0.1% ethyl acetate and 0.1% formic acid in chloroform. Flow was 2.5 ml/min. Sample peaks were

recorded and integrated with a Hewlett Packard model 3390A integrator. Retention times (minutes) under these parameters were as follows: isopsoralen, 5.1; psoralen, 6.1; xanthotoxin, 11.5.

Semipreparative Isolation and Characterization of Xanthotoxin Metabolites in Excreta of P. polyxenes. To obtain sufficient quantities of xanthotoxin metabolites for characterization studies, last-instar larvae of *P. polyxenes* were continuously fed a diet of fresh parsley twigs that had been dipped in an acetone solution (2.5 mg/ml) of unlabeled xanthotoxin. Preliminary studies had indicated that the use of [^{14}C]xanthotoxin was not required for these studies because the two major xanthotoxin metabolites (see below) were easily visualized on TLC plates when viewed under long-wavelength UV light.

Excreta samples (1.0 g) from these worms were added to 15 ml of water, acidified to pH $\leq$ 2.0, then extracted five times with 15- to 20-ml volumes of ethyl acetate. The combined organic extracts were dried over sodium sulfate, concentrated to dryness, and the residue dissolved in acetone for direct application to TLC. Samples were applied as bands to TLC (0.25 or 0.50 mm gel thickness), and plates were developed in the ethyl acetate-methanol-acetic acid solvent mixture. The two major metabolite bands were scraped, and the least polar major metabolite (metabolite 1, R_f 0.46) was eluted with ethyl acetate containing 1% acetic acid. The more polar major product (metabolite 2, R_f 0.12) was eluted from the gel with methanol.

The solution that contained metabolite 1 was concentrated to dryness by rotary evaporation and finally a gentle stream of nitrogen. The residue was dissolved in a small amount of hot ethyl acetate, and metabolite 1 was crystallized by the addition of hexane. The crystalline material was of excellent purity on the basis of TLC and subsequent spectral analysis (see below).

Attempts at crystallizing metabolite 2 by a variety of techniques were unsuccessful. Metabolite 2 exhibited some instability during manipulation as evidenced by its degradation to products that remained at or streaked from the origin on TLC, and for these reasons, it was methylated with an ether–ethanolic solution of diazomethane prior to further attempts at purification. The methylated metabolite 2 was subsequently purified by application to TLC with development in a solvent mixture of ethyl acetate–glacial acetic acid (99:1). The product was recovered by eluting the gel with ethyl acetate, and it readily crystallized from ethyl acetate–hexane in good purity as evidenced by its chromatographic and spetral behavior.

Nuclear magnetic resonance (^{1}HNMR) studies were done in deuteriomethanol on a JEOL FX-90Q instrument. Chemical shifts are reported as parts per million downfield from tetramethylsilane using the central deuteriomethanol peak (3.30 δ) as an internal standard. Mass spectral analyses were

done using a Varian/MAT CH-7 magnetic scan spectrometer. Sample volatilization was effected by heated direct insertion probe, and the ionization voltage was set at 70 eV.

RESULTS

Fate of [^{14}C]*Xanthotoxin In Vivo.* Larvae of *P. polyxenes* and *S. frugiperda* treated with [^{14}C]xanthotoxin at 5 μg/g or 100 μg/g eliminate almost all of the dosage with 24 hr posttreatment, although it is apparent that armyworm larvae retain a somewhat greater percentage of radiocarbon in the gut and in body tissues than do butterfly larvae (Table 1). There are, however, no apparent dose-dependent differences in excretion rates in either insect species.

TLC analysis of extracts of excreta showed that very little unmetabolized xanthotoxin is eliminated by these larvae—levels of xanthotoxin per se never exceed 10% of the administered dose in either species although levels of xanthotoxin in excreta of armyworms are somewhat higher than in butterfly larvae (Table 1). TLC resolution of metabolites in excreta extracts shows that detected routes of xanthotoxin metabolism are qualitatively the same in both species. Four metabolites are seen, of which two (metabolites 1 and 2) comprise by far the bulk of excreta radiocarbon in *P. polyxenes.* Armyworm excreta contains greater amounts of radiocarbon that are not extracted by ethyl acetate from the water–residue slurry, indicating that the rate of metabolism to more polar products is somewhat greater in *S. frugiperda* than in *P. polyxenes.*

Although studies in which treated insects and their excreta were analyzed 24 hr after dosing do not demonstrate major species differences in xanthotoxin disposition rates, further studies in which analyses were done at shorter intervals posttreatment showed clear and highly significant differences in xanthotoxin kinetic behavior between the two species. Data from these studies (Table 2) show that the rates of xanthotoxin absorption from the gut are rapid and are not appreciably different between species (as indicated by disappearance of radiocarbon from the gut and contents); however, radiocarbon excretion is much more rapid and body burdens of radiocarbon are much lower in *P. polyxenes* than in *S. frugiperda.* Thus, *P. polyxenes* excretes 50% of the administered radiocarbon within 1.5 hr after dosing, while *S. frugiperda* eliminates only about 1% within 1.5 hr. The result of these dramatic differences in excretion rates is that *S. frugiperda* contains >50% of the administered radiocarbon in body tissues 1.5 hr after dosing, whereas the comparable value for *P. polyxenes* is <4% (Table 2).

Analysis of body tissues (entire carcass exclusive of gut and contents) confirms that xanthotoxin is metabolized much more rapidly in *P. polyxenes*

TABLE 1. DISTRIBUTION OF RADIOCARBON 24 HOURS AFTER ORAL TREATMENT OF *Papilio polyxenes* AND *Spodoptera frugiperda* LARVAE WITH [^{14}C]XANTHOTOXIN AT 5 μg/g OR 100 μg/g[a]

	% of total administered ^{14}C as indicated metabolite or fraction (±SD)							
	Excreta[b, c]						Carcass	
Species and dosage level	Xanthotoxin	Metabolite 1	Metabolite 2	Unknown a	Unknown b	Water soluble/ unextractable[d]	Gut and contents	Body
P. polyxenes								
5 μg/g	1.3 ± 0.2	21.4 ± 8.8	66.6 ± 8.4	1.7 ± 0.4	1.7 ± 0.2	8.3 ± 0.9	0.2 ± 0.2	0.2 ± 0.2
100 μg/g	4.7 ± 0.2	25.3 ± 6.4	60.8 ± 6.8	2.7 ± 0.2	3.6 ± 0.4	8.9 ± 0.8	0.3 ± 0.1	0.2 ± 0.1
S. frugiperda								
5 μg/g	4.6 ± 0.8	38.2 ± 4.2	6.0 ± 0.6	6.5 ± 1.4	5.5 ± 3.2	27.8 ± 3.8	1.7 ± 0.8	2.4 ± 0.9
100 μg/g	9.8 ± 2.0	36.2 ± 1.8	9.5 ± 1.2	6.5 ± 1.2	3.7 ± 0.6	29.4 ± 2.2	1.6 ± 0.4	1.6 ± 0.4

[a] Last instar larvae. All insects starved 2 hr prior to dosing, except that *S. frugiperda* larvae treated at 100 μg/g were starved 6 hr.
[b] Data from total excreta eliminated during the 24-hr period after dosing.
[c] Products resolved by silica TLC developed in ethyl acetate–methanol–glacial acetic acid (150:50:2). R_f values as follows: xanthotoxin, 0.67; metabolite 1, 0.46; metabolite 2, 0.12; unknown a, 0.26; unknown b, 0.51.
[d] Radiocarbon remaining in the water/residue slurry after extraction with ethyl acetate.

TABLE 2. RADIOCARBON DISTRIBUTION AFTER ORAL TREATMENT OF *Papilio polyxenes* AND *Spodoptera frugiperda* WITH [^{14}C]XANTHOTOXIN AT 5 μg/g

Species and analysis interval posttreatment (hr)	% of administered radiocarbon in indicated fraction (±SD)		
	Excreta	Gut and contents	Body
P. polyxenes			
1.5	50.3 ± 6.8	39.9 ± 7.7	3.6 ± 1.0
3	62.4 ± 4.2	24.9 ± 5.4	3.3 ± 1.8
6	77.6 ± 0.8	3.4 ± 2.2	0.2 ± 0.1
12	77.6 ± 6.0	0.2 ± 0.1	0.1 ± 0.1
24	100.9 ± 5.6	0.2 ± 0.2	0.2 ± 0.2
S. frugiperda			
1.5	0.9 ± 1.1	32.4 ± 4.2	54.6 ± 4.1
3	18.3 ± 2.7	26.4 ± 2.1	36.7 ± 4.7
6	41.6 ± 16.5	10.7 ± 2.2	15.0 ± 4.6
12	59.7 ± 8.0	4.6 ± 3.1	4.3 ± 2.2
24	87.8 ± 6.7	1.8 ± 0.8	2.4 ± 0.9

than in *S. frugiperda* (Table 3). In armyworms, as much as 41% of the administered radiocarbon is recovered as unmetabolized xanthotoxin in body tissues 1.5 hr after dosing, but this parameter never exceeds 1% in butterfly larvae. At comparable analysis intervals, the levels of xanthotoxin per se in body tissues of *S. frugiperda* (when expressed as percentage of administered radiocarbon) range from 58- to >300-fold higher than those experienced by *P. polyxenes*. As indicated in Table 3, the metabolites detected in body tissues of both species are the same as those seen in excreta (Table 1).

TLC analyses of the gut and its contents from the treated worms (Table 4) again show the range of [^{14}C]components seen in excreta and body tissues. Somewhat surprisingly, only a minor amount (2.5% of dose) of radiocarbon in *P. polyxenes* gut samples analyzed 1.5 hr after treatment is in the form of unmetabolized xanthotoxin—the data in Tables 1, 3, and 4 thus indicate that >95% of an oral 5 μg/g dose of xanthotoxin in *P. polyxenes* is metabolized within 1.5 hr after dosing. In *S. frugiperda*, as might be predicted, unmetabolized xanthotoxin comprises the major radioactive component in gut samples at the early analysis intervals (Table 4).

Action of P. polyxenes, *Gut Contents on* [^{14}C]*Xanthotoxin.* Because of the very rapid rate of [^{14}C]xanthotoxin degradation observed in the in vivo studies with *P. polyxenes*, it was speculated that microbial populations or other enzymatic systems within the gut lumen might be primarily responsible

TABLE 3. COMPARATIVE DISTRIBUTION OF RADIOCARBON IN BODY TISSUES (WHOLE BODY EXCLUSIVE OF GUT AND CONTENTS) AFTER ORAL TREATMENT OF *Papilio polyxenes* AND *Spodoptera frugiperda* WITH [^{14}C]XANTHOTOXIN AT 5μg/g

Species and analysis interval posttreatment (hr)	% of administered radiocarbon as indicated metabolite or fraction (±SD)					
	Xanthotoxin	Metabolite 1	Metabolite 2	Unknown a	Unknown b	Water soluble/ unextractable
P. polyxenes						
1.5	0.7 ± 0.3	0.7 ± 0.1	1.4 ± 0.4	0.1 ± 0.1	0.1 ± 0.0	0.6 ± 0.2
3	0.1 ± 0.0	0.7 ± 0.3	1.9 ± 0.4	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1
6	<0.1	<0.1	<0.1	<0.1	<0.1	0.1 ± 0.0
12	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
S. frugiperda						
1.5	41.0 ± 4.7	4.2 ± 3.0	1.3 ± 1.0	0.8 ± 0.4	1.0 ± 0.5	6.2 ± 3.1
3	20.0 ± 2.5	6.0 ± 1.6	1.2 ± 0.4	0.8 ± 0.2	1.5 ± 0.4	7.1 ± 2.2
6	3.5 ± 2.0	5.0 ± 1.6	0.7 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	4.6 ± 1.1
12	0.4 ± 0.1	0.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	2.9 ± 1.0

TABLE 4. DISTRIBUTION OF RADIOCARBON IN GUT TISSUE AND CONTENTS AFTER ORAL TREATMENT OF *Papilio polyxenes* AND *Spodoptera frugiperda* WITH [^{14}C]XANTHOTOXIN AT 5μg/g

Species and analysis interval posttreatment (hr)	% of administered radiocarbon as indicated metabolite or fraction (±SD)					
	Xanthotoxin	Metabolite 1	Metabolite 2	Unknown a	Unknown b	Water soluble/ unextractable
P. polyxenes						
1.5	2.5 ± 0.4	11.7 ± 1.8	21.5 ± 1.6	0.6 ± 0.1	0.7 ± 0.3	2.8 ± 0.1
3	0.5 ± 0.1	6.8 ± 2.0	15.6 ± 2.1	0.6 ± 0.1	0.3 ± 0.0	1.1 ± 0.1
6	<0.1	0.6 ± 0.2	2.1 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.1
12	<0.1	<0.1	<0.1	<0.1	<0.1	0.1 ± 0.1
S. frugiperda						
1.5	17.3 ± 4.3	5.9 ± 2.6	1.7 ± 1.0	1.2 ± 0.3	1.4 ± 0.6	4.9 ± 1.0
3	11.8 ± 2.0	6.2 ± 1.3	1.2 ± 0.3	0.9 ± 0.2	1.1 ± 0.3	5.2 ± 1.6
6	2.0 ± 1.4	4.2 ± 1.2	0.5 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	2.8 ± 0.5
12	0.4 ± 0.1	1.0 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	2.8 ± 1.2

for xanthotoxin degradation in this insect. However, in vitro studies in which gut contents of *P. polyxenes* were incubated with [^{14}C]xanthotoxin showed that this is apparently not the case. TLC analysis of extracts of samples in which the entire gut contents from individual *P. polyxenes* larvae were incubated with [^{14}C]xanthotoxin in buffer for 12 hr showed that essentially no degradation of the radiochemical had occurred. These findings lead to the conclusion that such degradation is of no or, at most, minor significance in the metabolism of xanthotoxin by *P. polyxenes.*

In Vitro Metabolism of [^{14}C]*Xanthotoxin by Midgut and Whole Body Tissue Preparations.* Xanthotoxin is metabolized in vitro by midgut and body tissue preparations of both *P. polyxenes* and *S. frugiperda* to the same metabolites and in the same approximate proportions as is observed in in vivo studies (Table 5). Under the in vitro parameters studied, xanthotoxin metabolism by *P. polyxenes* midgut tissue is quite rapid and only 27% of the initial [^{14}C]xanthotoxin remains unmetabolized after 4 hr incubation. Body tissue of *P. polyxenes* also metabolize xanthotoxin rapidly, but body tissues of *S. frugiperda* are not as active and midgut tissue is even less so (Table 5). Transformations of [^{14}C]xanthotoxin observed in these studies are clearly metabolic in origin, as evidenced by studies in which heat deactivation of tissues prior to the incubations resulted in essentially no breakdown of the [^{14}C]xanthotoxin present (Table 5).

Degradation of Unlabeled Xanthotoxin, Psoralen, and Isopsoralen by P. polyxenes *Midgut Tissue In Vitro.* Studies in which these furanocoumarins were incubated with midgut tissue of *P. polyxenes* indicated that both linear (xanthotoxin, psoralen) and angular (isopsoralen) furanocoumarins are substrates for the midgut metabolizing system (Table 6). About 40–45% of xanthotoxin and psoralen remain unmetabolized after 4 hr, and the lesser degree of degradation observed with isopsoralen suggests that angular furanocoumarins may be somewhat more resistant to enzymatic degradation in *P. polyxenes* midgut tissue. Levels of unmetabolized furanocoumarins quantitated in heat-deactivated samples (Table 6) reflect the range of values seen in recovery experiments aimed at validating the HPLC analytical method used; thus, it is likely that essentially no degradation of the parent psoralens or isopsoralen occurred in the boiled tissue samples. In these studies, the use of unlabeled compounds precluded the identification of metabolites generated, so it was not possible to determine if the metabolism of isopsoralen occurred by pathways analogous to those determined for xanthotoxin (see below).

Fate of Isopsoralen in P. polyxenes *In Vivo.* HPLC analysis of body tissues (entire carcass less gut and contents) of *P. polyxenes* larvae treated with unlabeled isopsoralen at 5 μg/g showed that detectable quantities of this angular furanocoumarin occur in body tissues soon after treatment. Of the

TABLE 5. METABOLISM OF [^{14}C]XANTHOTOXIN BY MIDGUT TISSUE[a] AND BODY TISSUES[b] OF *Papilio polyxenes* AND *Spodoptera frugiperda* IN VITRO[c]

Species and tissue	% radiocarbon as indicated product or fraction (±SD)					
	Xanthotoxin	Metabolite 1	Metabolite 2	Unknown a	Unknown b	Water soluble/ unextractable[d]
P. polyxenes						
Midgut	27.3 ± 12.6	39.7 ± 9.8	25.5 ± 4.4	2.8 ± 0.4	1.4 ± 0.2	3.2 ± 0.5
Midgut (boiled)[e]	98.6 ± 0.0		0.8 ± 0.0[f]			0.6 ± 0.2
Body	33.6 ± 13.0	18.6 ± 7.0	37.5 ± 10.2	1.7 ± 0.4	2.9 ± 1.4	5.6 ± 1.2
Body (boiled)[e]	97.9 ± 0.8		0.6 ± 0.8[f]			1.5 ± 0.6
S. frugiperda						
Midgut	89.1 ± 3.0	2.7 ± 0.4	3.2 ± 3.0	1.7 ± 0.6	1.1 ± 0.2	2.2 ± 0.5
Midgut (boiled)[e]	99.2 ± 0.8		0.6 ± 0.8[f]			0.2 ± 0.2
Body	64.3 ± 3.6	10.9 ± 2.4	3.1 ± 0.3	3.2 ± 0.6	2.3 ± 0.6	16.2 ± 3.9
Body (boiled)[e]	99.1 ± 0.4		0.5 ± 0.4[f]			0.4 ± 0.2

[a] Unhomogenized midgut tissue.
[b] Entire insect body exclusive of gut and contents.
[c] Samples incubated for 4 hr at 32°C in phosphate buffer (see text).
[d] Radiocarbon not extracted by ethyl acetate from the water/tissue slurry.
[e] Enzymatic activity of tissue destroyed by heat prior to incubation.
[f] Cumulative values for all metabolites other than xanthotoxin.

TABLE 6. METABOLISM OF UNLABELED XANTHOTOXIN, PSORALEN, AND ISOPSORALEN BY MIDGUT TISSUE[a] OF *Papilio polyxenes* IN VITRO

Compound	% unmetabolized after 4 hr (±SD)[b]
Xanthotoxin	40.3 ± 13.3
Xanthotoxin (boiled tissue)[c]	95.2 ± 1.8
Psoralen	43.6 ± 11.1
Psoralen (boiled tissue)[c]	102.0 ± 7.1
Isopsoralen	68.8 ± 5.0
Isopsoralen (boiled tissue)[c]	95.0 ± 4.2

[a] Unhomogenized midgut tissue.
[b] As quantitated by high performance liquid chromatography (see text).
[c] Enzymatic activity of tissue destroyed by heat prior to incubation.

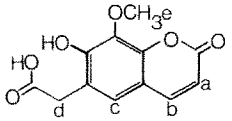
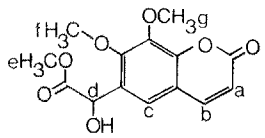
total administerd isopsoralen, 3.7 ± 1.6% and 0.4 ± 0.3% were found outside the gut wall at 1.5 and 3 hr posttreatment, respectively. These values are higher than those quantitated for xanthotoxin in similar studies (0.7% and 0.1%, Table 3), but much lower than levels of xanthotoxin in bodies of xanthotoxin-treated armyworms (41.0% and 20.0%, Table 3).

Characterization of Xanthotoxin Metabolites. Two of the four xanthotoxin metabolites were definitely characterized. The structures of metabolite 1 and of the dimethyl derivative of metabolite 2 are shown in Table 7, along with pertinent NMR and mass spectral data leading to the indicated structural assignments.

The proton NMR spectrum of metabolite 1 was simple and easily interpreted. Protons a, b, c, and the *O*-methyl protons e were readily assigned upon comparison of these NMR data with those from xanthotoxin itself. The only other NMR signal observed for the metabolite was a two-proton resonance at 3.65 ppm which was assigned as the methylene protons d. Mass spectral data for metabolite 1 supported the assigned structure. The molecular ion was at m/z 250, and the major fragmentation patterns (Table 7) were fully consistent with the metabolite being a simple hydroxy acid resulting from cleavage of the furan ring.

Similar to the situation with metabolite 1, NMR data from the dimethyl derivative of metabolite 2 indicated that the structure of the metabolite was a dihydroxy acid, shown in Table 7 as the dimethyl derivative. The proton assignments were very straightforward. Mass fragmentation patterns confirmed the structure of the product as shown (Table 7). Spectral data for metabolites 1 and 2 were fully consistent with those reported earlier for these compounds isolated as xanthotoxin metabolites from mammals (Kolis et al., 1979; Schmid et al., 1980).

TABLE 7. NMR AND MASS SPECTRAL DATA FOR METABOLITES OF XANTHOTOXIN IN *Papilio polyxenes* AND *Spodoptera frugiperda*

Metabolite and structure	NMR data[a]				Mass spectral data[b]		
	Proton	PPM	Coupling (Hz)	Integration	*m/z*	Relative intensity	Fragment
Metabolite 1 	a	6.19	d (10	1	250	94	$[M]^+$
	b	7.82	d (10	1	232	82	$[M-H_2O]^+$
	c	7.17	s	1	204	100	$[M-H_2O, CO]^+$
	d	3.65	s	2	175	88	$[M-H_2O, CO, CHO]^+$
	e	3.95	s	3			
Metabolite 2 (dimethyl derivative) 	a	6.34	d (10)	1	294	45	$[M]^+$
	b	7.89	d (10)	1	279	1	$[M-CH_3]^+$
	c	7.39	s	1	278	4	$[M-CH_3, H]^+$
	d	5.40	s	1	235	100	$[M-CO_2CH_3]^+$
	e	3.72	s	3	220	43	$[M-CO_2CH_3, CH_3]^+$
	f	4.00	s	3	207	50	$[M-CO_2CH_3, CO]^+$
	g	3.99	s	3	192	56	$[M-CO_2CH_3, CO, CH_3]^+$

[a]Samples run in deuteriomethanol.
[b]Electron impact, 70 eV.

No major attempts were made to isolate or otherwise study in detail unidentified metabolites a and b because these products were quantitatively minor in almost all samples (Tables 1, 2, 4, 5).

DISCUSSION

Our studies on the fate of photosensitizing furanocoumarins in psoralen-tolerant (*Papilio polyxenes*) and psoralen-sensitive (*Spodoptera frugiperda*) insect species have established that the rate of metabolic detoxification is the major determinant of tolerance. Although larvae of both insect species metabolize single oral doses of xanthotoxin (8-methoxypsoralen) almost completely prior to excretion, the rate of metabolism and subsequent excretion of the metabolites is much higher in the psoralen-tolerant *P. polyxenes* than in the psoralen-sensitive *S. frugiperda* (Table 2). In *P. polyxenes,* much, and perhaps almost all, of an oral xanthotoxin dose appears to be metabolized in the midgut tissue prior to absorption into body tissues. The result is that appreciable levels of intact photosensitizer do not reach the general body circulation where detrimental light-induced interactions with dermal or subdermal body tissues would be expected to occur. In armyworms, however, the relatively slow rate of xanthotoxin metabolism is such that as much as 40% of the initial dose reaches body tissues unmetabolized (Table 2).

On the basis of the structural characterization of metabolites 1 and 2, it is clear that oxidative opening of the furan ring is the major mechanism of metabolic degradation for xanthotoxin (and presumably other psoralens) in *P. polyxenes*. Although xanthotoxin degradation in both *P. polyxenes* and *S. frugiperda* appears to be qualitatively the same, there are appreciable quantitative differences, particularly in that armyworms generate lesser amounts of metabolite 2 and considerably greater amounts of water soluble and/or unextractable metabolites. Although metabolites 1 and 2 have not previously been reported as xanthotoxin degradation products in insects or any other invertebrate species, they have been isolated as products of xanthotoxin metabolism in mammals Kolis et al., 1979; Schmid et al., 1980). We do not know the pathways involved in the generation of the xanthotoxin metabolites characterized, but others have postulated in mammalian studies that a short-lived epoxide precursor may be involved in the formation of these two products (Kolis et al., 1979). We did obtain good (although perhaps not conclusive) evidence in our studies that metabolite 1 is not a precursor of metabolite 2. Solvent extracts of excreta from *P. polyxenes* larvae treated orally with purified [^{14}C]metabolite 1 contained almost exclusively unmetabolized 1 with no traces of metabolite 2.

Limited studies in *P. polyxenes* with isopsoralen were done to evaluate

potential differences in the disposition of linear and angular furanocoumarins in this insect. Although *P. polyxenes* is highly tolerant to the toxic effects of linear furanocoumarins (psoralens), angular furanocoumarins (isopsoralens) are antifeedants and are toxic to this species (Berenbaum and Feeny, 1981)—a puzzling observation in view of the fact that isopsoralens are generally considered to be much less photoactive than psoralens (Scott et al., 1976). Psoralens and isopsoralens differ only in the angle of fusion of the furan and coumarin rings, and because the furan ring of psoralens is the major site of metabolism by *P. polyxenes,* we hypothesized that isopsoralen might be immune or highly resistant to metabolic attack. Our data with isopsoralen do not lend appreciable support to that hypothesis. Isopsoralen is, in fact, metabolized by midgut tissue of *P. polyxenes* in vitro, although at a somewhat slower rate than observed for either xanthotoxin or psoralen (Table 6). Further, in vivo studies indicate that, at most, ~4% of an oral 5 μg/g isopsoralen dose can be detected in body tissues 1.5 hr after treatment. These data suggest that resistance to metabolic detoxification in the isopsoralen series may not explain the sensitivity of *P. polyxenes* to these compounds.

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CHARACTERIZATION OF A SEX PHEROMONE IN THE BLUE CRAB, *Callinectes sapidus:* Crustecdysone Studies

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Abstract—The molting hormone/sex pheromone hypothesis of Kittredge and Takahashi (1972) and Kittredge et al. (1971) was explored in *C. sapidus*. Two concentrations of crustecdysone (5×10^{-5} M and 5×10^{-6} M) were presented to male crabs in a bioassay system in which courtship behavior was monitored. The results demonstrate that crustecdysone does not stimulate courtship in this species. The physical properties of crustecdysone were also compared to those of the partially purified sex pheromone derived from pubescent females. Using HPLC and mass spectral analysis, no correspondence of crustecdysone with the bioactive material could be shown. These results, in conjunction with the findings of others, do not support an evolutionary relationship between the molting hormone and sex pheromone communication in the Crustacea.

Key Words—Crab, *Callinectes sapidus,* sex pheromone, crustecdysone, molting hormone, HPLC, mass spectrometry, bioassay, DCI, desorption chemical ionization.

INTRODUCTION

Chemical communication functions importantly in coordinating the mating behavior of a number of crustacean species (Dunham, 1978). Although several studies have examined the chemical nature of the signal compounds

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involved, to date none have been isolated and structurally characterized (e.g., Christofferson, 1970; McLeese et al., 1977).

Kittredge et al. (1971) reported that the crustacean molting hormone, crustecdysone (Figure 1; synonyms: β-ecdysone, 20-hydroxyecdysone, ecdysterone, isoinokosterone), induces precopulatory behavior in males of *Pachygrapsus crassipes, Cancer antennarius,* and *Cancer anthonyi.* In addition, although details of the experiments are not presented, they note an overlap in pheromone activity among different crab species; namely, the pheromone released by *P. crassipes* stimulates courtship behavior in *C. antennarius,* and *Cancer magister* males are excited by the pheromone of *Cancer productus.* Based on this apparent lack of pheromone specificity and the induction of precopulatory behavior by crustecdysone, they conclude that either crustecdysone is the sex pheromone for these species or has a structure which is sufficiently similar to the natural compounds to mimic their actions. From these findings, the investigators postulate that the molting hormone served as a substrate for sex pheromone evolution (Kittredge and Takahashi, 1972; Kittredge et al., 1971). They speculate this was effected via release of molting hormone by females to the external environment with a concomitant externalization of crustecdysone receptors by males. These two events permitted pheromone communication which, because of its reproductive advantage, was subsequently fixed in the genome.

Although the concept of this evolutionary link between pheromone communication and an internal (molting hormone) communication system is frequently cited in the literature (e.g., Shorey, 1976), the supporting evidence is nevertheless controversial. In addition to the Kittredge et al. (1971) report, two other studies have described apparent mating-related behaviors in other crustacean species following crustecdysone exposure. In the first of these, Hammoud et al. (1975) examined the effects of crustecdysone on male precopulatory behavior (coupling) in the amphipods *Gammarus pulex* and *Gammarus fossarum.* They noted that males exhibited precopulation towards previously "nonattractive" females which had been treated by the external application of a crustecdysone solution. The behavior was main-

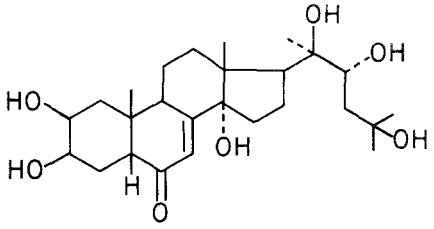

FIG. 1. Structure of crustecdysone.

tained for only a few minutes, however, and was species specific (i.e., males did not couple with treated females of the other species). In another study, Rudd and Warren (1976) tested male rock lobsters, *Jasus lalandii,* with crustecdysone and found that a "searching" activity was induced. This "searching" they consider to be similar to that exhibited by males exposed to water from tanks containing females in proecdysis. In contrast to these reports, the studies of Atema and Gagosian (1973) and Gagosian and Atema (1973) with *Homarus americanus* do not support the crustecdysone hypothesis. When known ecdysones and ecdysone metabolites were presented to males of this species, although alert responses were observed in some test animals, none of the compounds elicited definitively sexual behaviors. Similar findings were reported for the shore crab, *Carcinus maenas;* Seifert (1982) was unable to demonstrate any behavioral responses to crustecdysone (10^{-7} M) in this species. Moreover, contrary to the hypothesis that crustecdysone should only be released from females just prior to a nuptial molt (Kittredge and Takahashi, 1972), Seifert (1982) found that ecdysone excretion (as measured by radioimmunoassay) occurs in both sexes of *C. maenas* juveniles throughout the molting cycle.

Dunham (1978) has argued that important controls were not included in the Kittredge et al. (1971) study, and therefore their conclusion of a molting hormone/sex pheromone relationship in Crustacea was premature. In particular, it was not unambiguously shown that the behaviors exhibited with exposure to crustecdysone were, in fact, premating behaviors as opposed to novel stimulus responses. [A similar criticism is applicable to the Rudd and Warren (1976) study as well.] Indeed, Takahashi (1974) has subsequently noted that *P. crassipes* exhibits an "alert" posture which closely resembles the mating stance used in the Kittredge et al. (1971) bioassay. Since this "alert" posture is apparently stimulated by "glutamate and asparate as well as other substances," it would seem particularly important to control for such responses and to use blind observation procedures. Another subsequent finding which confounds the crustecdysone hypothesis is that, whereas *C. antennarius* and *C. anthonyi* were reported to exhibit concentration vs. "mating stance" relationships for crustecdysone which were similar to those determined for *P. crassipes* (Kittredge et al., 1971), Takahashi (1974) was unable to clearly demonstrate any sexual responses with crustecdysone exposure in *C. magister.*

As has been previously shown (Gleeson, 1980), males of *C. sapidus* display a specific courtship behavior when stimulated by a pheromone released by pubescent females. In light of the controversy surrounding the molting hormone/sex pheromone hypothesis, the objective of the present study was to examine the behavioral effects of crustecdysone on *C. sapidus* males. In addition, as a part of a project to characterize the structure of the

pheromone(s) released by the female of this species, we have compared a number of the physical properties of crustecdysone with those of the partially purified pheromone fraction derived from the urine of pubertal females.

METHODS AND MATERIALS

Urine Collection. Urine from pubertal females was used as a raw material source of the pheromone. Collections were made by directly aspirating urine from the antennal gland pores using Pasteur pipets which had been modified by tapering and fire polishing the tips. Samples collected were immediately frozen for storage in glass vials. Several vials of urine were subsequently pooled to provide sufficiently large batches of homogeneous material for chemical fractionations and bioassay tests.

Bioassay Procedures. All behavioral studies were performed in a semi-enclosed wet lab located adjacent to the Little Annemessex River at the University of Maryland's Marine Products Laboratory in Crisfield, Maryland. Water was continuously pumped from the river for circulation through the holding and bioassay tanks. During these tests salinities ranged from 15‰ to 19.5‰ and water temperatures ranged from 22 to 31°C.

Animals were obtained from local commercial sources and sustained on a diet of fish. Males used for bioassay were initially screened for responsiveness in a holding tank into which water from a tank containing pubertal females was introduced. Only those males exhibiting courtship behavior, as defined previously (Gleeson, 1980), were held for the subsequent bioassay testing. The criteria used in defining a courtship response were: (1) a courtship display: chelae extended in the lateral position, swimming appendages (fifth pereopods) rotated anterodorsally and waved from side to side above the carapace, and walking legs (second to fourth pereopods) extended such that the body is elevated to a near maximum height above the substrate; or (2) an approach towards another crab with chelae extended in the lateral position followed by an attempt to cradle-carry the approached individual.

Bioassays were performed using batteries of 6 to 12, 30-liter, glass aquaria each of which was fitted with a flow-through water system in which filtered (to 10 μm), aerated water was introduced at the surface and siphoned out at the bottom. The flow rate was approximately 1.4 liters/min. Each aquarium contained a single "test" male, previously screened for responsiveness to the pheromone, and an additional unresponsive male which had been rendered anosmic by antennule ablation (Gleeson, 1980, 1982). The latter animal served as an object for the orientation of courtship display and/or coupling behavior exhibited by the test crab. The aquaria were screened from

the observer by a partition with small viewing ports for observing behavior in each tank. All tests were performed between 0700 and 1800 hr under ambient daylight conditions.

Samples to be screened by bioassay were examined using 50-μl aliquots, each of which was brought to a volume of 1 ml in filtered seawater and presented to a test male via ejection through a standardized length of polyethylene tubing. The end of the tube was affixed directly to the carapace of the test animal with wax (Tackiwax, Central Scientific Co.) and oriented such that the ejected sample was expelled immediately beneath and between the antennules. Using this approach the experimenter had relatively precise control over the time and placement of the stimulus. Test animal activity was noted over a 2-min period following ejection of a water blank, then observed for another 2-min period following stimulus ejection, and the presence or absence of courtship behavior recorded.

The protocol used in guiding the chromatographic isolation of the pheromone from urine has been to present a series of six different samples to each test animal over an 8-hr period (one sample every 1.5 hr). The order in which these presentations are made to individual animals is randomized and not known to the observer. A series is presented to at least 24 different crabs over a 2 to 4-day period, and the number of pheromone responses associated with each of the six samples cataloged. In each test series, unfractionated urine is run as an "internal standard" against which four fractionated samples plus a recombined fraction (combined aliquots from the fractionated urine) can be compared. Since all fractionated samples are lyophilized and reconstituted to volumes equivalent to unfractionated urine, this approach permits direct comparison of response frequencies for monitoring activity in the fractions. The recombined material serves as a check on potential synergistic effects and/or activity losses resulting during the fractionation process.

For the crustecdysone assays, two solutions (10^{-3} M and 10^{-4} M) were made up in glass-distilled water using crystalline β-ecdysone (Sigma Chemical Co.). These solutions were then divided into 50-μl fractions which were immediately frozen and held at $-70°$C until bioassayed. Since each of the 50-μl aliquots was diluted with seawater just prior to presentation to a test crab, the maximum concentration of crustecdysone expelled in the region of the antennules was 5×10^{-5} M and 5×10^{-6} M, respectively. In addition to the two crustecdysone solutions, three urine standards and a distilled-water control were presented to each animal. As in the protocol described above, the order in which samples were presented to individual animals was randomized and unknown to the observer.

Pheromone Purification. Partial purification of the pheromone was accomplished by high-pressure liquid chromatography (HPLC) of raw,

bioactive urine on a Whatman reverse-phase silica preparative column (M-9, ODS 3, 25 cm) using various mixtures of water and methanol as eluant. Organic compounds were detected by ultraviolet (UV) absorbance at 220 nm, and all fractions were monitored for pheromone activity using the bioassay described above. Analytical HPLC was performed using a Whatman PXS 10/25 ODS 3 column (25 cm), eluting with 70:30 methanol–water at a flow rate of 1 ml/min. Thin-layer chromatography was carried out on Merck cellulose plates (0.25 mm thick × 173 mm long) which were developed with 70:30 methanol–water. Visualization was accomplished with ultraviolet light, sulfuric acid charring, or vanillin–sulfuric acid–ethanol spray followed by heating.

Mass Spectrometry. Mass spectra were determined on a Finnigan model 4500 mass spectrometer equipped for obtaining desorption ionization (DCI) spectra. DCI spectra were obtained using methane as the ionizing reagent gas; methane pressure was maintained constant between 0.1 and 0.3 torr. The ionizer temperature was 80° C and spectra were measured from 50 to 800 amu with an acquisition rate of one spectrum per second. Samples to be analyzed were dissolved in water and applied to a rhenium filament which was inserted, after the water had evaporated, into the ionization chamber of the mass spectrometer. Data acquisition was begun just prior to insertion of the filament into the ionization chamber. The filament was heated by passing a current through it at a rate of increase of 50 mA/sec to a final value of 1000 mA. This corresponds to a heating rate of approximately 50° C/sec with a final temperature of 1000° C. The rhenium filament was cleaned between runs by passing a high current (1350 mA) through it for approximately 10 sec.

RESULTS AND DISCUSSION

Repeated chromatography of pheromone-containing *C. sapidus* urine by preparative HPLC produced a fraction with all the biological potency of raw urine and which contained three substances as detected by UV absorbance. Thin-layer chromatography confirmed this finding; the components had R_f values of 0.23, 0.65 and 0.69. Comparison of the HPLC retention time of crustecdysone with the retention times of components of the purified pheromone fraction showed no correspondence between crustecdysone and the bioactive fraction (Figure 2). Under the analytical conditions used, crustecdysone had a retention time of 4.4 min, whereas the bioactive fraction elutes as an unresolved cluster with a retention time of 3.6 min.

Mass spectrometry was used to search for the presence of crustecdysone in the partially purified bioactive material obtained from preparative HPLC. The mass spectrum of crustecdysone was determined by the DCI technique, a

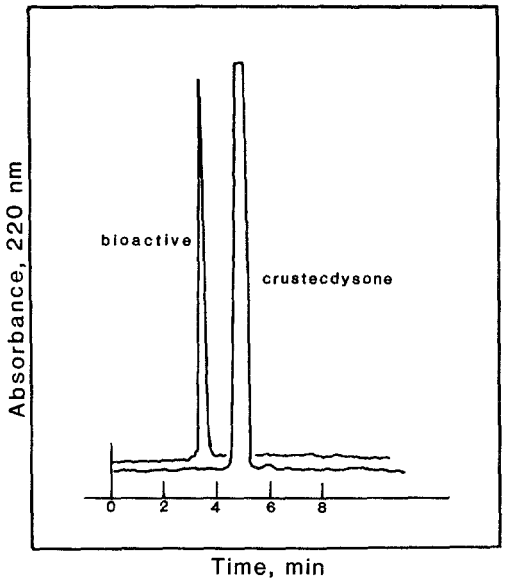

FIG. 2. Comparison of the HPLC retention time of crustecdysone with that of the partially purified pheromone from pubertal *C. sapidus* females.

method known to facilitate ionization of unstable and/or polar, nonvolatile substances of relatively high molecular weight (Cotter, 1980). Under DCI conditions, crustecdysone displayed a mass spectrum characterized by a strong pseudomolecular ion [$(P^+ + 1)$, 481 amu] and by successive losses of water molecules from the parent ion [$(P^+ + 1) - 18$, $(P^+ + 1) - 36$, $(P^+ + 1) - 54$, $(P^+ + 1) - 72$]. Components of the purified bioactive HPLC fraction were partially separated by heating on the DCI filament during the course of the mass spectral run. Comparison of the fragmentation patterns of crustecdysone with fragmentation patterns of components of the bioactive fraction revealed no correspondence between the two sets of spectra. A limited mass search was performed by computer to examine each spectrum determined for the bioactive mixture for the simultaneous occurrence of the five major fragment ions of crustecdysone (481, 463, 445, 427, and 409 amu). If a spectrum were found to contain these fragments, it would have been strong evidence that crustecdysone might be present in the bioactive fraction. In fact, with a detection threshold of at least 10^{-6} M, no spectrum was found to contain that particular combination of fragment ions or any subset of the ion group. This lack of spectral correspondence indicated that crustecdysone could not be detected in the bioactive HPLC fraction by this very sensitive mass spectrometric technique.

TABLE 1. COURTSHIP RESPONSES OF *C. sapidus* MALES FOLLOWING CRUSTECDYSONE PRESENTATION

Stimulus[a]	Courtship responses (No. of animals)
Urine stock A	12
Urine stock A (replicate)	10
Urine stock B	12
Distilled H_2O blank	0
Crustecdysone (5×10^{-5} M)	0
Crustecdysone (5×10^{-6} M)	0

[a] Each stimulus was presented to each test male. N=27.

The results of experiments in which the behavioral effects of crustecdysone were examined are shown in Table 1. Samples from two different stocks of urine (derived from pubertal females) were incorporated as internal standards to monitor the courtship response frequencies of test males. Samples from urine stock A were presented twice to each animal. A total of 27 males were tested, 17 of which exhibited definitive courtship behavior at least once following a urine sample presentation. No courtship responses to the distilled water blank nor to either of the crustecdysone samples were observed. An analysis of these response distributions using the Cochran Q test (Siegel, 1956) yielded a highly significant difference ($P < 0.001$) among the stimuli tested. "Alert" responses were occasionally seen following crustecdysone presentation. These responses were defined as an obvious change in behavior (excluding a courtship response) which occurred immediately following sample ejection; e.g., an increase in the rate of antennule flicking. Four animals exhibited low-intensity "alert" responses at each of the crustecdysone concentrations tested; however, a comparable "alert" frequency (five animals) was observed for the distilled-water blank. The "alert" responses are not surprising in that distilled water represents a potential chemical stimulus in seawater (i.e., via dilution of salts and/or other constituents of seawater).

These behavioral results, in conjunction with our data on the physical/chemical characteristics of the partially purified pheromone of *C. sapidus*, do not support the hypothesized molting hormone/sex pheromone role of crustecdysone (Kittredge and Takahashi, 1972; Kittredge et al., 1971), and therefore ally with other studies (Atema and Gagosian, 1973; Gagosian and Atema, 1973; Seifert, 1982) which do not substantiate such an evolutionary relationship in the Crustacea.

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WOODMICE (*Apodemus sylvaticus*) CAN DISTINGUISH CONSPECIFIC FROM HETEROSPECIFIC ODORS IN THE FIELD

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Abstract—In order to eliminate a common source of bias from trap-choice experiments in the field, trapping data from a study on the olfactory biology of the woodmouse were screened, and only those in which a direct choice existed were analyzed further. Woodmice choose conspecific odor to no odor but make no distinction between heterospecific odor and no odor. When choosing between conspecific and heterospecific odors, a significant majority choose conspecific.

Key Words—*Apodemus sylvaticus,* odor, conspecific, heterospecific, trapping, choice, preference.

INTRODUCTION

Ecologists interested in rodent populations have recently become concerned about the effect residual odors from previous occupants of live traps have on subsequent captures (e.g., Boonstra et al., 1982; Stoddart, 1983). In at least one study, population estimates based on recapture data were shown to be widely influenced by the odorous status of the traps (Stoddart, 1982a). Separate workers have concentrated on various aspects of rodent olfactory biology (Daly et al., 1978, 1980; Mazdzer et al., 1976; Montgomery, 1979; Stoddart, 1982b; Wuensch, 1982) using a variety of experimental methodologies. Cross comparisons and generalizations are difficult to make, as it is not always clear what criteria have been employed to reduce experimental bias introduced by the nonavailability of certain traps.

In general, field workers have adopted two procedures for investigating rodent response to odors. Either tainted sawdust, cotton wool, or similar

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absorbent material is introduced into the traps (Daly et al., 1978; 1980; Mazdzer et al., 1976; Stoddart, 1976; 1980; Wuensch, 1982), or the traps are allowed to become soiled as a consequence of normal field use and their subsequent performance examined (Boonstra and Krebs, 1976; Boonstra et al., 1982; Montgomery, 1979; Stoddart, 1982a,b). Both procedures have been adopted in the present study, and the data presented are the number of captures in each category of trap when a choice of traps was available. Serious bias is introduced into studies on trap choice if more than one member of a set of traps is occupied by any species, or if for some other reason the investigator is uncertain whether or not a true choice existed for the captive at the time of its capture.

We have conducted a study into the involvement of residual odor in the trap entry behavior of the European woodmouse *Apodemus sylvaticus* using an improved technique of trap-choice analysis to investigate whether woodmice can distinguish between conspecific and heterospecific odors under natural conditions.

METHODS AND MATERIALS

The field work was carried out in an overgrown meadow flanked on three sides by substantial woody hedgerows. The location was at Rogate, in the county of Sussex, England. The meadow contained a dense growth of grass (mostly *Dactylis glomerata, Arrhenatherum elatius,* and *Agrostis stolonifera*) and has not been grazed or cut for almost two decades. The hedgerows were made of oak (*Quercus rober*), hazel (*Corylus avellana*), holly (*Ilex aquifolium*), lime (*Tilia vulgaris*), and bramble (*Rubus fruticosus*), together with low, woody herbs. A grid of 70 trapping points spanned the study site in a 10 × 7 arrangement, with the distance betweeen rows being 10 m. Two aluminium Longworth live-traps, labeled A and B, were placed at each trapping point and positioned as closely together as the local topography allowed. Their entrances were never more than 5 cm apart. The relative position of each trap to the other was determined anew each time the traps were set by reference to random numbers, in order to overcome any bias introduced by a given individual mouse always entering, say, the left-hand member of a pair of traps. Care was taken in washing traps, and the source of hay (for nesting material) and grain (for sustenance) was kept constant throughout. Traps were designated "tainted" if they contained animal residues (resulting from either experimental introductions or from normal trapping), or "odorless" if they were clean. Tainted traps were further described as "conspecific" if they contained residues of *A. sylvaticus* or "heterospecific" if they contained residues of other small mammals from the study area (i.e., *Microtus agrestis, Clethrionomys glareolus, A. flavicollis,*

Micromys minutus, Sorex araneus, S. minutus). Having labeled the traps, it was possible to record their capture history and hence established a sequential pattern of trap entry.

Data were subjected to a retrospective examination in which the species of rodent caught in each trap on day t + 1 was compared with the species caught in the same trap on day t. Only those captures on day t + 1 which were made when a choice of trap categories existed on day t were included in this analysis. Of a total of 1306 captures of woodmice sustained during 101 nights of trapping in 22 sessions between February 1, 1982, and February 11, 1983, only 409 (31.3%) satisfied our strict screening procedure and were analyzed further. (See Figure 1.)

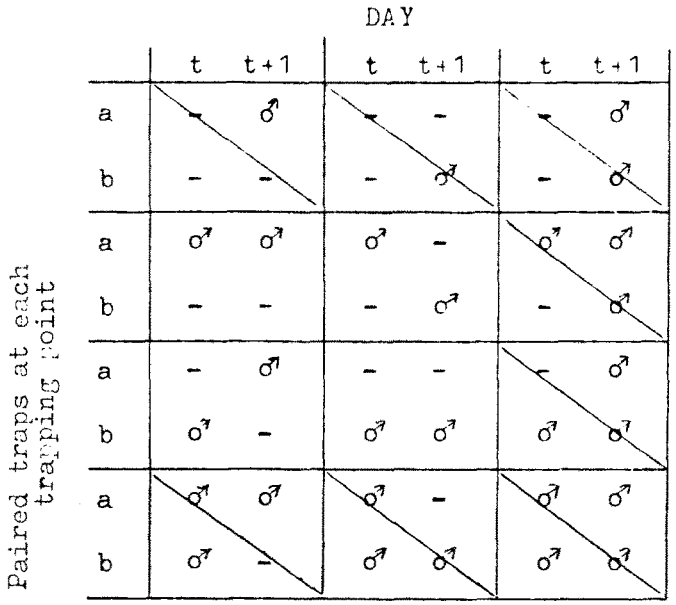

FIG. 1. The cells of the above matrix denote the range of possible captures on day t and the following day, day t + 1, for male mice. Only those occasions on which a trap entry on day t + 1 was preceded on day t by an entry providing a choice are included in the final analysis. Data from those cells crossed with a diagonal line are excluded because either no choice was available and/or it was not known which individual entered first on day t + 1 and exercised a preference. As further explanation, consider the top left-hand cell in which day t yielded no captures while on day t + 1 a male entered trap a. It exercised no choice of trap type, because no choice of trap types was available. Note that a similar matrix applies to females, and to males and females combined, for every preference combination shown in Table 1.

TABLE 1. OBSERVED AND EXPECTED NUMBER OF CAPTURES OF *Apodemus sylvaticus* WHEN VARIOUS CHOICES OF ODOR-TAINTED TRAPS WERE AVAILABLE[a]

Traps available on day t	Captures on day t + 1	
	Observed	Expected
A Tainted	244	204.5
Odorless	165	204.5
	409	409
	$\chi^2 = 15.3$	$P = <0.001$
B Conspecific	150	110
Odorless	70	110
	220	220
	$\chi^2 = 29.1$	$P = <0.001$
C Heterospecific	94	94.5
Odorless	95	94.5
	189	189
	$\chi^2 = 0.005$	$P =$ NS
D Conspecific	38	26.5
Heterospecific	15	26.5
	53	53
	$\chi^2 = 10.0$	$P = <0.01$

[a]The 'Expected' value refers to random trap entry. By restricting the data to those in which a genuine choice of trap types existed on day t, no allowance needs to be made for bias introduced by the size of individual range, species overlap, etc.

RESULTS

Table 1 shows the preference for traps made by woodmice when presented with various choices. It is apparent from Table 1A that odor-tainted traps sustain significantly more captures than odorless traps. When the exact nature of the odor is examined, it appears that significantly more captures are effected in conspecific-tainted traps rather than odorless (Table 1B). When the choice is between traps tainted with heterospecific odor and odorless traps, however, no such difference in the distribution of captures is seen (Table 1C). When the mice are faced with a direct choice between traps tainted with heterospecific or conspecific odor, a significantly high proportion are caught in the trap smelling of conspecifics (Table 1D).

DISCUSSION

These data demonstrate beyond doubt that woodmice can distinguish between conspecific and heterospecific odorous residues left in live-traps and

base their choice of trap upon this discrimination. Males and females responded alike over the trapping period described. It is not known whether woodmice exhibit a neophobia to odorless traps, although such neophobias are known for some small mammals (Shillito, 1963). Nor is it known whether or not heterospecific odor is perceptible to woodmice; it would not appear to be aversive, as certain other odors have been shown to be to some small mammals [e.g., weasel anal gland odor to *Microtus agrestis* (Stoddart, 1976), jaguar urine and tiger urine to *M. agrestis* (Stoddart, 1980, 1982a), shrew odor to *M. pennsylvanicus* (Boonstra et al., 1982)]. It appears that heterospecific odors are "neutral" to *A. sylvaticus* and do not influence trap choice.

It should be stressed that these data represent preferences observed once an individual has "decided" to enter a trap. the extent to which residual odors influence that decision in *A. sylvaticus* is not known, although Stoddart (1982a) showed that conspecific odors inhibited trap entry in some *M. agrestis*. An abiding problem yet to be faced by field workers is that there is no simple way of determinining which traps have been actively avoided by an individual rodent and which have been passively avoided or simply were not encountered.

The analytical procedure herein described is important because it allows an examination to be made of some of the complex social interactions found in a community of small mammals. Calhoun (1963) analyzed the results of many rodent ecological studies and theorized that the psychologically most dominant species would be undeterred in its pattern of movement by signals emanating from a less dominant species which, in its turn, would only be restricted by signals from the dominant. Residual odors at defecating sites, runways, and temporary nests would serve admirably for this purpose. Since *A. sylvaticus* seems oblivious to the odors of other species and trap entry is not deterred by heterospecific odor, it is possibly the dominant member of the community. Stoddart (1983) has shown that when traps are introduced into a previously untrapped location, *A. sylvaticus* takes several days to get used to the traps and be caught in large numbers. *M. agrestis,* by contrast, shows much less initial reticence. Our procedure provides a powerful tool for investigating this little understood aspect of small mammal community dynamics.

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EXTREME INTRASPECIFIC CHEMICAL VARIABILITY IN SOLDIER DEFENSE SECRETIONS OF ALLOPATRIC AND SYMPATRIC COLONIES OF *Longipeditermes longipes*

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Abstract—The chemical defense secretions of major and minor soldiers of over 18 colonies of the primitive glue-squirting nasute termite *Longipeditermes longipes* (Isoptera, Termitidae, Nasutitermitinae) were analyzed chormatographically. The colonies, collected from four rainforest sites in peninsular Malaysia, showed monoterpene patterns rich in pinenes and limonene but with few qualititative differences between colonies. In marked contrast, the diterpene chemistry is high variable, and includes tricyclic (trinervitane), tetracyclic (rippertane), bicyclic (secotrinervitane), and a new spirotetracyclic (longipane) skeleton. Three new natural products are included in this remarkable and unprecedented example of structural diversity among different colonies of a single species.

Key Words—Termites, Isoptera, Termitidae, *Longipeditermes longipes*, defense secretions, intraspecific variability, diterpenes.

INTRODUCTION

Soldiers of the evolutionary advanced Nasutitermitinae have evolved nozzle-shape heads for squirting terpenoid chemicals for defense. Over 500 species of

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this subfamily are known worldwide, and nearly 100 selected species have been subjected to detailed studies with respect to the terpenoid chemistry of the defensive secretions (Prestwich, 1979c, 1982, 1983, 1984; Goh et al., 1982; Baker and Walmsley, 1982). The diterpene natural products can serve as useful chemotáxonomic characters in genus (and sometimes species) differentiation as well as in the understanding of termite phylogeny and evolution (Prestwich et al., 1981; Prestwich, 1979a, 1982). Prestwich and Collins (1981) have shown that *Nasutitermes* and *Subulitermes* contain identical diterpenes; intermediate mandibulate nasutes lack the characteristic diterpenes but instead contain macrocyclic lactones (Prestwich, 1982) and a variety of mono- and sesquiterpenes (Baker et al., 1982a,b). *Leucopitermes*, another member of the alleged "*Subulitermes* branch" in the diphyletic scheme, also possesses trinervitanes (Goh, unpublished). The nasutes have been proposed to be monophyletic instead of diphyletic based on the unique evolutionary occurrence of the cembrene-derived nasute diterpenes.

We now report a detailed study of the defensive secretion of *Longipeditermes longipes*, a long-legged primitive nasute termite with free-ranging foraging habits in the lowland Malaysian rainforests. An analysis of the diterpenes, three of which are previously unreported, revealed a surprising case of intraspecific variation in structural types produced by allopatric and sympatric colonies. This discovery provides insight into the biogenesis of these compounds and their relationship to the evolution of defensive secretions of the subfamily of Nasutitermitinae.

METHODS AND MATERIALS

Termite Materials. The soldier termites materials used for the present studies were all collected within closed-canopy dipterocarp forests from localities as follows: (1) Gombak forest reserve (3° 20′N, 101° 46′E; 500 m a.s.l.), a hill dipterocarp forest in the state of Selangor; (2) Forest Research Institute (3° 10′N, 101° 30′E; 50 m a.s.l.), a forest research station at Kepong in the state of Selangor; (3) Pasoh forest reserve (2° 58′N, 101° 55′E; 100 m a.s.l.), a lowland dipterocarp forest which has been the site for intensive ecological studies under the auspices of the International Biological Programme; and (4) Taman Negara (4° 20′N, 102° 25′E; 1,000 m a.s.l.); a wildlife and forest reserve in the state of Pahang.

The termites were collected with minimum disturbance, placed in plastic bags, and chilled to −10° C, after which they were decapitated and the heads were crushed and extracted in hexane or carbon disulfide.

Analytical Methods. Gas chromatography (GC) was performed on a Tracor 560 instrument fitted with a flame ionization detector. 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 obtained at 70 eV with source and interface temperature at 150° and 210°, respectively, and the data were accumulated by a Kratos DS50 data system. A 5-ft × 3-mm glass column with 3% OV-17 on 100/120 Gas Chrom Q was used to analyze the terpenoids. A 10-ft × 1/8-in. stainless-steel column packed with Carbowax 20 M on 120/140 Gas Chrom Q was used to analyze the monoterpenes. Retention times of the diterpenes were obtained with reference to *n*-alkanes, which were spiked into the chromatograms by coinjection with the samples. Results are given in Table 2–5.

The amounts of monoterpenes and diterpenes secreted by minor soldiers were 15–42 μg and 30–60 μg per soldier, respectively. Major soldier termites provided 30–120 μg monoterpenes and 250–500 μg diterpenes per soldier. Total secretions account for about 4% of the minor soldier and 7% of the major soldier based on the fresh weight of the termites. Isolation of the individual compounds from the crude hexane extracts (ca. 100 mg each time) was aided by a Waters 440 HPLC instrument with a 25-cm × 1/4-in. Ultrasphere silica gel column. Elution was by a gradient of 2–10% ethyl acetate in pet. ether (bp 60–80° C). Isolation samples were checked by GC or TLC on 5 × 10-cm silica gel 60 plates (0.25 mm thickness).

Longipen-3α-ol (*II*). [^{1}H]NMR ($CDCl_3$, 100-MHz): δ5.13, 5.00 (2H, two br s, H-17), 4.02 (1H, dd, 10, 4.5, H-3), 2.70 (1H, br d, 7, H-16), 1.25-2.3 (ca. 18H, multiplet), 0.96 (3H, d, 7, H-20), 0.94 (3H, s, H-18) and 0.92 (3H, d, 6, H-19). At 300 MHz the cluster of methyl absorptions was resolved and the olefinic proton at 5.13 was resolved as a doublet of 1.5 Hz. [^{13}C]NMR resonances are tabulated in Table 1. EI-MS, *m/z* (rel. int.), 288 (22, M^+), 273 (43, M^+—CH_3), 270 (53, M^+—H_2O), 255 (85, M^+—H_2O—CH_3), 213 (25, $C_{16}H^+_{21}$), 175 (22, $C_{13}H^+_{19}$), 173 (35, $C_{13}H^+_{17}$), 161 (35, $C_{12}H^+_{17}$), 159 (35, $C_{12}H^+_{15}$), 145 (35, $C_{11}H^+_{13}$), 135 (50, $C_9H_{11}O^+$), 133 (35, $C_{10}H^+_{13}$), 121 (53, $C_9H^+_{13}$), 119, (37, $C_9H^+_{11}$), 109 (47, $C_8H^+_{13}$), 105, (49, $C_8H^+_9$), 91 (60, $C_7H^+_7$), 81 (60, $C_6H^+_9$), 77 (29, $C_6H^+_5$), 69 (28, $C_5H^+_9$), 67 (36, $C_5H^+_7$), 55 (74, $C_4H^+_7$), 43 (40, CH_3CO^+), and 41 (100, $C_3H^+_5$). Calcd. for $C_{20}H_{32}O$: 288.245; found 288.246. Acetylation of II (acetic anhydide in pyridine at 90° for 8 hr) gave the acetate derivative showing characteristic [^{1}H]NMR (C_6D_6) resonances at δ5.4 (1H, dd, 10.6, 4.7 H-3), 5.22, 5.11 (2H, two br s, H-17), 2.68 (1H, br d, 7, H-16), 1.83 (3H, s, OAc), 1.16 (6H, overlapping std, H-18 and H-19) and 1.09 (3H, d, 7.1, H-20). Silylation of II (1 mg, 20 1 HMDS, 20 μl TMCS and 60 μl pyridine, 100° C, 8 hr) gave the silyl derivative which has mass spectrum *m/e* 360 (57, M^+), 345 (32, M^+-CH_3), 270 (91, M^+-Me_3SiOH), 255 (64, M^+-Me_3SiOH-CH_3), 207 (36, $C_{12}H_{19}OSi^+$), and 73 (100, Si^+Me_3). Calcd. for $C_{23}H_{40}OSi$ and $C_{12}H_{19}OSi$: 360.285 and 207.121, respectively; found: 360.296 and 207.121, respectively.

Oxidation (Jones reagent, acetone, 25°, 20 hr) gave the C-3 ketone in

TABLE 1. ^{13}C NMR SPECTRA OF NEW DITERPENES[a]

Carbon	Compound II	III	IV
C-1	43.64 s	39.71 d	44.68 d
C-2	36.48[a] t	39.25[a] t	78.48 d
C-3	74.17 d	71.93 d	74.86 d
C-4	48.42 s	40.19 s	39.19 s
C-5	43.48[a] t	36.88[a] t	37.03[a] t
C-6	36.79[a] t	36.52[a] t	37.03[a] t
C-7	61.25[b] d	127.89[b] d	128.01[b] d
C-8	39.82 d	133.53[c] s	134.11[c] s
C-9	28.80[a] t	25.31[a] t	22.39[a] t
C-10	30.90[a] t	25.31[a] t	25.51[a] t
C-11	61.10[b] d	126.08[b] d	127.89[b] d
C-12	35.69 d	132.39[c] s	132.96[c] s
C-13	30.90[a] t	25.84[a] t	25.51[a] t
C-14	34.93[a] t	37.56[a] t	39.94[a] t
C-15	152.90 s	150.74 s	146.49 s
C-16	56.87 d	46.43[a] t	47.88[a] t
C-17	111.91 t	103.11 t	106.14 t
C-18	26.59[d] q	21.52[d] q	19.31[d] q
C-19	19.49[d] q	15.08[d] q	15.50[d] q
C-20	21.05[d] q	14.22[d] q	14.67[d] q

[a] Chemical shifts (in ppm, TMS = 0) of II in $CDCl_3$ and of III and IV in C_6D_6. Assignments with identical superscripts are interchangeable. Multiplicites were assigned using the attached proton test (Patt and Schoolery, 1982) at 75 MHz on a Nicolet NT 300 instrument.

which the following [^{1}H]NMR shifts were observed at 300 MHz: δ2.97 (d, ^2J = 14.0 Hz, ^4J = 0.9D Hz), 2.84 (d, 8.3 Hz, H-16), 1.08 (3H, s, H-18); 0.95 (d, 6.5 Hz, H-19), 0.82 (d, 7.2 Hz, H-20).

Secotrinervita-7(8),11(12),15(17)-trien-3α-ol (III). [^{1}H]NMR ($CDCl_3$): 5.29 (1H, br t, 7.5, H-11), 4.86 (1H, br t, 7.5, H-7), 4.65, 4.57 (2H, two br s, H-17), 4.03 (1H, dd, 10, 5.2, H-3), 2.66 (2H, br d, 13.1, H-16), 2.27 (4H, d, 3.5, H-9 and H-10), 1.2-2.7 (ca. 2H, multiplet), 1.58 (6H, br, s, H-19 and H-20), and 0.84 (3H, s, H-18). The 20-MHz [^{13}C]NMR (C_6D_6) of III is given in Table 1. EI-mass spectrum of III gave peaks at *m/e* 288 (20, M^+), 270 (20, M^+—H_2O), 255 (30, M^+—H_2O—CH_3), 159 (15, $C_{12}H^+_{15}$), 136 (25, $C_{10}H^+_{16}$), 135 (27, $C_9H_{13}O^+$), 121 (30, $C_9H^+_{11}$), 119 (30, $C_9H^+_{11}$), 107 (30, $C_8H^+_{11}$), 105 (30, $C_8H^+_9$), 93 (35, $C_7H^+_9$), 91 (40, $C_7H^+_7$), 81 (55, $C_6H^+_9$), 79 (40, $C_6H^+_7$), 77 (25, $C_6H^+_5$), 67 (45, $C_5H^+_7$), 55 (70, $C_4H^+_7$), and 41 (100, $C_3H^+_5$). Calcd. for $C_{20}H_{32}O$: 288.245; found: 288.255.

Silylation of compound III (1 mg, 20 μl HMDS, 20 μl TMCS and 60 μl pyridine, 100°C, 8 hr) afforded the silyl derivative which has strong mass

spectrum peaks at *m/e* 360 (100%, M^+), 345 (9, M^+—CH_3), 270 (17, M^+—Me_3SiOH), 255 (13, M^+—Me_3SiOH—CH_3), 207 (28, $C_{12}H_{19}OSi^+$), and 73 (100%, Si^+Me_3). Calculated exact masses for the silyl derivative $C_{23}H_{40}OSi$ and an expected fragment $C_{12}H_{19}OSi$ are 360.285 and 207.121, respectively; found: 360.276 and 207.112.

Seotrinervita-7(8),11(12),15(17)-trien-2β,3α-diol (*IV*). This exhibited characteristic [^{1}H]NMR ($CDCl_3$) signals at δ5.15 (1H, br d, 8.9, H-11), 4.82 (1H, br t, 8.0, H-7), 4.71, 4.68 (2H, two br s, H-17), 3.16 (1H, dd, 11.1, 8.5, H-2) 3.45 (1H, d, 8.6, H-3), 2.93 (2H, br d, 11.1, H-16), 2.30 (4H, d, 3.5, H-9 and H-10), 1.5 (6H, ss, H-19 and H-20), and 0.84 (3H, s, H-18). The 20-MHz [^{13}C]NMR (C_6D_6) data for IV is given in Table 1. EI-MS *m/e* 304 (8, M^+), 286 (31, M^+-H_2O), 271 (M^+-H_2O-CH_3), 175 (24, $C_{12}H_{15}O^+$), 161 (12, $C_{12}H^+_{11}$), 135 (63, $C_9H_{11}O^+$), 121 (37, $C_9H^+_{13}$), 119 (29, $C_9H^+_{11}$), 109 (27, $C_8H^+_{13}$), 107 (57, $C_8H^+_{11}$), 105 (33, $C_8H^+_9$), 95 (33, $C_7H^+_{11}$), 93 (33, $C_7H^+_9$), 91 (54, $C_7H^+_7$), 81 (51, $C_6H^+_9$), 55 (65, $C_4H^+_7$), and 41 (100%, $C_3H^+_5$). Calculated mass for $C_{20}H_{32}O_2$: 304.245; found, 304.251

Acetylation of the compound (IV) afforded a diacetate which gave characteristic [^{1}H]NMR ($CDCl_3$, 100 MHz) resonances at δ5.15 (1H, d, 8.9, H-11), 4.71, 4.68 (2H, two br s, H-17), 4.69 (1H, br t, 8.9, H-7), 3.0 (1H, d, 10.5, H-3), 2.01 (6H, two overlapping s, OAc), 1.66 (6H, two br s, H-19 and H-20), and 0.90 (3H, s, H-18).

Silylation of compound IV (1 mg, 20 μl, HMDS, 20 μl TMCS, and 60 μl pyridine at 100° C, 8 hr) afforded the silyl derivative which has mass spectral peaks at *m/e* 448 (36, M^+), 433 (3, M^+—CH_3), 358 (64, M^+—Me_3SiOH), 343 (7, M^+—Me_3SiOH—CH_3), 268 (21, M^+—2 Me_3SiOH), 253 (7, M^+—2 Me_3SiOH—CH_3), 207 (26, $C_{12}H_{19}OSi^+$), 196 (13, Me_3Si^+OCH—$CMeCH_2CH_2CH$=C=CH), 169 (17, $C_9H_{17}OSi^+$), 147 (31, $C_{11}H_{15}^+$), 145 (13, $C_{11}H_{13}$), 143 (58, Me_3Si^+OCH=$CMeCH_2$), 133 (14, $C_{10}H_{13}^+$), 131 (11, C_9H_{15+}), 121 (9, C_9H_{13+}), 119 (17, $C_9H_{11}^+$), 107, (12, $C_8H_{11}^+$), 105 (14, $C_8H_9^+$), 95 (12, C_7H_{11+}), 93 (16, $C_7H_9^+$), 91 and 73 (100%, Me_3Si^+). Calculated exact masses for the silyl derivative $C_{26}H_{48}O_2Si_2$ and expected fragments of $C_{12}H_{19}OSi$ and $C_{11}H_{20}OSi$ are 448.319, 207.121, and 196.128 respectively; found: 448.322, 207.119, 196.122.

Mono- and dihydroxyditerpenes (I and V through X) were identified by their ^{1}H-NMR and mass spectra as well as by comparison of their retention times with those previously identified (Prestwich, 1979c). Di- and triacetoxyditerpene derivatives (XI and XII) were identified by their mass spectra and compared to those reported (Vrkoč et al., 1978).

RESULTS AND DISCUSSION

Longipeditermes longipes colonies were collected in Malaysian rainforests over a fairly widespread region in peninsular Malaysia. The defense

TABLE 2. RANGE OF VARIATION OF MONOTERPENE COMPOSITION (%) IN *Longipeditermes longipes* SOLDIERS

	Composition (%)													
	IA[b,c]		IB		II[d]		IIIA[d]		IIIB[d]		IVA[d]		IVB	
Compound[a]	mi	mj	mi	mj	mi	mj	mi	mj	mi	mj	mi	mj	mi	mj
α-Pinene	15–28	11–38	34	44	26–35	30–47	41–42	21–24	40–48	60–61	27–28	24–51	36	47
Camphene	1–5	1–7	5	2	4–6	6–8	2–3	3–4	2–4	1–2	1–2	0–1	6	7
β-Pinene	6–47	13–48	30	8	4–35	6–15	18–33	8–14	17–22	16–17	41–59	8–8	29	16
Myrcene	0–2	0–2	7	6	12–22	3–15	0–2	0–3	0–1	1–3		0–1	6	2
Δ^3-Carene	0–1	0–2			0–1	0–1					1–2	0–2	1	1
α-Terpinene	3–18	1–12	3	2	0–1	0–1	6–9	10–16	0–2	1–2	1–3	3–10	2	2
Limonene	9–34	11–40	14	23	11–31	15–28	16–29	45–47	28–33	14–15	7–18	30–40	13	15
β-Phellandrene	0–1	0–1			0–7	0–2						0–1		
γ-Terpinene	3–10	2–11	3	2	0–1	0–3	0–1	0–1	0–1	0–1	1–3	1–2	2	3
Terpinolene	4–7	3–13	4	3	4–5	6–12	1–3	0–3	0–3	2–4	2–4	6–7	5	7

[a] Listed in increasing order of retention time on 10′ 10% Carbowax 20M on Gas Chrom Q.
[b] IA & B—Gombak Reserve, Selangor; II—Forest Reaserach Institue, Kepong, Selangor; IIIA & B—Pasoh Forest Reserve, Negeri Sembilan; IVA & B-Taman Negara, Pahang; mi and mj denote minor and major soldiers, respectively
[c] Range for 8 colonies.
[d] Range for 2 colonies.

secretions were chemically analyzed for mono- and diterpenoids, and the results are summarized in Tables 2–5. Monoterpene hydrocarbons consisted primarily of pinenes and limonene, with several other structures also found in significant quantities (Table 2). The monoterpenes exhibit a "broad spectrum" pattern in contrast to many other nasute termites with marked preference for the relatively more toxic α-pinene (Goh et al., 1982; Eisner, 1976; Hrdý et al., 1977).

The chemistry of the diterpenoids obtained (Table 3) shows the most impressive variability yet to be produced by a single species of termite. Diterpenes can have novel bi-, tri-, and tetracyclic structures (including the methyl-shifted rippertenol (I). *Longipeditermes* diterpenes show a remarkable structural diversity in having four of the five known skeletal types (Figure 1). The spirotetracyclic longipenol (II) and bicyclic secotrinervitatriene mono- and diols (III and IV) are new natural products not observed previously in other nasutes. Only the kempane skeleton has not been found in this species.

The identifications of the known rippertenol I and trinervitadiene derivatives V–XII were made by spectral (GC-MS, [^{1}H]NMR, [^{13}C]NMR) (Table 4) and chromatographic (GC, TLC) comparisons (Table 5) with authentic materials (Prestwich et al., 1976a, 1980; 1979a; Vrkoč et al., 1978a,b).

The structure of the new compound II has been assigned on the basis of extensive 2-D J[^{1}H]- and [^{13}C]NMR studies (M. Tempesta and G. Prestwich, unpublished results). In its proton NMR, longipenol II (*m/e* 288) shows three CH_3 signals (two d, one s), a hydroxymethine coupled to two nonequivalent hydrogens, and two terminal olefinic protons. In the [^{13}C]NMR [using the attached proton test of Patt and Shoolery (1982)], two quaternary, six methine, seven methylene, three methyl carbons, and a characteristic pair of 1,1-disubstituted olefinic carbons were present. The novel spirotetracyclic cembrene-derived structure is that shown as II. The determination of the stereochemistry of II will be described in detail elsewhere.

The secotrinervitane alcohols III (*m/e* 288) and IV (*m/e* 304) show six olefinic carbons and show methyl, allylic, and vinylic protons consistent with the structures shown. This structural type was discovered simultaneously as a minor constituent of *Nasutitermes principes* by Braekman et al. (1980) (see also Dupont et al., 1981). The major *N. princeps* (and *N. lujae*) secotrinervitene XIII could be converted to the compound III (also found in *N. lujae*) by dehydration ($POCl_3$, pyridine) and deacetylation (K_2CO_3, CH_3OH) (J. C. Braekman, personal communication; Braekman et al., 1983). The spectral data for the *L. longipes*- and *N. lujae*-derived samples of III were identical in every respect. The deduction of the structure of diol IV follows directly from the similarity of the [^{13}C]- and [^{1}H]NMR data, with the exception of a new carbinyl H-2 signal coupled (axial–axial) to H-3 (J = 8.5 Hz) and H-1 (J = 11 Hz).

TABLE 3. BROAD DISTRIBUTIONS OF DITERPENE COMPOSITION (%) IN *Longipeditermes longipes* SOLDIERS

Compound[a]	Composition (%)													
	IA[b,c]		IB		II[d]		IIIA[d]		IIIB[d]		IVA[d]		IVB	
	mi	mj	mi	mj	mi	mj	mi	mj	mi	mj	mi	mj	mi	mj
Secotrinervita-7(8),11(12),15(17)-trien-3α-ol (III)	15–19	23–32												
Ripperten-3α-ol (I)	15–19	23–32	4	19	2–3	11–16	10–13	13–20	4–5	4–6	36–38	42–57	17	47
Longipenol (II)	51–65	28–36					35–36	32–38			53–55	32–50		
Trinervita-1(15),8(9)-dien-2β-ol (VI)			6	20	2–3	12–13	35–36	32–38	10–18	14–22	2–3	3–6	27	16
2-Oxotrinervita-1(15),8(19)-dien-3α-ol (V)			10	9	3–4	2–3			3–6	2–4			7	2
Secotrinervita-7(8),11(12),15(17)-trien-2β,3α-diol (IV)	2–11	2–20												
Trinervita-1(15),8(19)-dien-2β,3α-diol 2-*O*-acetate (VIII)			5	8	4–10	2–6	2–4	2–6	4–6	7–12			6	15
Trinervita-1(15),8(19)-dien-2β,3α-diol (IX)			44	36	26–42	35–38	8–10	5–8	39–40	26–34	3–5	2–5	22	10
Trinervita-1(15),8(9)-dien-2β,3α-diol (VII)			8	6	2–4	10–15	2–4	1–2	14–19	21–23	1–2	0–2	9	7
Trinervita-1(15),8(19)-dien-2β,3α,-9α-triol 9-0-acetate (X)			10	2	20–48	13–18	0–2		13–16	6–16	0–1	0–1	12	3
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol 2,3-O-diacetate (XI)			8		2–3	2–3			0–1	0–1				
Trinervita-1(15),8(19)-dien-2β,3α,9α-triol triacetate (XII)			3											
Others	1–6	0–5	2		0–8	0–1	0–1	0–3	0–2	0–2				

[a] Listed in increasing order of retention times on 5′ 3% OV-17 column. Percent compositions are from uncalibrated FID detector response.
[b] IA & B—Gombak Forest Reserve, Selangor; II—Forest Research Institute, Kepong, Selangor; IIIA & B—Pasoh Forest Reserve, Negeri Sembilan; IVA & B—Taman Negara, Pahang; mi and mj are minor and major soldiers, respectively.
[c] Range for 8 colonies.
[d] Range for 2 colonies.
[e] Decomposed in GC column.

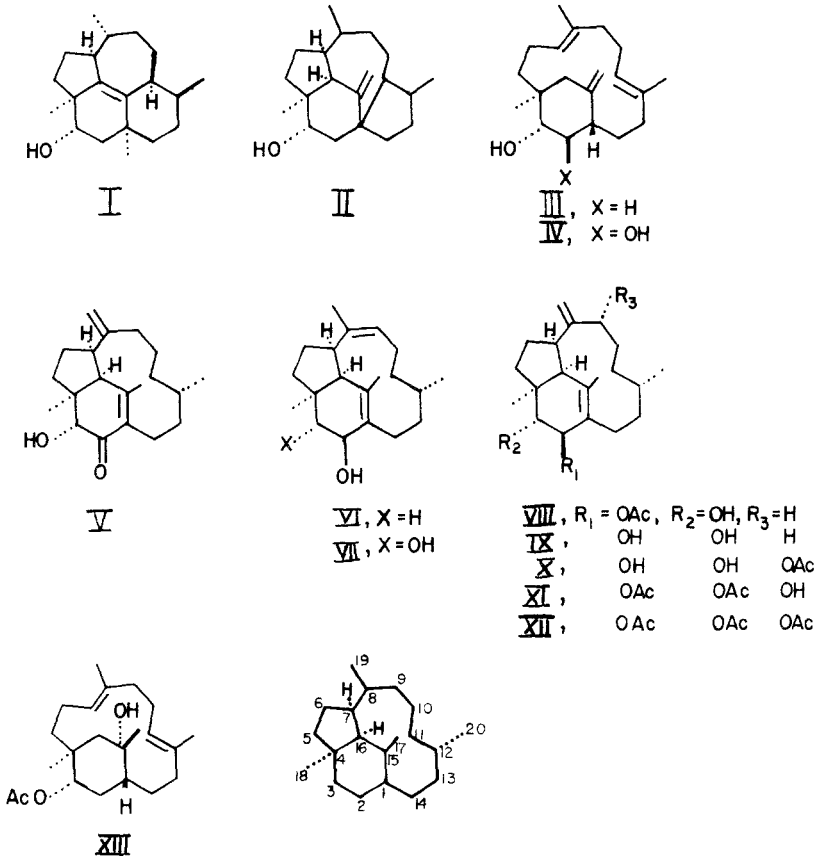

FIG. 1. Diterpenes isolated from *Longipeditermes longipes*. The assignment of structure II is tentative.

Interspecific variation of terpene constituents in the rather poorly defined genus *Nasutitermes* (Prestwich, 1979b; Gush, 1983; Bentley and Prestwich, unpublished) in the Neotropics and in Africa is extremely common. Similarly, the genus *Trinervitermes* of East, West and South Africa shows compositional and structural variation (Prestwich, 1979a, 1983, 1984; Braekman, personal communication; Theaker and Prestwich, unpublished). Intraspecific variation in allopatric colonies (i.e., from different geographical populations) is now well established for several species of *Trinervitermes* in East Africa (Prestwich, 1978; Prestwich and Chen 1981) and *Nasutitermes* in Central America (Bently et al., 1982; Gush, 1983; Prestwich, 1983). In none of these advanced nasutes, however, did we ever observe colony–colony variability in structural types, although minor inter- and intracolony compositional variations can be measured and analyzed statistically (Prestwich, 1983).

TABLE 4. CHARACTERISTIC 100 MHz [^{1}H]NMR DATA FOR *Longipeditermes* DITERPENES

	Compound								
Proton[a]	I	II	III	IV	VI	VII	VIII	IX	X
H-2	—	—	—	3.16dd (11.1,8.5)	3.92dd (7.4;6)	4.0d (8)	5.37 br d (9)	3.95dq (8.9;2)	3.76d (8)
H-3	3.60dd (10.5;7.5)	4.02dd (10;4.5)	4.03dd (10;5.2)	3.45d (8.6)		3.76d (9)	3.98d (9)	3.80d (9)	4.02d (9)
H-7	2.54t (9)		4.86 br t (7.5)	4.82 br t (8.0)			3.14dt (11;9)	3.10dt (13;9)	3.20dd (12;10)
H-9			2.27d (3.5)	2.30d (3.5)	5.14ddq (12;6;6)				5.50 br t (6)
H-11			5.29 br t (7.5)	5.15 br d (8.9)					
H-16		2.70 br d (7)	2.66 br d (13.1)	2.93 br d (13)			2.40 br d (11)	2.40 br d (12)	
H-17	1.12s	5.13 br s 5.00 br s	4.57 br s 4.65 br s	4.71 br s 4.68 br s	1.72s	1.67d (1.8)	1.78s	1.74d (1.7)	1.68 br s
H-18	0.97s	0.94s	0.84s	0.84s	0.92s	0.97s	1.06s	1.0s	0.99s
H-19	0.92d (6.5)	0.92d	1.58s	1.50s	1.58s	1.49s	4.79 br s 4.95 br s	4.75 br s 4.91 br s	5.16 br s 5.25 br s
H-20	0.84d (6.5)	0.96d (7)	1.58s	1.50s	0.84d (6.3)	0.90d (6.6)	0.93d (6.6)	0.91d (6.3)	0.91d (6.6)
OAc							2.19s		2.02s

[a]Chemical shifts ( ) in $CDCl_3$ and coupling constants J in Hz (given in parentheses).

TABLE 5. CHROMATOGRAPHIC PROPERTIES OF *Longipeditermes longipes* DITERPENES

Compound	Oxgenated positions	OV-1[a]	OV-17[a]	R_f color[b]
V	C2=0, C3—OH	22.8	25.5	0.61 (lilac, fades to orange)
II	C3—OH	22.2	24.6	0.59 (greenish blue, fades to greenish grey)
III	C3—OH	21.8	24.1	0.56 (deep purple)
XII	C2—OAc, C3—OAc, C9—OAc	29.0	31.0	0.55 (orange)
VI	C2—OH	22.3	24.7	0.54 (violet, fades to greenish grey)
I	C3—OH	21.8	24.8	0.48 (sky blue)
XI	C2—OAc, C3—OAc, C9—OH	27.1	28.8	0.42 (greenish blue)
VIII	C2—OAc, C3—OH	23.5[c]	26.2[c]	0.36 (pink)
IX	C2—OH, C3—OH	23.5	26.2	0.23 (pink)
IV	C2—OH, C3—OH	23.2	25.9	0.18 (greyish purple, fades to orange)
VII	C2—OH, C3—OH	24.0	26.7	0.16 (greyish blue)
X	C2—OH, C3—OH, C9—OAc	24.5	27.4	0.14 (deep purple)

[a] 5-ft × 3-mm 3% OV-17 or 6-ft × 3-mm 3% OV-1 on 100/120 Gas Chrom Q; retention indices relative to *n*-alkanes.
[b] TLC on silica gel G. Solvent, ethyl acetate–dichloromethane (5:95, v/v); stained by vanillin (Prestwich, 1979c).
[c] Compound decomposed in the GC column.

The soldier secretions of the monotype genus *Longipeditermes* are exceptional in showing colony–colony structural variability. Whereas one colony may be dominated by bicyclic secotrinervitanes and the spirotetracyclic longipenol, nearby colonies may afford trinervitanes, kempanes, or rippertanes. Degree of oxidation is also variable, with mono-, di-, and trioxygenated diterpenes appearing in different colonies. Within a colony, however, only compositional and not structural differences are found in the diterpene components.

The diterpenes presently isolated from *Longipeditermes* are of interest in relation to earlier postulated biogenetic schemes (Prestwich, 1979a; Prestwich et al., 1981c). These compounds are now known to be biosynthesized by nasute termites independently of the dietary source (Prestwich et al., 1981a; Prestwich and Chen, 1981). It may also be noted that *Longipeditermes*, a wood feeder, produces the common trinervitadienediol IX which is also secreted by *Hospitalitermes umbrinus*, a specialist feeder on lichen (Chuah et al., 1982). Cembrene-A, previously isolated from *N. exitiosus* (Birch et al., 1972) and from *Cubitermes* (Wiemer et al., 1979), or its 3,4-epoxide would be an obvious choice as a precursor to the observed diterpenoids. As shown in Figure 2, a one-step cyclization of (*R*)-cembrene-*trans*-3,4-epoxide would yield directly

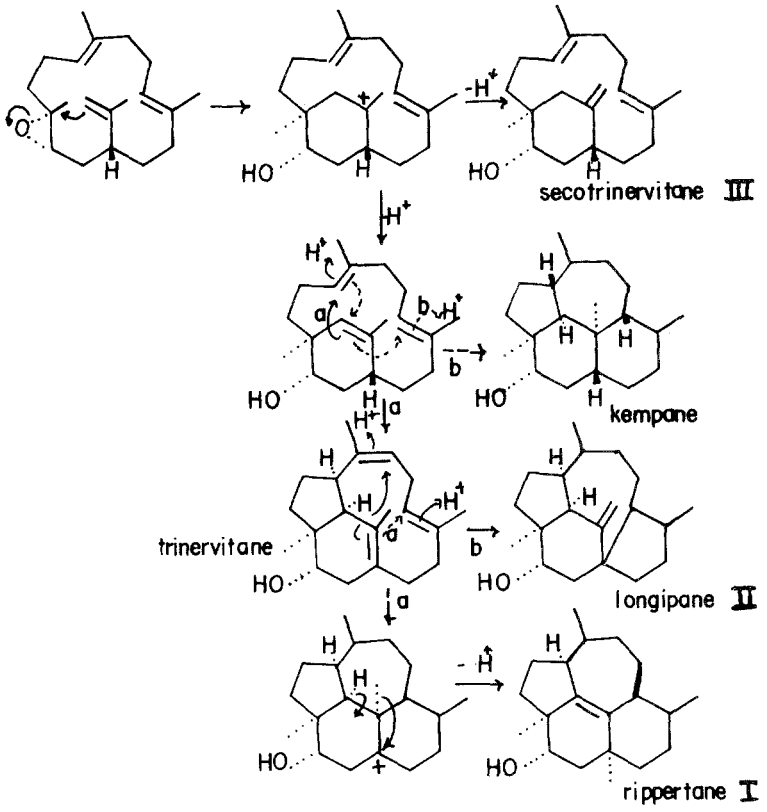

FIG. 2. Biosynthetic interrelationships among the bi-, tri-, tetracyclic, and spiro-tetracyclic diterpenes.

the secotrinervitatrienol III. Interestingly, after isomerisation III has the double bonds positioned for a further cyclization to give longipenol II. The methyl-shifted rippertenol (I) would arise similarly via stepwise cyclization using bicyclic secotrinervitatrienol and tricyclic trinervitadienol as intermediates.

A large number of unique nasute termite diterpenes are now known, but the exact functions of these compounds are not fully understood. It is generally believed that they act as a solute to retard the rate of evaporation of the monoterpenes (Eisner et al., 1976) which are relatively more toxic (Hrdý, 1977). Diterpenes also form the sticky base of the defense secretion, and the hydroxylated derivatives appear well-suited for stickiness based on the model of hydrophobic–hydrophilic interactions (Prestwich, 1979b). The instability

of some of the diterpenoids (e.g., trinervitadiendiol IX) toward air oxidation (Vrkoč et al., 1978) also aids in the resinification of the secretion. However, a number of diterpenes are present in the secretion as the acetylated or propionated derivations (Prestwich, 1979b; Prestwich et al., 1981b). The use of these more stable acetate or propionate derivatives may be preferable for less-active squirters which, for the most part, remain protected inside covered trails [e.g., *Bulbitermes singaporensis* (Prestwich et al., 1981c) and *N. matangensis* (Prestwich et al., 1981b)]. In contrast, active squirters such as the free-ranging *Longipeditermes* and *Hospitalitermes* would use hydroxylated diterpenes and in relatively large quantities. Both major and minor soldiers of *Longipeditermes* participate actively in defense, converging in large numbers to any site of disturbance. Apart from defensive use, the composition of diterpenes for many colonies appears sufficiently distinct to allow for a possible role in nestmate recognition.

The genus *Longipeditermes* occupies an interesting position in the evoluation of Nasutitermitinae. Prestwich and Collins (1981) have summarized arguments that mandibular regression may be concurrent with the evolution of defense secretion. Together with the uninvestigated *Hirtitermes* and *Angularitermes* (Figure 3), *Longipeditermes* is among the first nasutitermitines to evolve the ability to manufacture diterpenes for defense. It is fascinating that the diterpene structural variety in this primitive glue-squirter includes all the known skeletal types, including the novel longipane skeleton, which is unknown in other nasute secretions. We propose that the primitive state for nasute diterpene production involves the biosynthesis of numerous different diterpene forms, each under the control of a different enzyme. The enzyme complex is the result of large genetic variation in the early nasutes, and the secretion of *Longipeditermes* shows the ancestral characteristic of possessing numerous biosynthetic "intermediates" and all possible "end products." In contrast, the more advanced genera like *Trinervitermes* and *Subulitermes*, which lack mandibular conical points, have a derived secretion type, dominated by trinervitanes and in general conservative with respect to intraspecific variation. The highly variable genus *Nasutitermes* has many degrees of mandibular point regression, and a correspondingly high variability in diterpene types. In this genus, no dramatic intercolony variation has ever been found. The evolutionary forces driving the nasute secretion towards reliance on a limited repertoire of structure are unknown. They may include selection imposed by predation, or incipient speciation resulting from recognition of intercolonial chemical differences and eventual reproductive isolation of the genetically homogeneous types. Although the chemical nature of nasute secretions has become well studied, the underlying questions of the biogenesis of the secretion and its importance in nasute ecology and evolution are as yet unanswered.

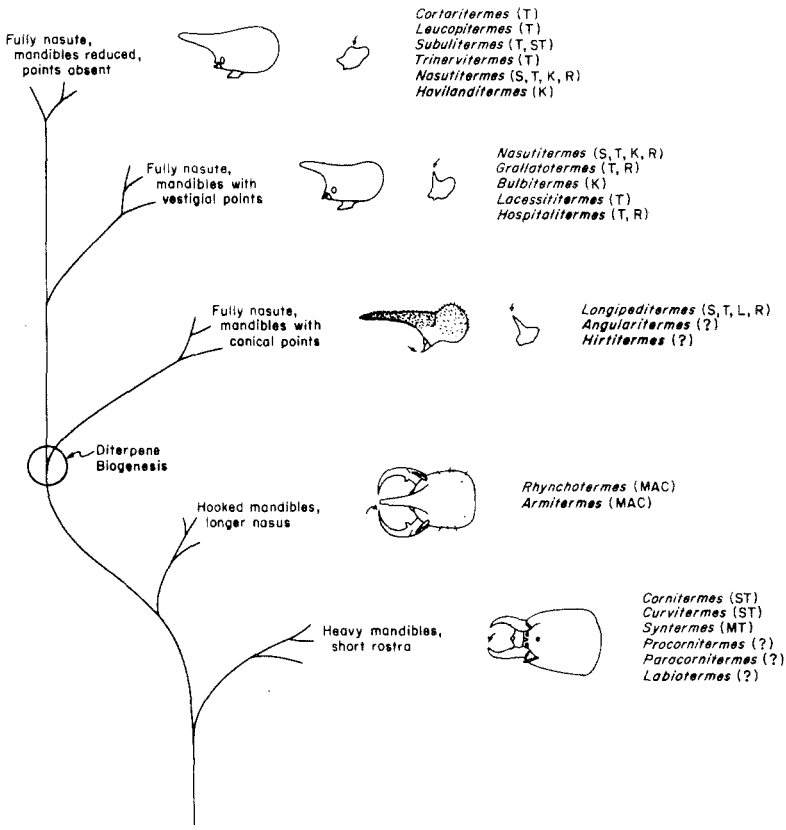

FIG. 3. Proposed monophyletic relationships of nasute termites (Prestwich and Collins, 1981). The circle indicates the diterpene-producing ancestor. Chemical key: diterpenes, K = kempane, L = longipane, R = rippertane, S = secotrinervitane, T = trinervitane; monoterpenes, MT; sesquiterpenes, ST; macrolides, MAC, not investigated, "?".

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METABOLISM OF USCHARIDIN, A MILKWEED CARDENOLIDE, BY TISSUE HOMOGENATES OF MONARCH BUTTERFLY LARVAE, *Danaus plexippus* L.

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Abstract—Midgut and fat body homogenates of monarch butterfly larvae, *Danaus plexippus* L. (Lepidoptera:Danaidae), were examined for microsomal monooxygenase activity using *p*-chloro-*N*-methylaniline *N*-demethylation and for the ability to metabolize a milkweed (*Asclepias* spp.) cardenolide (C_{23} steroid glycoside), uscharidin. All homogenates tested had both *N*-demethylation and uscharidin biotransformation activities. Both transformations required NADPH. The monooxygenase inhibitors sesamex, SKF525A, and carbon monoxide inhibited *N*-demethylation but not uscharidin biotransformation. Subsequent subcellular fractionation revealed the uscharidin biotransformation occurs in the soluble fraction and not the microsomal fraction, while *N*-demethylation occurs in the microsomal fraction and not the soluble fraction. The larval NADPH-dependent microsomal monooxygenase apparently is not involved in the metabolism of uscharidin.

Key Words—Cardenolide, uscharidin, metabolism, monarch butterfly, *Danaus plexippus,* Lepidoptera, Danaidae, milkweed, *Asclepias, N*-demethylation, mixed function oxidase, monooxygenase.

INTRODUCTION

Milkweeds, *Asclepias* spp. (Asclepiadaceae), contain a complex mixture of cardenolides, C_{23} steroid gylcosides (Duffey and Scudder, 1971; Roeske et al., 1976; Nelson et al., 1981). Monarch butterfly larvae, *Danaus plexippus* L. (Lepidoptera: Danaidae) ingest cardenolides as they feed on milkweeds. Some of these cardenolides are sequestered and stored by the larvae and

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remain in the insect tissues through development into the adult. Sequestration of these xenobiotics by the monarch may deter some vertebrate predators due to their bitter taste and emetic and toxic properties (Brower and van Zandt Brower, 1964; Parsons, 1965; Roeske et al., 1976; Fink and Brower, 1981). Cardenolides inhibit Na^+-K^+-activated adenosine triphosphatase and have relatively low mammalian LD_{50} values (intraperitoneal LD_{50}, male Swiss-Webster mouse: 6.5–12 mg/kg for various cardenolides; data from Benson, 1978). The chemical ecology of this cardenolide-based plant–herbivore–predator interaction is a subject of considerable interest (Brower and van Zandt Brower, 1964; Parsons, 1965; Brower, 1969; Roeske et al., 1976; Calvert et al., 1979; Fink and Brower, 1981; Marty et al., 1982).

Extracts of frass and tissues of monarch butterflies reared on *Asclepias curassavica* L. contain relatively polar cardenolides, most of which are present in the plant. However, these extracts lack some of the less-polar plant cardenolides. One possible explanation for this observation is that the insects metabolize the less polar cardenolides to more polar ones (Seiber et al., 1980). Biotransformation of cardenolides resulting in a change in polarity of the molecule may influence disposition and sequestration of these plant chemicals because the polarity of a compound plays a major role in its disposition in an organism. This ultimately will determine the effectiveness as a defense against predators.

Microsomal monooxygenases are involved in the metabolism of many lipophilic xenobiotics, including many plant allelochemicals. Such NADPH-dependent enzyme systems have been studied in insects using a wide variety of substrates (Krieger et al., 1971, Wilkinson and Brattsten, 1972; Chang and Hodgson, 1975; Krieger et al., 1976; Anderson, 1978; Yu and Terriere, 1978; Brattsten, 1979). These enzyme systems are prime candidates for examination relative to the metabolism of milkweed cardenolides (Krieger et al., 1971). In the present study, we investigated the possible role of microsomal monooxygenases in the metabolism of uscharidin, a milkweed cardenolide present in *A. curassavica* but not stored by monarch larvae.

METHODS AND MATERIALS

Fat body and midgut homogenates of monarch butterfly larvae were examined for *p*-chloro-*N*-methylaniline (PCMA) *N*-demethylation and the ability to metabolize the milkweed cardenolide, uscharidin. The effects of SKF525A, sesamex, CO, N_2 atmosphere, and NADPH were examined. Additionally, subcellular fractionation by differential centrifugation was used to determine the locus of activity of each biotransformation.

Animals. Ultimate instar larvae were obtained from milkweed, *Asclepias*

fascicularis Dcne., growing in the Sacramento Valley and northern coastal valleys of California. *A. fascicularis* contains low levels to nil cardenolide. Larvae reared on this milkweed provide tissues with minimal background cardenolide interference. The larvae were transported to the laboratory along with aerial portions of their foodplant. Larvae were used within 24 hours of collection. Only midgut and fat body of actively feeding larvae were examined for biotransformation activity because monooxygenase activity levels have been shown to drop sharply in lepidopterous larvae after the larval feeding period has ended (Krieger and Wilkinson, 1969; Thongsinthusak and Krieger, 1974; Krieger et al., 1976).

Chemicals. Nicotinamide adenine dinucleotide phosphate sodium salt (NADP), reduced nicotinamide adenine dinucleotide (NADH), D-glucose-6-phosphate monosodium salt, glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, Missouri), *p*-chloro-*N*-methylaniline hydrochloride, *p*-chloroaniline hydrochloride (PCA), and *p*-dimethylaminobenzaldehyde (PDAB) (gold label, Aldrich Chemical Co., Milwaukee, Wisconsin) were purchased. Uscharidin, calotropin, and calactin were extracted and purified to single chromatographic spots (TLC) by Dr. Mark Lee from *Asclepias curassavica* L. (Lee, 1983). Additional purification by thin-layer chromatography was performed prior to use. All solvents used were Nanograde or Resianalyzed. Acetonitrile used in HPLC was distilled in glass (Burdick and Jackson, Muskegon, Michigan).

Homogenate Preparation. Fat body and gut homogenates were prepared as described by Marty et al. (1982). Tissues were ground in 0.1 M phosphate buffer, pH 7.7, at 30°C. Each assay tube contained 0.5 ml homogenate added to 0.5 ml reaction mixture. Final concentrations of reaction mixture constituents were 0.23 mM NADP, 2.7 mM glucose-6-phosphate, and 2.8 mM KCl.

The subcellular fractionation experiments utilized tissue homogenates made in 0.2 M phosphate buffer, pH 7.7, at 30°C (Hansen and Hodgson, 1971). The homogenates were centrifuged at 12,000 g_{max} for 15 min, 0–4°C. The resulting pellet, designated mitochondrial fraction, was kept on ice prior to examination for enzyme activity. The supernatant liquid was filtered through glass wool and centrifuged at 122,000 g_{max} for 60 min. The resulting supernatant liquid was designated soluble fraction. The microsomal pellet was resuspended in 0.2 M phosphate buffer. Each fraction and a portion of the original homogenate were examined for PCMA *N*-demethylation and uscharidin biotransformation activity. The reaction mixture for these fractions contained 150 mg glucose-6-phosphate, 40 mg NADP, 20 mg KCl, 70 mg $MgCl_2$, and 50 units glucose-6-phosphate dehydrogenase in 100 ml 0.01 M phosphate buffer, pH 7.7, at 30°C. Each assay tube consisted of 0.5 ml fraction added to 0.5 ml reaction mixture. The final concentrations of

reaction mixture constituents were 0.23 mM NADP, 27 mM glucose-6-phosphate, 1.4 mM KCl, 2 mM Mg^{2+} and 0.25 units/ml glucose-6-phosphate dehydrogenase in 0.1 M phosphate buffer. Blanks were prepared by boiling the assay tubes for 2 min. Reagent blanks consisted of reaction mixture plus buffer.

Enzyme Assays. Assay tubes were preincubated in a water bath shaker at 30° C for 4 min. Substrate or substrate plus inhibitor was added and reaction allowed to proceed for 15 or 20 min. Final concentration of all inhibitors was 7.5×10^{-4}M. *N*-Demethylation reactions were stopped by the addition of 3 ml PDAB, 20 mg/ml in acidified aqueous ethanol (95% ethanol–4 H_2SO_4, 31.7:1, final concentration of H_2SO_4, 0.046 M). Uscharidin biotransformation assays were stopped by the addition of 1 ml of acetone. When gaseous inhibitors were used, the tubes were stoppered with serum caps and flushed with gas for 8 min at a flow rate of 85 ml/min per tube while on ice. To examine CO as an inhibitor, air and CO were mixed in a 5:1 ratio.

PCMA *N*-demethylation was measured as in the procedure of Kupfer and Bruggeman (1966) with modifications for increased sensitivity (Dohn, 1980). This method uses a final concentration of H_2SO_4 that is nine times less than the original protocol. The resulting increase in sensitivity of PCA detection allows measurement of PCMA *N*-demethylation in preparations with low monooxygenase activity. All protein was measured by the method of Lowry (Lowry et al., 1951) using BSA as a standard.

Uscharidin biotransformation was examined by thin-layer chromatography (TLC) of methanolic extracts of each assay tube. TLC plates were EM silica gel 60 with preconcentrating zone. Each plate was developed twice in ethyl acetate–methanol 97:3 or three times in $CHCl_3$–MeOH–$OCHNH_2$ 90:6:1. Cardenolides were visualized by spraying the plates with a 0.4% solution of 2,2',4,4'-tetranitrodiphenyl in toluene followed by 10% KOH in 50% aqueous methanol (Nelson et al., 1981). HPLC was performed on select samples for confirmation of product identity and quantitation. The HPLC system consisted of Waters model 6000A pumps and accompanying Waters data module and system controller, a Rheodyne model 7125 syringe loading sample injector, a 10-μm pore size μBondapak C18 25-cm $\times$ 10-mm column and a Waters model 450 variable wavelength detector set at 217 nm. The solvent system was 31% acetonitrile in water running at a flow rate of 1.7 ml/min. Solvents were filtered and degassed before use.

RESULTS

All gut and fat body homogenates tested had PCMA *N*-demethylation and uscharidin biotransformation activity. Typically, the gut homogenates were more active than the corresponding fat body homogenates for both

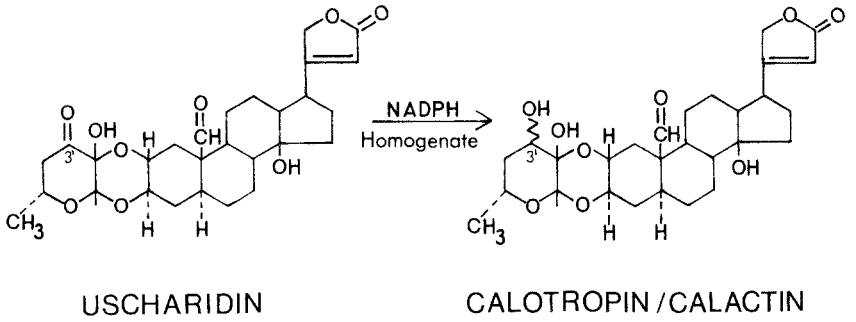

FIG. 1. Biotransformation of uscharidin. The products calotropin and calactin are configurational isomers. Calotropin has an α-OH at C-3′ and calactin has a β-OH at C-3′.

biotransformations as measured by specific activities. This has been observed for monooxygenase-catalyzed reactions in many Lepidoptera insects (Wilkinson and Brattsten, 1972; Krieger et al., 1971).

Uscharidin was biotransformed in the gut homogenates into two more-polar cardenolides which cochromatographed by TLC with the cardenolides calotropin and calactin (Figure 1). The fat body homogenates produced one more-polar cardenolide which cochromatographed by TLC with calotropin. The identities of the products, calotropin and calactin, were later confirmed by HPLC. When calotropin was used as a substrate, no cardenolide metabolites including uscharidin were produced by gut or fat body homogenates. This indicates that the reaction is unidirectional and that calotropin and calactin are probably final products.

The monooxygenase inhibitors sesamex, SKF525A, and CO were examined for their effects on uscharidin biotransformation and PCMA *N*-demethylation. Thin-layer chromatography of extracts of uscharidin biotransformation assays revealed no apparent change in the production of metabolites in the presence of these inhibitors. In contrast, PCMA *N*-demethylation was inhibited by all three inhibitors. Sesamex inhibited *N*-demethylation activity by 45% in the gut homogenates ($N = 3$) and by 52% in the fat body homogenates ($N = 3$) (Table 1). SKF525A also inhibited *N*-demethylation by 66% in gut homogenates and by 30% in fat body homogenates (Table 1).

TLC of extracts of uscharidin biotransformation assays performed in an air–CO 5:1 ($CO–O_2$ 1:1) atmosphere indicated that CO had not inhibited uscharidin metabolism. This observation was confirmed subsequently by HPLC. Table 2 gives specific activities for PCMA *N*-demethylation and uscharidin biotransformation in the presence and in the absence of CO. Monooxygenase-catalyzed *N*-demethylation was inhibited by 43% in gut

TABLE 1. INHIBITION OF PCMA *N*-METHYLATION BY SESAMEX AND SKF525A

Tissue	*N*[a]	Sesamex	SKF525A	Specific activities *N*-Demethylation[b]	Inhibition (%)
Gut	3	+	−	0.184 ± 0.015	45
Gut	3	−	−	0.333 ± 0.021[c]	
Fat body	3	+	−	0.043 ± 0.002	53
Fat body	3	−	−	0.091 ± 0.005[c]	
Gut	3	−	+	0.151 ± 0.034	66
Gut	3	−	−	0.441 ± 0.084[c]	
Fat body	3	−	+	0.075 ± 0.003	30
Fat body	3	−	−	0.107 ± 0.004[c]	

[a]Number of homogenates tested.
[b]Expressed as nanomoles PCA produced per milligram protein per minute.
[c]Specific activities without inhibitor are significantly greater than in the presence of inhibitor, $P < 0.01$, Student's t test.

homogenates and by 61% in fat body homogenates. If monooxygenase catalysis were involved in uscharidin metabolism, inhibition by carbon monoxide would have occurred.

The effects of a N_2 atmosphere on uscharidin biotransformation was then examined. PCMA *N*-demethylation was used as an indicator of monooxygenase activity in the same homogenates. As expected, *N*-demethylation was inhibited greatly. The gut homogenates under N_2 produced 14% as much total PCA as those under air (4.3 ± 0.85 nmol PCA

TABLE 2. INHIBITION BY CARBON MONOXIDE

			Specific activities	
Tissue[a]	*N*[b]	CO	*N*-demethylation[c]	Uscharidin biotransformation[d]
G	3	−	840 ± 156[e]	1.97 ± 0.65
G	3	+	476 ± 81	2.10 ± 0.54
F	3	−	222 ± 29[e]	1.38 ± 0.21
F	3	+	85 ± 3	1.36 ± 0.20

[a]G = gut homogenate; F = fat body homogenate.
[b]Number of homogenates tested.
[c]Expressed as picomoles PCA produced per milligram protein per minute.
[d]Expressed as nanomoles calotropin and calactin (gut) or nanomoles calotropin (fat body) produced per milligram protein per minute.
[e]Specific activity in the absence of carbon monoxide is significantly greater than in the presence of CO, $P < 0.02$, Student's t test.

produced per tissue under N_2 as compared to 31.5 ± 0.70 nmol PCA produced per tissue under air, $N = 2$). Similar results were obtained for fat body homogenate (under N_2, 4.8 ± 3.2) nmol PCA were produced per tissue; under air, 28 ± 1.4 nmol PCA were produced per tissue, $N = 2$). In contrast, TLC of extracts of uscharidin biotransformation assays performed in air or under N_2 showed no differences in production of metabolite. Metabolism of uscharidin to calotropin and calactin is apparently a reductive rather than an oxidative process.

Subcellular fractionation using differential centrifugation was used to localize the uscharidin biotransformation activity. Table 3 gives specific activities for homogenate, mitochondrial, soluble, and microsomal fractions. There is a lack of measurable *N*-demethylation activity in the soluble fraction of gut and fat body. As expected, the microsomal fraction had the most activity. In contrast, the uscharidin biotransformation activity is concentrated in the soluble fraction. The activity of the soluble fraction of the gut is 20 times greater than the microsomal fraction and the activity of soluble fraction of the fat body is 22 times greater than the corresponding microsomal fraction. The mitochondrial and microsomal fraction activities did not differ significantly from each other.

Both biotransformations require an NADPH-generating system. Spe-

TABLE 3. SPECIFIC ACTIVITIES OF SUBCELLULAR FRACTIONS

Tissue[a]	Fraction	N[b]	Uscharidin biotransformation[c]	N[b]	*N*-Demethylation[d]
G	Homogenate	4	1.6 ± 0.49	4	0.933 ± 0.164
G	Mitochondrial	4	0.32 ± 0.20	4	0.251 ± 0.053
G	Microsomal	4	0.21 ± 0.14	4	2.42 ± 0.919[e]
G	Soluble	4	4.1 ± 0.99[e]	4	not detectable
F	Homogenate	4	0.88 ± 0.22	4	0.334 ± 0.023
F	Mitochondrial	4	0.33 ± 0.11	4	0.166 ± 0.031
F	Microsomal	4	0.26 ± 0.10	4	0.258 ± 0.079[f]
F	Soluble	4	1.90 ± 0.47[e]	4	not detectable

[a]G = gut; F = fat body.
[b]Number of fractions tested.
[c]Expressed as nanomoles calotropin and calactin (gut) or nanomoles calotropin (fat body) produced per milligram protein per minute.
[d]Expressed as nanomoles PCA produced per milligram protein per minute.
[e]Soluble fraction specific activity is significantly greater than microsomal and mitochondrial fractions, $P < 0.01$, Student's t test. Microsomal fraction specific activity is significantly greater than mitochondrial fraction, $P < 0.01$, Student's t test; no PCA was produced by the soluble fraction.
[f]Microsomal fraction specific activity is greater than mitochondrial fraction, $0.05 < 0.10$, Student's t test; no PCA was produced by the soluble fraction.

TABLE 4. SPECIFIC ACTIVITIES IN PRESENCE AND ABSENCE OF NADPH AND NADH

Tissue[a]	N[b]	NADPH	NADH	Uscharidin biotransformation[c] (soluble fraction)	PCMA N-Demethylation[d] (homogenate)
G	3	+	−	4.19 ± 0.45[e]	0.561 ± 0.004[e]
G	3	−	+	0.28 ± 0.30	
G	3	−	−	0.11 ± 0.04	not detectable
F	3	+	−	2.65 ± 0.58[e]	0.212 ± 0.020[e]
F	3	−	+	0.91 ± 0.21	
F	3	−	−	0.86 ± 0.22	0.069 ± 0.015

[a]G = gut; F = fat body.
[b]Number of homogenates (N-demethylation) or soluble fractions (uscharidin biotransformation) tested.
[c]Expressed as nanomoles calotropin and calactin (gut) or nanomoles calotropin (fat body) per milligram protein per minute.
[d]Expressed as nanomoles PCA formed per milligram protein per minute.
[e]Specific activities in the presence of NADPH regenerating system are significantly greater than in the presence of NADH or in the absence of added cofactor, $P < 0.01$, Student's t test.

cific activities are given in Table 4 for uscharidin biotransformation in gut and fat body soluble fractions in the presence of an NADPH-generating system, in the presence of NADH, and in the absence of any added cofactor. Specific activities for PCMA N-demethylation in the presence and in the absence of an NADPH generating system are also given. The uscharidin biotransformation activity is 38 times greater in the gut soluble fraction and four times greater in fat body soluble fraction when an NADPH-generating system is present than when no cofactors are added. Addition of NADH (0.56–5.6 mM) did not significantly increase specific activity in either gut or fat body soluble fractions. PCMA N-demethylation activity of gut homogenates was not measurable in the absence of an NADPH-generation system. N-demethylation activity was three times greater in fat body homogenate in the presence of an NADPH-generating system than in its absence.

DISCUSSION

The cardenolides in milkweeds, *Asclepias* spp., may limit utilization of these plants by some herbivores (Feeny, 1976). Monarch butterfly larvae feed solely on milkweeds in the family Asclepiadaceae. Consequently, the larvae are continually exposed to cardenolides. The levels of exposure can be high because some milkweeds contain large amounts of cardenolide [Roeske et al., (1976) reported 6.3 mg digitoxin equivalents/g dried buds in *A. curassavica*].

Cardenolides are chemically diverse and possess a wide range of polarities. Many are lipophilic enough to be substrates of monooxygenase enzymes. Several cardenolides in the digitalis series such as digitoxin and digitoxigenin, which are of comparable polarity to uscharidin and other milkweed cardenolides, are substrates of mammalian microsomal monooxygenases (Talcott and Stohs, 1972; Richards and Lage, 1977; Schmoldt et al., 1980; Castle, 1980). Monarch butterfly larvae have low levels of monooxygenase activity as measured in tissue homogenates by PCMA *N*-demethylation and aldrin epoxidation (Marty et al., 1982).

This study showed that monarch larval tissue homogenates biotransform the milkweed cardenolide, uscharidin, to more polar cardenolide products. However, NADPH-dependent microsomal monooxygenases are not responsible for catalyzing this biotransformation. The biotransformation does not occur in the microsomal fraction but rather in the soluble fraction in contrast to *N*-demethylation, an enzymatic transformation characteristic of the microsomal monooxygenases.

Uscharidin biotransformation by tissue homogenates requires NADPH and is blocked by boiling the tissue and by adding denaturing solvents to the homogenates. The products are formed by reduction of a keto group on the glycosidic portion of the molecule (Figure 1). Gut tissue metabolizes uscharidin to the configurational isomers calotropin and calactin. Only one of these isomers, calotropin, is formed by fat body. This indicates that stereoselectivity is a characteristic of the enzyme(s) responsible for the biotransformation in the fat body but not in the gut.

There are numerous examples of enzymatic reduction of endogenous and xenobiotic carbonyl compounds by the group of enzymes called ketone reductases (references in Falsted and Bachur, 1980). Although not well characterized, these enzymes seem to use NADPH rather than NADH as a cofactor and most are localized in the soluble fraction. These enzymes have been studied in vertebrates. Perhaps similar enzymes exist in insects and are responsible for the metabolism of uscharidin.

Monarch larvae do not store all cardenolides present in their food plants (Roeske et al., 1976). The more-polar plant cardenolides are present in the insects' tissues (Roeske et al., 1976; Seiber et al., 1980; Brower et al., 1982). Perhaps the less-polar cardenolides are rapidly metabolized to more-polar cardenolides that are then stored by the insect. This appears to be true for the cardenolide uscharidin and may be true for other cardenolides as well.

It is unlikely that microsomal monooxygenases are important in the biotransformation of milkweed cardenolides in monarch butterfly larvae. Many of the milkweed cardenolides are not metabolized by the larvae. Of the cardenolides investigated, the relatively few apolar cardenolides that are present in the plant but not in the insect are probably metabolized to other

cardenolides by virtue of hydrolyses and reductions (Brower et al., 1982). In this regard, it is of interest that the monarch does not store the cardenolides uscharin and uscharidin from *A. curassavica* or labriformin and labriformidin from *A. eriocarpa*. The sugars attached to uscharin and labriformin are identical and have a thiazoline ring attached at C-3′. Both can be hydrolyzed to yield the C-3′ ketones, labriformidin and uscharidin. These in turn could be reduced to the corresponding secondary alcohols, desglucosyrioside and calotropin/calactin (Brower et al., 1982). We have shown that uscharidin is enzymatically reduced by larval tissues to the secondary alcohols calotropin and calactin which are stored in the larval tissues. Perhaps the reduction of labriformidin, which is not stored in larval tissues, to the secondary alcohol, desglucosyrioside, which is stored, is a similar enzymatic transformation.

The physicochemical properties of the parent compound and its metabolic derivatives will determine whether a particular cardenolide is stored or excreted by a given organism. Thorough pharmacological studies on the relative bitterness, emeticity, and general toxicities of cardenolides to potential predators will be necessary to further elucidate the biological significance of cardenolide metabolism and storage by monarch larvae.

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CHEMICAL INDUCTION OF FEEDING IN CALIFORNIA SPINY LOBSTER, *Panulirus interruptus* (RANDALL): Responses to Molecular Weight Fractions of Abalone

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Abstract—Molecular weight fractions of abalone muscle were tested for the ability to induce appetitive feeding and locomotor behavior in the spiny lobster, *Panulirus interruptus*. Fractions of <1000, 1000–10,000 and >10,000 daltons were isolated by ultrafiltrations and gel chromatography from a seawater extract of abalone muscle. The two lower-molecular-weight fractions (<1000, 1000–10,000) were the least stimulatory of the three fractions tested, and both were ineffective as feeding stimulants. Solutions combining any two of the three isolated fractions produced behavioral activity equal to that caused by whole extract; thus, no single fraction was essential to the stimulatory capacity of abalone. The >1000-dalton fraction was also highly stimulatory, meaning that large and not small molecules were essential in initiating feeding. Finally, a 75% ethanol-insoluble component of the <10,000 fraction was effective, while the ethanol-soluble portion was not. Since the insoluble material consisted predominantly of peptides and polypeptides, it is probable that these molecules act as principal stimulants in abalone muscle.

Key Words—Chemoreception, feeding, lobster, Crustacea, *Panulirus interruptus*.

INTRODUCTION

Chemical stimuli are dominant inducers of feeding in crustaceans, serving to activate probing and grasping reflexes (Maynard and Dingle, 1963; Hazlett, 1968; Fuzessery and Childress, 1975; Pearson and Olla, 1977; Pearson et al.,

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1979; Derby and Atema, 1982), while also directing patterns of locomotion (McLeese, 1973b; Reeder and Ache, 1980; Devine and Atema, 1982). Identification of the responsible stimulatory compounds has followed two general approaches: In the first, chemicals readily available in purified form have been assayed without parallel tests of substances prepared from prey or carrion tissues (e.g., Crisp, 1967; McLeese, 1970; Kay, 1971; Shelton and Mackie, 1971; Allen et al., 1975; Hindley, 1975; Allison and Dorsett, 1977; Hartman and Hartman, 1977; Ache et al., 1978). These investigations have been valuable in determining effects of molecular structure on attractivity. In the second approach, extracts of natural tissues and their component fractions have been tested (Mackie and Shelton, 1972; Mackie, 1973; McLeese, 1973a; Carr and Gurin, 1975; Carr, 1978). These studies have shown that several classes of compounds are necessary for complete induction of feeding behavior.

It is often assumed that soluble, low-molecular-weight substances are the primary feeding stimulants of crustaceans, but this view must be questioned, since in most studies only low-molecular-weight substances have been assayed without tests of the stimulatory abilities of macromolecules. Amino and organic acids (e.g., McLeese, 1970; Kay, 1971; Hindley, 1975; Ache et al., 1978), sugars (e.g., Hartman and Hartman, 1977; Robertson et al., 1981), nucleotides (Shelton and Mackie, 1971), and other low-molecular-weight nitrogenous compounds, such as betaine and trimethylamine oxide (e.g., McLeese, 1970; Mackie, 1973; Carr, 1978) have been the subject of intensive investigations, while to our knowledge, only studies by Carr and Gurin (1975) and Carr (1978) have directly investigated influences of molecular weight fractions of natural attractants. A macromolecular fraction (>1000 daltons) was actually tested in only one of these studies (Carr and Gurin, 1975).

We have tested molecular weight fractions (<1000, 1000–10,000, >10,000 daltons), prepared from abalone (*Haliotis* spp.) extract, for ability to cause feeding in the California spiny lobster *Panulirus interruptus* (Randall). Abalone is available to *Panulirus* as carrion, and it is eaten alive by *Panulirus* in laboratory experiments (Carlberg, 1975). When available, it is the preferred bait used by southern California commercial lobster fisherman. Field experiments have already demonstrated that abalone muscle is attractive to lobsters, when presented in dialysis membranes releasing substances <10,000 daltons, but not when releasing substances <1000 daltons (Zimmer-Faust and Case, 1982b). Since trapping experiments cannot be used to determine stimulant effects on the various behavioral subcomponents of searching and feeding, the present laboratory studies were undertaken. They show the importance of bait tissue macromolecules to both foraging and feeding in *Panulirus*.

METHODS AND MATERIALS

Collection and Maintenance of Animals. Lobsters were captured in traps or by SCUBA and brought immediately to the laboratory where groups of 10 were held in 3000-liter aquaria for 14 days prior to experiments. A continuous, single-pass seawater flow (5-μm filtered) through each holding tank maintained aeration and ambient sea temperature (15–18° C). Incoming animals were tatoo marked (Kuris, 1971), and carapace length, sex, and reproductive status were recorded. Only hard-shelled animals of 60–68 mm carapace lengths were used in experiments. They were fed abalone muscle, mackerel muscle, and opened mussels and deprived of food for 24 hr before testing.

Test Apparatus and Procedures. These were nearly identical to those previously described (Zimmer-Faust and Case, 1983). Animals were individually tested for responses to chemical solutions in rectangular aquaria, $30 \times 30 \times 13$ cm, a size allowing precise stimulus flow control without inhibiting behavior. Lighting was provided by 25-watt, red incandescent bulbs in diffusing housing 50 cm above the aquaria. Illumination was completely confined to the aquaria by opaque blinds provided with observation ports. The surrounding laboratory was kept dark to reduce visual disturbances. Seawater (980 ml/min) entered each aquarium from a head-tank maintained under constant hydrostatic pressure and stimulants were introduced from a reservoir (1.4 ml/sec) by a three-way valve. Dilution associated with stimulus delivery was 1.02×10^{-3} ($\pm 0.13 \times 10^{-3}$ SD) times original concentrations, as determined by fluorometric measurement of fluorescein dye dilution. Peak dye concentrations were attained 19 sec (± 4 SD) after initial dye input and homogeneous distribution was established 30 sec after dye introduction (Zimmer-Faust and Case, 1983).

Lobsters were tested only once in 48 hr for a maximum of five times during a 14-day period. Animals were put in experimental aquaria 90–120 min prior to testing and usually settled within 30–40 min. Observations of behavior were initiated 1 min before chemical introduction and were continued for 3 min after introduction, according to a double blind protocol. Selections of chemical solutions and their order of presentation were made using a random-numbers table with the exception that identical solutions were never repetitively tested on the same animal. Order of stimulus presentation did not influence the behavior of individuals, since it could be demonstrated that for each extract and fraction, the proportion of animals responding was unrelated to the test sequence. Each test was considered statistically independent and treated as a Bernoulli trial.

Preparation of Freeze-Dried Abalone Muscle Extract (FDAME). Abalone foot muscle tissue was lyophilized and ground to a fine powder, then 20 g were homogenized with 500 ml filtered seawater (FSW : 5-μm filtered). The homogenate was transferred to a flask and combined with an additional 400 ml of FSW, vortex-stirred at 4° C for 24 hr, and next centrifuged (16,300 g at 4° C for 30 min). The supernatant was decanted and filtered (Millipore membrane, 0.45-μm pore diameter). Retentates from both centrifugation and filtration were combined on the tared filter membrane and were dried to constant weight at 60° C. Stock solution concentration was calculated by subtracting dry weights of retentates from the original 20 g of tissue. Unless otherwise stated, all bioassays were performed using a single test concentration of FDAME, 6.00×10^{-3} g/liter, as corrected for dilution.

Preparation of Molecular Weight Fractions by Ultrafiltration. Trapping experiments in typical lobster habitats suggest that molecules >1000 daltons released from abalone muscle are attractive to lobsters (Zimmer-Faust and Case, 1982b). For the research described here, fractions were prepared from the standard FDAME. The <1000-, 1000–10,000-, and >10,000-dalton molecular weight fractions along with combinations of these fractions were tested for stimulatory capacity. Fractions were prepared using a pressure ultrafiltration vessel (Amicon model 402) and membranes having molecular weight cutoffs of 1000 daltons (Amicon UM-2) and 10,000 daltons (Amicon UM-10). Ultrafiltrations were performed at 50 psi (N_2) at 4° C. Ultrafiltrates (<1000 and <10,000) were collected and tested undiluted at original concentrations, while ultraretentates (>1000 and >10,000) were concentrated and rinsed with FSW to reduce the possibility of contamination by residual low-molecular-weight substances before dilution for tests, also at original concentrations. Recombinations (<1000 + >1000; <10,000 + >10,000) of ultrafiltrates together with their complementary ultraretentates were used to control for potential losses of attractivity caused by analytical procedures. Behavioral responses produced by recombined fractions were expected to equal those induced by whole extract (FDAME). A 1000- to 10,000-dalton fraction was prepared by first ultrafiltering FDAME using a UM-10 membrane, then collecting and ultrafiltering the UM-10 ultrafiltrate with a UM-2 membrane. Ultraretentate from this second ultrafiltration was concentrated, rinsed with FSW, then diluted to its original concentration for assay.

One liter of the <10,000 dalton fraction was diluted to 4 liters with absolute ethanol, then held at 4° C for 24 hr. The ethanol-insoluble material was collected by centrifugation (16,300 *g* at 4° C for 30 min), rinsed 2 times with 75% ethanol in FSW to remove residual soluble material, then resuspended to the original volume. The ethanol-soluble material was concentrated to dryness by rotary evaporation and resuspended to the

original volume. The subfractions were stored frozen (-20°C) in randomly assigned, numbered test tubes until testing. A schematic diagram of fractionation procedures is given in Figure 1.

Chemical extracts and fractions were considered stimulatory when the proportions of animals responding differed significantly from the proportion responding to seawater controls (chi-square test: $P < 0.05$).

Preparation of Molecular Weight Fractions by Gel Chromatography. Molecular weight fractions were also prepared by gel chromatography to provide a comfirmatory assay of small molecules. Three hundred grams of Sephadex G-10 gel were hydrated in boiling nanopure water for 1 hr, cooled (20° C), and packed in a glass column (60 × 5 cm) to a bed depth of 38 cm. Packing was protected by two Whatman GF/C filter papers and rinsed with 2 liters of nanopure water, prior to column use. A blue dextrin/fluorescein dye solution was used to visually calibrate elution volumes for high (>700 daltons) and low (<700 daltons) molecular weight fractions, respectively. When fractionating FDAME, 500 ml were carefully layered onto the column and washed into the resin by applying 25 ml of nanopure water. Nanopure water was then used to elute the fractions. High-molecular-weight substances (>700 daltons) emerged after 250 ml and were fully eluted at 750 ml. Low-molecular-weight substances (<700 daltons) began to emerge after 800 ml and were fully eluted only after application of 4500 ml. Both fractions were evaporated to dryness and then resuspended to original concentrations before testing. A recombined solution (<700 + >700) was prepared to control for analytical procedures.

Amino Acid Analysis. A lithium citrate step-gradient system (Pierce) for examination of physiological fluids was used with a microbore cation exchange column (0.4 × 25 cm) to quantify the amino acids found in the FDAME and subfractions. Postcolumn derivatization with Fluropa (Dionex) yielded fluorescent products. Physiological fluid standards (Pierce) were used to identify the individual amino acids, and norleucine was used as an internal standard for quantification of the component amino acids.

RESULTS

Description of Feeding and Locomotor Behavior and Selection of Behavioral Acts for Bioassay. To define aspects of behavior important in appetitive feeding and locomotion, we observed animals kept both in large tanks (1.3 × 0.74 × 0.20 m) and in smaller test aquaria as they responded to chemical extracts prepared from abalone muscle and mussels (*Mytilus californianus*). Responses to extracts were nearly identical to those occurring when whole food was placed near lobsters. No differences in behavior were observed associated with the two different types of tanks, although response latencies were often longer in the large tanks, as might be expected.

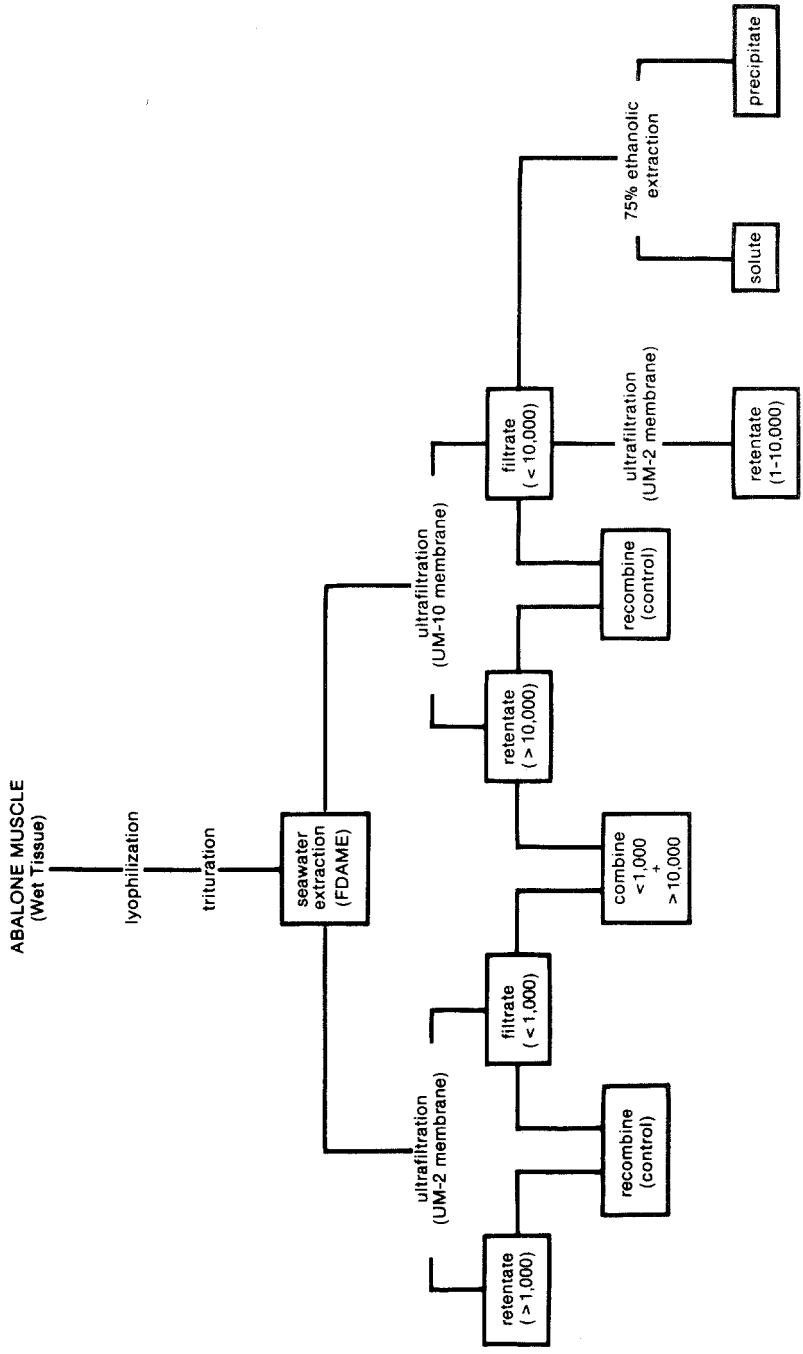

FIG. 1. A schematic diagram outlining procedures used in the preparation of molecular weight fractions by ultrafiltrations. Assayed solutions appear in boxes. Recombinations were performed to control for the use of both UM-2 and UM-10 membranes. Single fractions (<1000, 1000-10,000 and >10,000) and combinations of single fractions (<10,000, >1000, and <1000 + >10,000) were prepared for tests.

Inactive animals rested in shelters or in corners of aquaria with tails flexed and antennae contacting surfaces. Pereiopod movements were commonly associated with slight changes in body posture, while third maxillepeds frequently contacted other mouthparts. Occasionally, maxillepeds were extended, then flexed in a fanning motion, possibly associated with ventilation. Antennule flicking was relatively slow ($\leq$1 flick/sec) and occurred in bursts so that mean flick rates were not always indicative of short-term variability.

Following introduction of natural food or an effective chemical stimulus, antennule flicking increased but remained arrhythmic. Side-to-side sweeps of one or both antennules became frequent, and often lobsters pointed antennules in the direction of the prevailing current or towards a distant chemical source. Substrate probing movements with pereiopods were associated with increased antennular activity. Response sequences were frequently arrested at this point, particularly when only low concentrations (10^{-5}–10^{-7} g/liter FDAME) or small bits of food had been introduced. At higher stimulus intensities (e.g., $> 10^{-3}$ g/liter FDAME), lobsters continued to respond by rubbing opposing third maxillepeds together and by deflecting antennules downward into contact with third maxillepeds, leading to grasping and wiping of antennular flagellae. Wiping of one antennule typically alternated with wiping of the opposing antennule, in a series of successive acts. Induction of locomotion was commonly delayed for $\geq$60 sec after introduction of an effective stimulus, and was initiated only when early pereiopod probings failed to contact food.

For the present study, antennule flicking and wiping, leg probing, and locomotion were selected for assay. Both electrophysiological and behavioral investigations have shown that flicking is important to the detection of chemical stimuli (Snow, 1973; Pearson and Ola, 1977; Price and Ache, 1977; Pearson et al., 1979; Schmitt and Ache, 1979), while antennule wiping is believed to function either in cleaning and resetting antennule chemoreceptors (Snow, 1973) or in transferring stimulus molecules from antennules to mouthparts (Fuzessery and Childress, 1975). Probing and locomotion have obvious utility in food search. Feeding is defined here as the mutual occurrence of flicking, wiping, and probing, each within a 3-min test period. These acts are important either in detecting or in searching for immediately nearby food. Because locomotion is also important in distant food search, it is treated independently of feeding. Definitions of these behavioral elements appear in Table 1.

Appetitive Feeding Responses to Molecular Weight Fractions of FDAME. The stimulatory capacity of single fractions increased with molecular weight, but no single fraction evoked whole-feeding responses in a significant proportion of animals (Table 2). Feeding behavior approached

TABLE 1. DEFINITIONS OF BEHAVIORAL ELEMENTS IN APPETITIVE FEEDING AND LOCOMOTION BY *Panulirus*

Act	Definition
Feeding	
Antennule flicking	Vertical deflection of a lateral antennular flagellum to a position nearly contacting the medial flagellum. A response was defined as >1.0 flick per second.
Leg probing	Any nonlocomotor movement of a pereipod: either raking a dactyl across the substratum or elevating a dactyl to a position no longer in contact with the substratum.
Antennule wiping	A downward and vertical deflection of an antennule, resulting in simultaneous contact of both antennular flagellae with the third maxillepeds.
Locomotion	A laterally or anteriorly directed movement of the body to a distance $>\frac{1}{2}$ carapace length.

significance ($P = 0.06$) in the presence of the >10,000 fraction, but activity associated with the <1000 fraction was nearly identical to that of seawater controls. This last result is important because it shows that low-molecular-weight substances alone are ineffective feeding stimulants. It could be argued that the inability of single fractions to stimulate behavior resulted from losses of attractivity associated with the fractionation procedure. This did not appear to be the case, since recombined (control) fractions were highly stimulatory (Table 2). It was also possible that the failure of the <1000 fraction to stimulate feeding was because tests were conducted exclusively at a single, high concentration. Low-molecular-weight substances, specifically amino acids, have been demonstrated to be attractive when dilute, but nonattractive at high concentrations (Shelton and Mackie, 1971; Carr, 1978). For this reason, we tested the < 1000 fraction at concentrations of 6.00×10^{-5} g/liter and 6.00×10^{-7} g/liter. Only 22% ($N = 23$) and 18% ($N = 23$) of tested animals exhibited feeding at these concentrations, respectively, indicating that the lack of attractivity was unrelated to stimulus intensity.

We next tested combinations of two of the three single molecular weight fractions. Lobsters responded to these solutions equally as well as to the recombined (control) fractions, showing that combined attractants were essential to the production of full behavioral activity (Table 2). Of particular interest was the ability of the >1000 fraction to evoke whole-feeding responses since this demonstrated that molecules <1000 daltons are unnecessary for feeding to occur. Stimulants were found to vary widely in molecular weight, so that no single fraction (i.e., <1000, 1000–10,000, >10,000) was essential to induction of feeding.

TABLE 2. APPETITIVE FEEDING RESPONSES OF *Panulirus* TO FDAME AND MOLECULAR WEIGHT FRACTIONS[a]

Test solution	Proportion feeding	Number tested
Whole extract (FDAME)	0.40***[b]	60
Ultrafiltrations		
Single fractions		
<1,000	0.13	40
1000–10,000	0.13	40
>10,000	0.25	40
Recombined fractions (control)		
<1,000 + >1,000	0.35**	40
<10,000 + >10,000	0.33*	40
Combined fractions		
<10,000	0.35**	40
>1,000	0.38***	40
<1,000 + >10,000	0.40***	40
Ethanolic extraction (<10,000)		
Solute	0.18	40
Precipitate	0.40***	40
Gel filtrations		
<700	0.15	24
>700	0.33*	24
<700 + >700	0.37**	24
Seawater (control)	0.09	45

[a]Feeding was defined as the occurrence of probing, wiping, and increased flicking, each within a 3-min trial period.

[b]The difference is significant between proportions of animals responding to test versus control (seawater) solutions (chi-square test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

An additional test using products from the ethanolic extraction of the <10,000 fraction showed that the precipitate and not the solute was most stimulatory to lobsters (Table 2). Since the solute consisted predominately of small molecules, including amino acids (Table 3), its failure to induce behavior was consistent with previous trials. A final test of small molecules involved fractions prepared by gel chromatography. In this test, both the high-molecular-weight (>700 daltons) and recombined (<700 + >700) fractions were effective in stimulating whole-feeding responses. The <700 fraction was not effective (Table 2), thus confirming our observation that large, rather than small, molecules are essential to chemical induction of feeding.

Thus far only chemical initiation of the entire feeding sequence has been considered. We turn next to the ability of each fraction to elicit component

TABLE 3. CONCENTRATION (MILLIMOLES/LITER) OF PRINCIPAL[a] AMINO ACIDS IN FDAME AND SELECTED SUBFRACTIONS

					EtOH		
	FDAME	<1000	<10,000	>10,000	Sol.	Insol.	FSW
Tau	6	5.5	7.2	0.2	4.45	0.4	0
Asp	0.05	0.01	0	0	0.01	0.01	0
Thr	0.05	0[b]	0	0	0	0	0
Ser	0.05	0	0	0	0	0	0
Glu	0.3	0.05	0	0	0.04	0.03	0
Gly	0.5	0.45	0.4	0.01	0.2	0.02	0
Ala	0.4	3.0	0.3	0.01	0.2	0.02	0
NH_4^+	0.4	1.54	0.3	0	0.6	0	0
Orn	0.01	0.2	0.01	0	0.03	0	0
Lys	0.03	0.2	0.03	0	0.01	0.02	0
His	0.01	0	0.01	0	0	0	0
Arg	0.65	0.05	0.6	0	0.01	0	0

[a]The total comprises greater than 95% of total free amino acids.
[b]A zero value indicates concentrations less than 0.01 mM.

elements of feeding behavior and locomotion. All tested fractions caused increased rates of antennule flicking, indicating their detection by lobsters (Table 4). However, flicking did not always occur with other behavioral acts and was a poor indicator of the overall stimulatory capacity of a solution. In particular, low-molecular-weight fractions of <1000 and 1000–10,000 daltons were found highly effective in stimulating flicking, but were unable to elicit probing, wiping, or locomotion (Table 4). Fractions of >10,000 daltons and the solute of the ethanolic extraction of the <10,000 fraction were effective in causing flicking and probing but not wiping or locomotion. These latter two fractions were thus capable of triggering behavioral patterns important in searches for nearby food. Combined fractions, recombined (control) fractions, and the precipitate from the ethanolic extraction induced all four assayed behaviors (Table 4), demonstrating a capacity to initiate both appetitive feeding and exploratory locomotor behavior.

DISCUSSION

Molecular weight fractions prepared from a seawater extract of abalone muscle contributed nonspecifically to feeding induction. All combinations of two molecular weight fractions of the three available (<1000, 1000–10,000, >10,000) were able to induce whole-feeding responses. No single fraction was

TABLE 4. COMPONENT RESPONSES OF *Panulirus* TO FDAME AND MOLECULAR WEIGHT FRACTIONS

Test solution	Proportion responding				Number tested
	Detection (increased flicking)	Probing	Wiping	Locomotion	
Whole extract (FDAME)	0.97***[a]	0.83***	0.41***	0.35**	60
Ultrafiltrations					
Single fractions					
<1,000	0.90***	0.43	0.15	0.13	40
1000–10,000	0.95***	0.35	0.15	0.13	40
>10,000	0.95***	0.68**	0.28	0.15	40
Recombined fractions (control)					
<1,000 + >1,000	0.90***	0.60*	0.35**	0.25*	40
<10,000 + >10,000	1.00***	0.70***	0.33*	0.25*	40
Combined fractions					
<10,000	0.95***	0.63*	0.35**	0.23*	40
>1,000	0.93***	0.65**	0.40***	0.23*	40
<1,000 + >10,000	0.98***	0.60*	0.40***	0.23*	40
Ethanolic extraction (<10,000)					
Solute	1.00***	0.58*	0.20	0.07	40
Precipitate	0.95***	0.60*	0.42***	0.23*	40
Gel filtrations					
<700	0.95***	0.37	0.15	—[b]	24
>700	0.93***	0.67**	0.33*	—	24
<700 + >700	0.95***	0.64**	0.37**	—	24
Seawater (control)	0.67	0.35	0.11	0.07	45

[a]The difference is significant between proportions of animals responding to test versus control (seawater) solutions (chi-square test: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).
[b]Locomotion was not assayed in gel filtration experiments.

found either to be responsible for the induction of a specific behavioral component or to be essential to the overall stimulatory capacity of FDAME. Fractions of <1000 and 1000–10,000 daltons stimulated only antennule flicking, while a combination of these fractions (<10,000 daltons) evoked all four assayed behaviors. This demonstrated that stimulants of widely varied molecular weights act jointly to cause qualitative and quantitative changes in behavior.

The precipitate of the ethanolic extraction of a <10,000-dalton fraction, when resuspended in FSW, had nearly equal stimulatory effects to FDAME. The solute of this ethanolic extraction was only slightly stimulatory. These

findings differ from those for other lobsters, *Homarus gammarus* (Mackie and Shelton, 1972) and *H. americanus* (McLeese, 1973a), which respond maximally to ethanolic solutes and minimally to ethanolic precipitates of squid mantle and cod muscle tissues, respectively. Our data for *Panulirus* suggest that peptides and polypeptides are the most active components in FDAME, since these are the predominant components of the precipitate. However, the precipitate contains other substances, such as polysaccharides, which may also act as stimulants. Therefore, before the role of peptides and polypeptides is clearly defined, there must be tests on additional subfractionations.

In terms of sensitivity to macromolecules, *Panulirus* is similar to the shrimp, *Palaemonetes pugio,* which responds to proteins present in human blood serum and to molecular weight fractions >1000 daltons prepared from oyster mantle fluid and coquina (Carr and Gurin, 1975). The sand fiddler crab, *Uca pugilator,* has also been demonstrated to feed on sandy substrata containing seawater mixtures of protein (casein) and polysaccharide (dextrin, glycogen), but not on a substratum containing only seawater (Robertson et al., 1981). While these results for *P. pugio* and *U. pugilator* are most interesting, they do not show that these crustaceans are responsive to macromolecules from their natural foods.

The foot muscle of abalones, taken from the *Panulirus* habitat, was used in preparing molecular weight fractions for our studies. Abalones are available to *Panulirus* as carrion, and *P. interruptus* eats live abalone in laboratory tests (Carlberg, 1975). Field trapping experiments have shown that the chemical effluence from injured abalone or from excised foot is attractive to lobsters (Zimmer-Faust and Case, 1982b). Substances in the range of <10,000 daltons caused significant attraction in the field, while substances in the range of <1000 daltons by themselves did not. Our field and laboratory experiments agree, therefore, in showing that macromolecules released from abalone muscle are essential to induction of both feeding and foraging in *Panulirus.*

It has generally been assumed that low-molecular-weight substances are likely to be distant feeding stimulants because of their relatively high diffusion coefficients and solubilities in seawater (Ache et al., 1976). Mobile decapod predators sensitive to small molecules might effectively capitalize on food resources. Some appear to follow this model of behavior (Rittschof, 1980a), but others, such as *P. interruptus,* do not. Under natural conditions, *Panulirus* is attracted in greatest abundance to traps baited with abalone muscle only after tissues have been field-exposed for 24–48 hr, even though small molecules (primary amines) are released predominately over the first 0- to 3-hr interval (Zimmer-Faust and Case, 1982b). *Panulirus* is similar in

response latency to the hermit crabs *Clibanarius vittatus, Pagurus longicarpus, and P. duplicatus,* which are attracted to peptides and polypeptides, and arrive at gastropod predation sites 2 hr to several days after tissues first become available (Rittschof, 1980a, b). By delaying responses, hermit crabs avoid contact with early-arriving predators. Perhaps because *Panulirus* lacks defensive chelae, its sensitivity to macromolecules, coupled with its response latency, favors avoidance of predation while searching according to a predator-sensitive foraging strategy. Furthermore, because of their gradual dissipation, macromolecules may act as persistent markers of food (Mackie, 1975). Small molecules (<1000 daltons) are unlikely to serve in this capacity due to their rapid diffusion and selective assimilation by bacteria (Ogura, 1975). Finally, low solubilities of macromolecules in seawater should not diminish their ability to act as either contact or distant feeding stimuli, since ocean currents suspend and carry substances in the naturally turbulent habitat of *Panulirus.*

We have found *Panulirus* particularly responsive to high (>700, >1000 daltons) and not to low (<700, <1000 daltons) molecular weight fractions of abalone muscle. Results were corroborated by field-trapping experiments. These findings differ from what is commonly assumed for the decapod crustacea, namely that low-molecular-weight substances control induction of feeding. However, much of the support for a dominant role of small molecules has come from behavioral investigations of the stimulatory capacity of small molecules without direct tests of macromolecules or of macromolecular fractions (e.g., McLeese, 1970; Kay, 1971; Shelton and Mackie, 1971; Mackie, 1973; Allen et al., 1975; Fuzessery and Childress, 1975; Hindley, 1975; Hartman and Hartman, 1977; Carr, 1978; Zimmer-Faust and Case, 1982a). Macromolecules have been occasionally assayed, but often these have not been present in the natural foods of the animals under investigation (e.g., casein, egg albumin, hemoglobin: Ache et al., 1978; Robertson et al., 1981). Consequently, the capacity of macromolecules to induce food search and feeding has rarely been adequately assessed. We know of no other behavioral investigations, besides those of Carr and Gurin (1975) and the present study, which have actually tested the stimulatory abilities of both low- and high-molecular-weight fractions. Both of these studies find macromolecular fractions to be highly stimulatory. Macromolecules may yet be identified as generally important in the stimulation of food search among decapod crustacea, especially for animals like *Panulirus* that alternate periods of foraging with inactivity. For these, sensitivity to macromolecules may permit location of food which might first become available several hours or days earlier during inactive periods. This could greatly facilitate their utilization of food resources.

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SEX PHEROMONE OF THE PEA MOTH, *Cydia nigricana* (F.) (LEPIDOPTERA: OLETHREUTIDAE)

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Abstract—The sex pheromone of the pea moth, *Cydia nigricana* (F.), was identified as (*E*, *E*)-8,10-dodecadien-1-yl acetate (*E*, *E*8,10-12: Ac) (approximately 0.1 ng/abdominal tip), in vacuum distillates of virgin female abdominal tips and volatiles emitted by calling females, from its chemical properties and capillary gas chromatography and mass spectrometry data. Synthetic *E*, *E*8,10-12: Ac and the natural pheromone elicited similar quantitative electrophysiological and behavioral responses from male moths. Other related compounds which also attract male moths in the field, *viz.*, (*E*)-10-dodecen-1-yl acetate, (*E*, *E*)-8,10-dodecadien-1-ol, and (*E*, *E*)-8,10-dodecadienal, were not detected in the pheromone and *E*, *E*8,10-12: Ac was not found in male moths.

Key Words—Pea moth, *Cydia nigricana* (F.), *Laspeyresia nigricana* Steph., Lepidoptera, Olethreutidae, sex pheromone, sex attractant, gas chromatography, mass spectrometry, single-ion monitoring, electroantennography, (*E*, *E*)-8,10-dodecadien-1-yl acetate.

INTRODUCTION

The pea moth, *Cydia nigricana* (F.), is a pest of economic importance in the main pea-growing areas of Britain and in parts of Europe and North America (Greenway et al., 1976; Thompson and Sanderson, 1977, and references therein). In common with other Lepidoptera, virgin female pea moths attract males by emitting a sex pheromone from an abdominal gland, and this natural pheromone, extracted from abdominal tips and incorporated into a slow-release formulation of natural rubber, was attractive for several days in the field (Lewis et al., 1975).

Electroantennographic studies and field tests have disclosed four attractants for male pea moths, as well as synergists and inhibitors (Greenway et al.,

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1982; Wall et al., 1976), which are, in order of increasing activity, (*E*, *E*)-8,10-dodecadien-1-ol (*E*, *E*8, 10–12:OH) (Lewis et al., 1975), sex pheromone of the related codling moth, *Cydia pomonella* (L.) (Beroza et al., 1974; Roelofs et al., 1971), (*E*)-10-dodecen-1-yl acetate (*E*10-12: Ac) (Wall et al., 1976), (*E*, *E*)-8,10-dodecadienal (*E*, *E*8,10-12: ALD) (Greenway and Wall, 1981a), and (*E*, *E*)-8,10-dodecadien-1-yl acetate (*E*, *E*8,10-12: Ac) (Greenway and Wall, 1980; Wall et al., 1976). Evaluation of these attractants to determine dose–response relationships, effects of weathering, and evaporation rates from slow-release formulations in the field has led to the development of a lure containing *E*10-12: Ac for monitoring pea moth populations in commercial dried pea crops (Greenway and Wall, 1981b; Greenway et al., 1981; Wall and Greenway, 1981). Further work is in progress to explore the use of the most potent attractant, *E*, *E*8,10-12: Ac, for monitoring the much smaller pea moth populations found in vining peas in Britain (Greenway and Wall, 1982).

This paper describes the chemical characterization of the sex pheromone of the pea moth in both abdominal tip extracts from virgin females and volatiles in the air entrained over "calling" virgin females.

METHODS AND MATERIALS

Collection of Female Sex Pheromone. Pea moths were reared individually in pea pods according to Lewis and Sturgeon (1978). Pheromone was obtained from virgin females (1–14 days old) by two methods.

Extracts were made by excising abdominal tips (terminal three segments) between 15.00 and 17.00 hr, the time of peak flight activity in the field (Lewis et al., 1975), and extracting them with redistilled dichloromethane (1 ml). The extracts were stored under N_2 at −20° C, while tips were added over a period of a few weeks and then filtered. For gas–liquid chromatography coupled with mass spectrometry (GC-MS), volatile components of filtered extract (>100 tips) were distilled *in vacuo* at 20° C into a U tube cooled with liquid N_2, from which they were recovered in redistilled *n*-hexane (1 ml).

Pheromone volatiles emitted by calling females were condensed in a U tube cooled with solid CO_2. Air was drawn in succession through granulated charcoal, a 500-ml conical flask containing virgin female moths, and the U tube by a small electric pump at approximately 20 ml min^{-1} between 12.00 and 17.30 hr (when the moths were calling) and at a very slow rate at other times. Moths were maintained at 20° C and a 16:8 light:dark photoperiod; newly emerged moths were added to the flask each morning, and dead moths removed. Every few days, condensate was rinsed from the U tube with dichloromethane (5 ml), the aqueous layer was removed and extracted with more dichloromethane (5 ml), and the combined organic layers were dried.

Extracts and condensates were stored under N_2 at $-20°C$ until required and were then evaporated, if necessary, to an appropriate volume in a gentle stream of N_2.

Liquid Chromatography (LC). A portion of extract, 25 female equivalents (FE), was evaporated and the residue dissolved in redistilled *n*-pentane (1 ml). This was chromatographed on a column of Florisil (100–200 mesh) (11 cm × 1-cm i.d.) eluted stepwise with pentane:diethyl ether from 100:0 to 0:100 (v/v) after Buser and Arn, (1975); 5-ml fractions were collected, then evaporated to a small volume in N_2, and portions were bioassayed by electroantennography (EAG) in comparison with equivalent fractions from a similar column eluted with solvents alone.

Gas Chromatography (GC). A Pye 105 gas chromatograph fitted with a flame ionization detector (FID) was used with a glass column, 2 m × 4-mm i.d., packed with 2.5% Carbowax 20M, on 80- to 100-mesh Chromasorb G-HP-DMCS (Applied Science Laboratories).

Electroanntenography (EAG). The apparatus (essentially as described by Wall et al., 1976) used the antennae of live males. EAG responses to synthetic chemicals, extracts, or fractionated extracts were elicited by puffing air (1 ml) into the air line through either glass capillaries containing GC-fractionated extracts or Pasteur pipet containing test material dosed onto a strip of filter paper or applied directly in a little solvent to the inside surface. In most tests, these responses were compared with those to a standard, *E,E*8, 10–12:OH (100 μg), and to solvent alone.

GC-EAG. Portions of distillate (1 FE) were injected onto a Flexsil capillary column (25 m × 0.2 mm) wall-coated with OV 101 (Phase Sep) in a Pye 304 gas chromatograph, with the column disconnected from the detector and projecting (1–2 cm) through the oven wall. The GC was either temperature programmed from 70 to 200°C at 6° min^{-1} or operated isothermally at 140°C. Fractions (1 min) were collected in glass capillaries (10 cm × 1.5-mm i.d.) cooled with solid CO_2/acetone.

GC-Mass Spectrometry (GC-MS). GC-MS was by splitless injection onto glass capillary columns (50 m × 0.25 mm) wall-coated with either UCON 5100 or heat-treated Carbowax 20M (Phase Sep) in a Pye 204 gas chromatograph, with helium (approximately 1 ml min^{-1}), linked directly to the ion source (220°C) of the mass spectrometer (MS) (VG Micromass 70-70F + Data System 2035), with electron impact ionization at 70 eV. Compounds were identified by comparing their mass spectra and retention times either with those of authentic compounds or with published data. For single-ion monitoring (SIM), portions of distilled extract (usually 5 FE) or of condensate were injected into the GC, which was temperature programmed from 25 to 150°C at 8° min^{-1}, and the effluent was analyzed with the MS tuned to maximum sensitivity to a particular m/z value (e.g., m/z 224, M^+ of

E,*E*8,10-12:Ac). Identity was confirmed on peak enhancement by coinjection of natural and synthetic materials.

Other laboratory and field behavioral tests were as described by Greenway et al. (1977, 1982) and Lewis et al. (1975). *E*,*E*8,10-12:Ac was synthesized as before (Greenway and Wall, 1981b).

RESULTS AND DISCUSSION

Fractionation of crude females *C. nigricana* tip extract after Buser and Arn (1975) and EAG of the fractions (Figure 1) suggested that the active

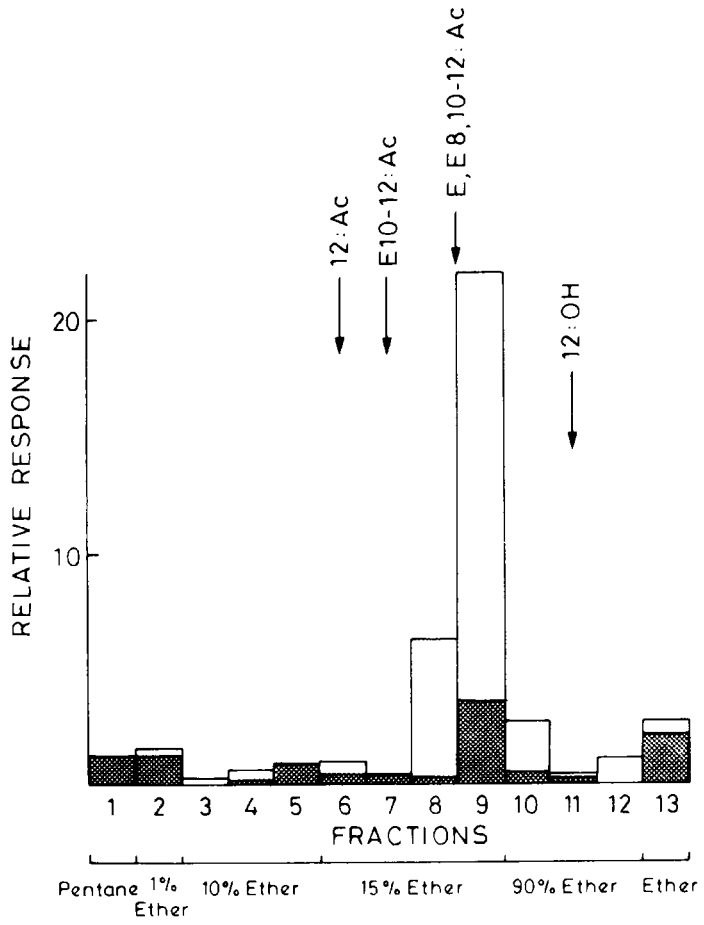

FIG. 1. Antennal (EAG) responses of male *C. nigricana* to fractionated female extract (4 FE) (open columns) and to solvent blanks (hatched columns) relative to *E*,*E*8,10-12:OH (100 μg). Elution positions of standard compounds were arrowed.

material was a long-chain unsaturated acetate. Only this fraction caused the characteristic "fanning" response from male moths. No components of lower, e.g., hydrocarbons, or higher polarity, typically alcohols, caused significant responses. Confirmation of an acetate as the biologically active material was obtained by hydrolysis of extract (12 FE) with aqueous ethanolic sodium hydroxide (EAG activity eliminated), followed by acetylation with acetyl chloride (EAG activity restored). Female distillate was separated by GC on an OV 101 capillary column and the fractions collected were tested for antennal activity by the EAG bioassay. Figure 2 shows the results of a typical temperature-programmed GC run encompassing the elution times of most previously identified lepidopteran sex pheromones. A single fraction, collected between 19 and 20 min, contained activity significantly above ($P < 0.001$) the biological responses to column effluent, which fitted well to an exponential decay curve (individual EAG preparations gave different background curves of the same general formula). Similarly, a single fraction with EAG activity above the column background was obtained from fractions collected during isothermal GC runs. Under both GC conditions, the retention times of the active fractions coincided with those of *E*,*E*8,10-12:Ac and differed from those of the other pea moth attractants (Greenway and Wall, 1981a; Lewis et al., 1975; Wall et al., 1976) as well as from those of

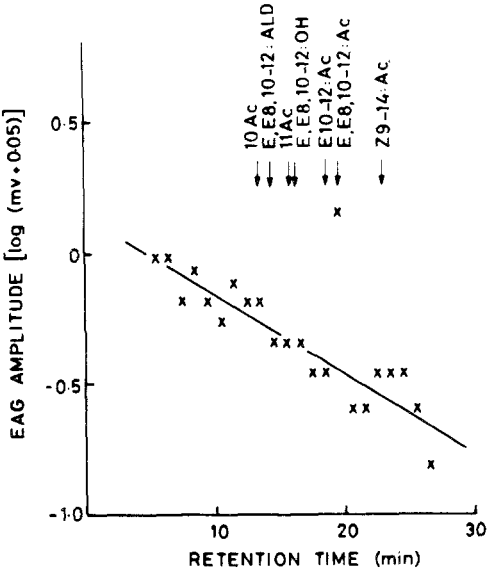

FIG. 2. Antennal (EAG) responses of male *C. nigricana* to 1-min GC fractions of female distillate from an OV101 capillary column programmed from 70 to 200°C at 6° min^{-1}. Responses to column effluent fitted the regression line shown. Retention times of standard compounds are arrowed.

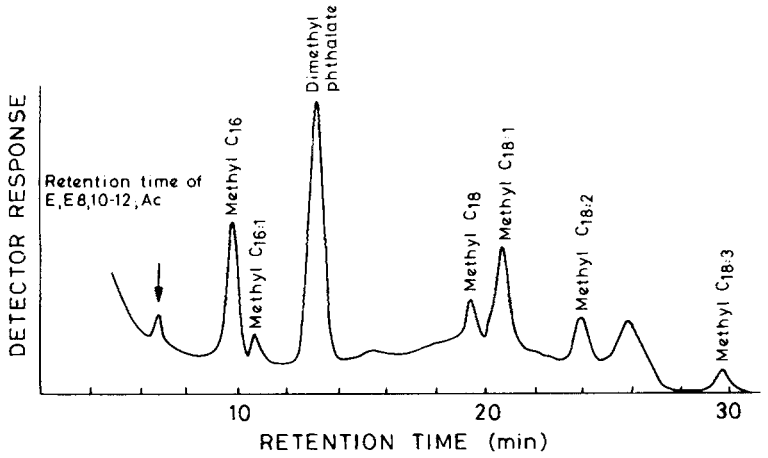

FIG. 3. GC-FID chromatogram of *C. nigricana* abdominal tip extract (10 FE). Carbowax 20M column at 182°C.

compounds in other lepidopteran pheromones (Figure 2). GC-FID of female extract on a Carbowax 20M column (Figure 3) and cochromatography with *E,E*8,10-12:Ac showed a minor component with the retention time of this compound; other compounds in this extract were identified by their retention times and by GC-MS as methyl esters of normal fatty acids, *viz.*, C_{16}, $C_{16:1}$, C_{18}, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$, with dimethyl phthalate (Buser and Arn, 1975). Comparison with injections of known amounts of *E,E*8,10-12:Ac indicated approximately 0.1 ng FE^{-1}. All the above evidence strongly indicates that the EAG and behaviorally active material is *E,E*8,10-12:Ac, and this conclusion is supported by previous quantitative EAG data (Wall et al., 1976) and because *E,E*8,10-12:Ac and the natural pheromone elicited the same excited behavior and typical fanning response in both laboratory and field tests (Greenway et al., 1977; Lewis et al., 1975). GC-MS of female distillate on OV 101 showed that a component in the EAG-active GC fraction (Figure 2) had the same MS (Figure 4A) as that of authentic *E,E*8,10-12:Ac (Figure 4B).

The presence of *E,E*8,10-12:Ac in the female pea moth was further indicated by single-ion monitoring (SIM) by GC-MS of prominent and/or characteristic ions in the mass spectrum of this compound on two capillary columns of both abdominal tip distillate and condensate from calling virgin females. The ions monitored were m/z 224 (M^+ of *E,E*8,10-12:Ac), 164 ($M^+ - CH_3COOH$) and 61 ($CH_3COOH_2^+$), both of which are weak but characteristic fragments in the spectra of acetates (Budzikiewicz et al., 1967), 67 ($C_5H_7^+$), 68 ($C_5H_8^+$), and 81 ($C_6H_9^+$), which have been found as prominent ions from various compounds containing the 1,3-pentadienyl group (Buser and Arn, 1975; Mudd, 1981). Single-ion peaks for all these fragments were found at the

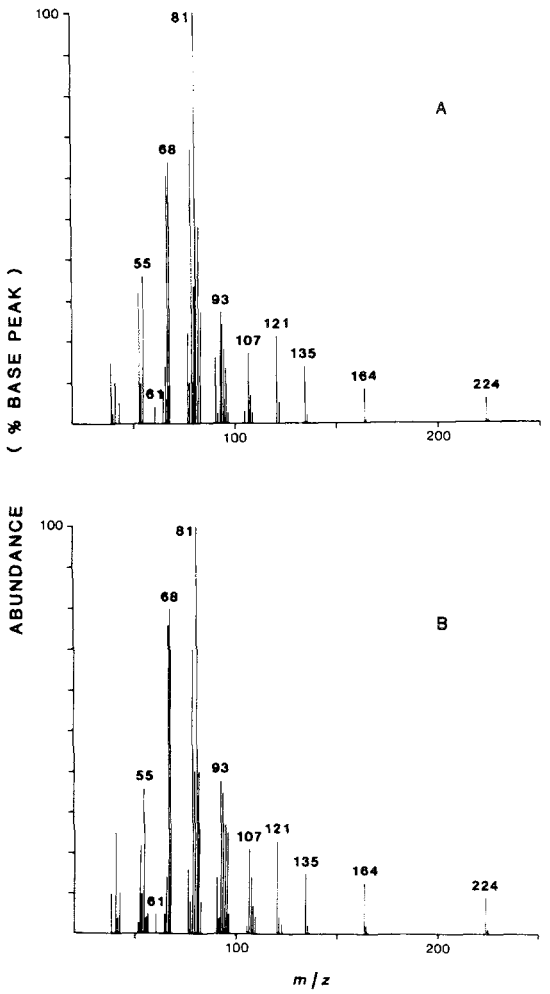

FIG. 4. Mass spectra of (A) the EAG-active component in female *C. nigricana* distillate and (B) synthetic *E*,*E*8,10-12:Ac.

retention time of *E*,*E*8,10-12:Ac on both capillary columns in both distillate (Figure 5a) and condensate (Figure 5c), and all peaks were enhanced by coinjection of *E*,*E*8,10-12:Ac (Figure 5b). These chromatographic data eliminated the possibility of other geometrical isomers of 8,10-dodecadien-1-yl acetate since they differ in their retention times on both columns from those of the (*E*,*E*) isomer (Hill et al., 1976; Roelofs et al., 1972); furthermore, these other isomers were not attractive in the field to male *C. nigricana*. Typical injections of distillate were of 5 FE and the single-ion peaks at the

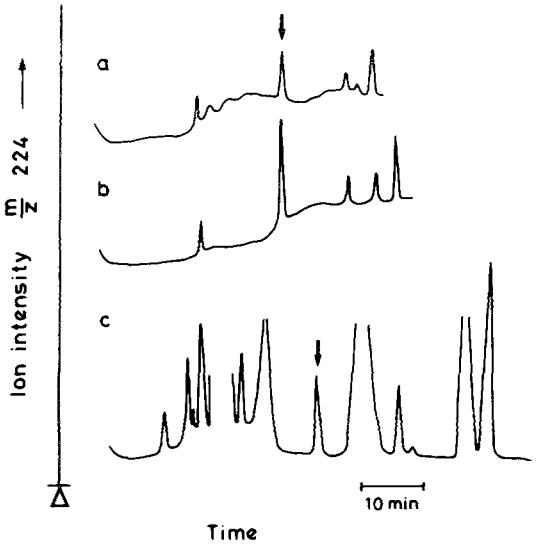

FIG. 5. Typical (GC-MS) single-ion chromatograms on a Carbowax 20M glass capillary column of (a) *C. nigricana* distillate (5 FE), (b) distillate (5 FE) enhanced with 0.5 ng of *E,E*8,10-12:Ac, and (c) female condensate on a UCON 5100 glass capillary column. The retention times of *E,E*8,10-12:Ac are arrowed.

retention time of *E,E*8,10-12:Ac were all equivalent to those from 0.5 ng of this compound, thus confirming both the identity of the compound and the quantity (average female content, 0.1 ng FE^{-1}), while SIM at *m/z* 224 showed that 100 moth hr of condensate contained approximately 1 ng of *E,E*8,10-12:Ac.

These results, together with the activity of *E,E*8,10-12:Ac in the field (Greenway and Wall, 1981b; Wall et al., 1976; C. Wall, personal communication) showed that this was the only significant component of the pea moth sex pheromone. EAG responses to distillate and condensate containing measured (by SIM) amounts of natural *E,E*8,10-12:Ac were compared with those to known amounts of synthetic attractant and found to be the same (Table 1). Distillate was also examined by SIM on capillary GC-MS for the possible presence of the other pea moth attractants, *E,E*8,10-12:OH (*m/z* 164, $M^+—H_2O$) and *E,E*8,10-12:ALD (*m/z* 180, M^+), and the possible monoene precursors of the diunsaturated pheromone, *viz.*, *E*8-12:Ac (*m/z* 166, $M^+—CH_3COOH$) and *E*10-12:Ac (*m/z* 166, $M^+—CH_3COOH$), which are an inhibitor and an attractant, respectively (Greenway et al., 1982), but no trace of any of these compounds or of other potential pheromone components was found. Nor was any *E,E*8,10-12:Ac found in an extract of male pea moths by SIM at *m/z* 224.

TABLE 1. ANTENNAL (EAG) RESPONSES TO DIFFERENT AMOUNTS OF *E,E*8,10-12:Ac AND TO PORTIONS OF *C. nigricana* DISTILLATE AND CONDENSATE

	EAG response[a] to		
*E,E*8,10-12:Ac (ng)	*E,E*8,10-12:Ac	Distillate	Condensate
0.01	0.05		
0.1	0.31	0.28 (1 μl)[b]	
0.5	0.54		
1.0	0.83	0.94 (10 μl)[b]	0.76 (2 μl)[b]

[a]Relative to *E,E*8,10-12:OH (100 μg). Standard error of difference between means = 0.06; error degrees of freedom = 12.
[b]GC-MS of *m/z* 224 showed *E,E*8,10-12:Ac of 0.1 ng μl^{-1} in distillate and 0.5 ng μl^{-1} in condensate.

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FIELD AND ELECTROANTENNOGRAM RESPONSES TO SEX PHEROMONE OPTICAL ISOMERS BY MONOPHAGOUS JACK PINE SAWFLIES (HYMENOPTERA: DIPRIONIDAE)

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Abstract—Several species of monophagous jack pine sawflies (Hymenoptera: Diprionidae) were tested in the field and by electroantennograms (EAG) for activity toward the optical isomers of a pine sawfly sex pheromone, the acetate and propionate esters of 3,7-dimethylpentadecan-2-ol. *Neodiprion rugifrons* and *Neodiprion dubiosus* were attracted to a mixture of the propionate esters of the 2S,3R,7R and 2S,3R,7S isomers, whereas *Neodiprion swainei* was attracted to the 2S,3S,7S propionate isomer. Samples containing the 2S,3R,7S propionate isomer elicited the strongest EAG responses in these three species and *Neodiprion nigroscutum*. The 2S,3S,7S propionate isomer was equally active (EAG) in the case of *N. swainei*.

Key Words—Pine sawflies, *Neodiprion* spp., Hymenoptera, Diprionidae, electroantennogram, sex pheromone, isomers, jack pine, *Pinus banksiana*, diprionol, 3,7-dimethylpentadecan-2-ol, acetate, propionate.

INTRODUCTION

The major component of the long-range sex pheromone of pine sawflies (Hymenoptera: Diprionidae) was identified by Jewett et al. (1976) as either the acetate or the propionate ester of diprionol, 3,7-dimethylpentadecan-2-ol. Various preparations of the eight optical isomers of this compound were tested in the field and by electroantennograms (EAG) for activity toward

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species of three genera (Matsumura et al., 1979; Kraemer et al., 1979, 1981, unpublished data; Kikukawa et al., 1982, 1983). Species belonging to the Old World genera *Diprion* and *Gilpinia* preferred the 2S,3R,7R isomer, whereas the New World *Neodiprion* species responded primarily to the 2S,3S,7S isomer. One species, *Neodiprion pratti pratti* (Dyar), also responsed to the 2S,3R,7R isomer. The choice of acetate or propionate ester also provides some degree of pheromone specificity. *Diprion similis* (Hartig) responds to the propionate of the 2S,3R,7R isomer, whereas *Gilpinia* responds most strongly to the acetate form of this isomer. Most *Neodiprion* species studied to date respond to the acetate of the 2S,3S,7S isomer. Some species, *Neodiprion nanulus nanulus* Schedl, *Neodiprion sertifer* (Geoffroy), and *Neodiprion taedae linearis* Ross, are also attracted to the propionate of this isomer in the field.

The present paper explores the field and EAG responses of four sympatric species, *Neodiprion rugifrons* Middleton, *Neodiprion dubiosus* Schedl, *Neodiprion swainei* Middleton, and *Neodiprion nigroscutum* Middleton, and the possible evolutionary relationships of these sawfly species to the sex pheromone isomers. The four sympatric species are essentially monophagous and are found in the jack pine (*Pinus banksiana* Lamb.) forests of eastern North America. With the exception of *N. nigroscutum*, these species often occur in outbreak. *Neodiprion rugifrons* and *N. dubiosus* are closely related. Becker et al. (1966) resolved Wisconsin populations of these species from the *Neodiprion virginianus* complex described by Ross (1955). *Neodiprion dubiosus* was found only in northern Wisconsin, whereas *N. rugifrons* was distributed throughout the range of jack pine in Wisconsin. Although *N. dubiosus* is common in Ontario and western Quebec, it is not clear how far north the range of *N. rugifrons* extends. The taxonomy, biology, and distribution of *N. swainei* and *N. nigroscutum* are discussed by Becker and Benjamin (1964, 1967). *Neodiprion swainei* is one of the most damaging diprionids in eastern Canada, whereas *N. nigroscutum* is "probably the rarest of Ontario Diprionidae" (Atwood, 1961), and outbreaks have never been reported in Wisconsin. All four species are members of the *lecontei* species-group. This group includes most of the pine sawfly species found in eastern North America (Ross, 1955).

METHODS AND MATERIALS

The three asymmetric centers of the carbon skeleton of diprionol (carbons 2, 3, and 7) allow eight (2^n, $n = 3$) possible optical isomers. The chiral configuration at these centers is designated either R or S. Samples containing a 1:1 mixture of different isomers are described by (R/S) at the appropriate carbon. The isomer samples were prepared by several groups of chemists. Because small amounts of optical impurities are found in some of these

preparations, code names were given (Tables 1–4). Those samples prepared by Tai et al. (1978) begin with the letter C (Tai et al. also prepared the samples in Table 1), those by Mori et al. (1978) begin with T, and those of Kikukawa et al. (1983) begin with K. In addition, Kikukawa et al. (1982) epimerized the 2-carbon position of Dr. Mori's isomers to form 1 : 1 mixtures of two isomers. Their code begins with the letters TM. The ester form of the isomer samples is designated with an A for acetate and a P for propionate. The eight optical isomers of diprionol are classified as either *erythro* or *threo* forms, depending on the chiral relationship between the 2- and the 3-carbon positions. The *erythro* isomers hae a 2S,3S or 2R,3R configuration, whereas the *threo* isomers have a 2S,3R or 2R,3S configuration. The four *erythro* isomers prepared by Mori et al. (1978) are at least 99% free of *threo* isomer contamination, whereas the isomer samples prepared by Tai et al. (1978) and Kikukawa et al. (1982) contain about 5% of the epimerization product at carbon 3 (Kikukawa et al., 1983).

Natural pheromone attractants were prepared after the method described by Jewett et al. (1978). Eighty virgin females were refluxed in 200 ml MeOH for 24 hr. The solvent was reduced to 50 ml by a rotary evaporator and 3 g KOH was added. This mixture was refluxed for 2 hr. Most of the methanol was removed by a rotary evaporator and the residue was transferred to a separatory funnel containing 250 ml hexane and 100 ml water. The mixture was swirled gently for 5 min and the aqueous layer removed. The hexane layer was passed through a funnel containing anhydrous Na_2SO_4 and the hexane evaporated. Half of the residue was acetylated with acetic anhydride (2 ml) in pyridine (4 ml) and the other half was propionated with propionic anhydride (2 ml) in pyridine (4 ml). These reaction mixtures were heated to 80°C in a water bath for 1 hr and transferred to a separatory funnel containing hexane (100 ml) and water (200 ml). The hexane layer was washed four times with water, dried over anhydrous Na_2SO_4, and concentrated to a volume of 8 ml, i.e., 10 female equivalents/ml.

We used white Pherocon II traps (Zoecon Corp., Palo Alto, Calif.) for field bioassays. Isomer samples were dissolved in 1.0-ml aliquots of distilled *n*-hexane and stored in sealed glass ampoules. Each ampoule was broken in the field and its contents were poured onto a 4.0-cm cotton dental wick held with forceps. The wick was suspended from the trap roof by a hair clip. The traps were randomized and attached to pine branches at breast height and about 10 m apart.

Taxonomic keys to the adult males of *Neodiprion* sawflies do not exist, and in some cases the identity of specimens trapped in the field could not be determined. Therefore, larval collections were made at the trapping locations to determine the species present. The Wisconsin field tests were conducted in jack pine windbreaks near Whitewater and Trempealeau. Only *N. rugifrons* larvae (one colony/tree in 1977) were found at the Whitewater site. In addi-

tion, this small area of 50–100 trees was isolated from other pines by at least 1 ml.

This larval population was subsequently reduced to near-zero after pesticide application by the property owner and further field tests were delayed until the population had partially recovered in 1980 and 1981. A total of eight and six larval colonies, respectively, was later found. Although the small population at this site limited the number of bioassays, it was unlikely that any other sawfly species were present. Both *N. rugifrons* and *N. swainei* were present at the Trempealeau site, in about equal numbers (3–5 colonies/tree in 1979 and 1980). The adult males of these two species are similar in appearance and could not be separated by taxonomic methods.

The unique oviposition behavior of *N. swainei* provided some temporal separation of the populations. *Neodiprion swainei* oviposits only on the emerging current-year needles of jack pine, whereas all other *Neodiprion* species oviposit on the previous year's foliage. This allows only one generation per year of *N. swainei*, whereas the *N. rugifrons* population at the Trempealeau site often has a second-generation flight period in early August (Wilkinson et al., 1966; Becker and Benjamin, 1964). By 1981 only one *N. rugifrons* colony was found at this site and field tests were discontinued. The Canadian field sites were in jack pine forests, near Lake Cousacouta, Quebec, where *N. swainei* was in outbreak (18 colonies/tree) and near Chapleau, Ontario, where *N. dubiosus* was known to be present. Several hundred colonies of *N. dubiosus* larvae has been collected from scattered outbreaks along highways 102 and 667, south of Chapleau, in the 2 years previous to the field tests. However, the extensive nature of the jack pine forests allowed for the possible presence of other species.

The EAG tests were performed with equipment and techniques described by Jewett et al. (1977). Uniform strips of filter paper impregnated with an isomer sample were placed inside Pasteur pipets. A 2-ml pulse of air was passed through a pipet and into a moisturized air stream (6 liters/min) directed at an antenna. The amount of each isomer impregnated on the filter-paper strips was kept constant (10 μg/isomer) and the EAG deflection compared. In those instances where an isomer sample contained two isomers, the quantity of sample tested was doubled, for both EAG and field tests. The EAG tests were performed on males reared from larvae collected at the field test sites. *Neodiprion nigroscutum* larvae were collected from Chapleau, Ontario, and from Mondovi and Lone Rock, Wisconsin.

RESULTS AND DISCUSSION

Field Tests

The initial field tests of *N. rugifrons* (1977) at the isolated Whitewater, Wisconsin, site indicated that the *threo* isomer configuration and the propio-

TABLE 1. NUMBERS OF *N. rugifrons* MALES CAPTURED IN ISOMER-BAITED STICKY-TRAPS (NOON TO NOON) NEAR WHITEWATER, WISCONSIN, 1977

Code	Isomer(s)	μg	7/19–21[a]	7/21–26	7/26–29	7/29–8/3	Total
A-I	2R,3R,7(R/S)-A	300	0	0	0	0	0
A-II	2S,3S,7(R/S)-A	300	0	0	0	2	2
A-III	2S,3R,7(R/S)-A/ 2R,3S,7(R/S)-A	300	3	0	0	0	3
P-I	2R,3R,7(R/S)-P	300	0	0	0	0	0
P-II	2S,3S,7(R/S)-P	300	1	4	0	1	6
P-III	2S,3R,7(R/S)-P/ 2R,3S,7(R/S)-P	300	9	13	3	4	29

[a]Trap positions were randomized after each count.

nate ester form were most important for this species (Table 1). The trap baited with the P-III sample caught 29 of the 40 captured males. The P-III sample is a 1:1:1:1 mixture of the 2S,3R,7R; 2S,3R,7S; 2R,3S,7R; and 2R,3S,7S isomers. Further tests of optically more pure samples at Trempealeau in 1978 and Whitewater in 1980 (Table 2) showed that a 1:1 mixture of the 2R,3S,7R

TABLE 2. NUMBER OF *N. rugifrons* MALES CAPTURED IN ISOMER-BAITED STICKY-TRAPS NEAR TREMPEALEAU, WIS., (8/2–8/19, 1978) (REPLICATE 1), AND NEAR WHITEWATER, WIS., (7/27–8/15, 1980) (REPLICATES 2 AND 3)[a]

			Replicate			
Code	Isomer(s)	μg	1	2	3	Total
TA-1	2R,3R,7R-A	25	0	0	0	0a
TA-2	2R,3R,7S-A	25	1	0	0	1a
TA-3	2S,3S,7R-A	25	2	0	0	2a
TA-4	2S,3S,7S-A	25	8	2	2	12b
CA-1	2S,3R,7(R/S)-A	50	0	1	0	1a
CA-2	2R,3S,7(R/S)-A	50	—[b]	0	0	0a
TP-1	2R,3R,7R-P	25	0	0	0	0a
TP-2	2R,3R,7S-P	25	1	0	0	1a
TP-3	2S,3S,7R-P	25	2	0	0	2a
TP-4	2S,3S,7S-P	25	10	1	0	11ab
CP-1	2S,3R,7(R/S)-P	50	57	15	18	86c
CP-2	2R,3S,7(R/S)-P	50	0	0	0	0a

[a]Statistical analysis by Duncan's new multiple range test after catches converted to percentage of total catch of replicate. Total trap catches followed by the same letter indicate that mean trap catches are not significantly different at the 0.05 level.
[b]Lost trap.

and 2R,3S,7S isomers, CP-2, was not active, whereas the traps containing a 1:1 mixture of the 2S,3R,7R and 2S,3R,7S isomers, CP-1, caught 86 males. Except for the traps baited with the acetate and propionate of the 2S,3S,7S isomer (TA-4 and TP-4), which caught a total of 12 and 11 males, respectively, no other isomer sample caught more than 2 males.

Traps baited with both the 2S,3R,7R-P and the 2S,3R,7S-P isomers, CP-I and P-III, caught the most *N. rugifrons* males, but traps with only one of these isomers, TMP-1 and TMP-2, caught few males. The P-III, TMP-1, and TMP-2 traps caught 43, 9, and 5 males, respectively (Trempealeau, Wis.; 8/2–8/19, 1978). Each trap contained equal amounts of the 2S,3R,7R and/or 2S,3R,7S isomer (50 μg). Additional tests of TMP-1 and TMP-2 at Trempealeau in 1979 produced similar results (Table 3). The *erythro* isomer components of TMP-1 and TMP-2 did not appear to inhibit attraction to the 2S,3R,7R-P and 2S,3R,7S-P isomers. Traps baited with a 1:1 mixture of TMP-1 and TMP-2 caught more males (33) than either TMP-1 or TMP-2 alone (1 and 3 males, respectively) (Table 3). These traps contained 25 μg of *threo* isomer(s). The CP-1-baited traps containing an equivalent isomer

TABLE 3. NUMBER OF *N. rugifrons* AND *N. swainei* MALES CAPTURED IN ISOMER-BAITED STICKY-TRAPS (NOON TO NOON) NEAR TREMPEALEAU, WISCONSIN, 1979

			5/5–6/7, Replicate		6/7–6/22, Replicate		
Code[a]	Isomer(s)	μg	1	2	1	2	Total
CP-1	2S,3R,7(R/S)-P	25	3	4	0	10	17
TMP-1	2(R/S),3R,7R-P	50	0	1	0	0	1
TMP-2	2(R/S),3R,7S-P	50	1	1	1	0	3
TMP-1/TMP-2		25/25	2	7	9	15	33
TP-4	2S,3S,7S-P	25	2	0	3	3	8
TA-4	2S,3S,7S-A	25	2	2	5	3	12
CP-1	2S,3R,7(R/S)-P	50	4	9	23	25	61
CA-1	2S,3R,7(R/S)-A	50	1	0	0	0	1
CP-2	2R,3S,7(R/S)-P	50	0	0	0	0	0
CA-2	2R,3S,7(R/S)-A	50	0	0	0	0	0
Rug-A			0	0	0	0	0
Rug-P			4	10	2	6	22
Swa-A			0	0	0	0	0
Swa-P			0	0	19	13	32

[a]Rug-A and Rug-P are the acetate and propionate preparations of *N. rugifrons* natural extract and Swa-A and Swa-P are the ester preparations of *N. swainei* natural extract. All preparations were tested at a quantity of 10 female equivalents.

quantity (25 μg) caught 17 males. Further tests with optically pure 2S,3R,7R and 2S,3R,7S isomer samples are needed. Because the CP-1 sample contained two isomers, it was tested at twice the quantity of the 2S,3S,7S isomer samples, TA-4 and TP-4. A test of these three samples in equal quantity (10 μg; three replicates) at the Whitewater site (June 1–23, 1981) again indicated that the CP-1 sample was the most attractive. The CP-1 traps caught a total of 19 males (6, 3, and 10), and the TA-4 traps caught only 3 males (1, 1, and 1). The TP-4 traps caught no males. Mixtures (1:1) of CP-1 and TA-4 and of CP-1 and TP-4 (10-μg total) caught only 4 and 1 males, respectively.

Because both *N. rugifrons* and *N. swainei* adults were present at the Trempealeau site in the spring of 1979 (Table 3), natural product extracts prepared from the virgin females of these two species were used to determine their respective flight periods. Becker et al. (1966) reported that the flight period of *N. swainei* occurs later than that of *N. rugifrons* and the other *Neodiprion* species which overwinter as prepupal larvae. These species are often present about 2 weeks before the expanding new foliage is available for *N. swainei* oviposition. Although the *N. rugifrons* extract-baited traps caught males throughout the trapping period, the *N. swainei* extract traps caught males only after June 7 (Table 3). Also, only the traps baited with the propionated extract caught males. The acetate form of the sex pheromone appeared to be inactive. A comparison of trap catch before and after June 7 indicates no change in the relative trap catch to the different isomer samples. Either *N. swainei* males respond to the same isomers as *N. rugifrons* or this species is not strongly attracted to any of the isomer samples tested. Field tests of *N. swainei* in Quebec (7/5–7/21, 1978) indicated that the 2S,3S,7S-P isomer was attractive. Two traps containing TP-4 caught 129 and 157 males, whereas no other trap caught more than 2 males. Because of the late placement of the traps, on July 5, it was unlikely that any other species of sawfly was present. The high *N. swainei* population later observed at this site (18 larval colonies/tree) and the relatively larger quantities of isomer tested (50 vs 25 μg) probably account for the large TP-4 trap catch in Quebec.

Field tests in Ontario indicted that *N. dubiosus* responds similarly to *N. rugifrons* (Table 4). The traps baited with CP-1 caught the most males (100). Optically more pure samples of the 2S,3R,7R-P and 2S,3S,7S-P isomers (KP-1 and KP-2, respectively) were available and used instead of TMP-1 and TMP-2. Traps baited with KP-1 caught a total of 41 males, whereas the KP-2 traps caught only 2 males. As with *N. rugifrons*, a 1:1 mixture of the 2S,3R,7R-P and 2S,3R,7S-P isomers (KP-1/KP-2) caught more males (72) than either alone. Although Table 4 lists only those isomer samples thought to be important, the other isomers were also tested at the same time (two replicates): TA-1, TP-1, TA-2, TP-2, TA-3, TP-3, CA-1, CA-2, and CP-2. No males were captured in these traps. Traps baited with the 2S,3S,7S-A isomer (TA-4 and KA-4) also failed to catch males, although

TABLE 4. NUMBERS OF *N. dubiosus* (D) AND UNIDENTIFIED SPECIES (X) CAPTURED IN ISOMER-BAITED STICKY-TRAPS 6/24–6/29/82[a]

			Replicate							
			1		2		3		Total	
Code	Isomer(s)	μg	D	X	D	X	D	X	D	X
TA-4	2S,3S,7S-A	10	0	0	0	0	0	0	0a	0a
KA-4	2S,3S,7S-A	10	0	0	0	0	0	0	0a	0a
TP-4	2S,3S,7S-P	10	1	2	0	1	0	12	1a	15ab
KP-4	2S,3S,7S-P	10	0	12	1	8	0	44	4a	64d
CP-1	2S,3R,7S(R/S)-P	20	23	0	38	0	39	0	100e	0a
KP-1	2S,3R,7R-P	10	21	0	4	0	16	0	41c	0a
KP-2	2S,3R,7S-P	10	1	0	0	0	1	5	2a	5a
KP-1/KP-2		10/10	17	0	29	0	26	1	72d	1a
KP-4/CP-1		10/20	11	2	18	5	2	1	31bc	8ab
KP-4/KP-2		10/10	1	8	0	10	2	11	3a	29c
KP-4/KA-4		10/10	1	4	0	12	0	8	1a	24bc
KA-4/KP-2		10/10	2	0	5	1	4	4	11ab	5a

[a]Statistical analysis by Duncan's new multiple range test after trap catches converted to percentage of total catch of replicate. Total trap catches followed by the same letter indicate that mean trap catches are not significantly different at the 0.05 level.

a few *N. rugifrons* were consistently captured in TA-4-baited traps in Wisconsin.

A second, unidentified, *Neodiprion* species was captured at the Ontario site in traps baited with the 2S,3S,7S-P isomer (Table 4). It was entirely black except for light brown labial and mandibular palps, legs, and tarsi. The black of the abdominal sternites was distinctive. With the possible exception of *Neodiprion abbotii* (Leach) (Hetrick 1956), the males of all described *Neodiprion* species found in eastern North America have a lower abdomen which ranges in color from yellow to brown to reddish. *Neodiprion abbotii* is a rare species, and although the larvae feed on a variety of hard pines, it has never been reported on jack pine, the only species of pine in the trapping area. An extensive search of the jack pine was made 1 month after the traps were removed. Several hundred colonies of *N. dubiosus* larvae and one colony of *nigroscutum* were collected. The male adults reared from these larvae all had brown abdominal sternites. The species with the black sternites may be a biotype of *N. swainei*. At the time of the larval collection, *N. swainei* would have been in either the egg or the first larval stage and thus easily overlooked. However, the major period of *N. swainei* adult flight probably occurred after the traps were removed, June 29. The new needles had emerged only about 5 mm from the fascicles. Although the identity of this species is uncertain, it

probably belongs to the jack pine-feeding group. No other species of pine were present and the preference for the propionate ester form was similar to the other species in this group. It is not clear why the KP-4 traps caught significantly more males (64) than the TP-4 traps (15). The KP-4 sample may contain 5% of the 2S,3R,7S *threo* isomer due to the synthetic process used, whereas the TP-4 sample is at least 99% free of *threo* isomer contamination (Kikukawa et al., 1983). The small amount of 2S,3R,7S isomer in the KP-4 sample may be synergistic. However, a 1:1 mixture of the 2S,3S,7S-P and 2S,3R,7S-P isomers (KP-4/KP-2) caught fewer males (29) than KP-4 alone (64). Traps with a 1:2 mixture of KP-4 and CP-1 caught only 8 black abdomen males and 31 *N. dubiosus*. Each species may be inhibited by the isomer(s) attractive to the other at these ratios.

The identification of sawfly males trapped in the field was a major problem with the jack pine-feeding species. It is clear that three isomers are important in this group (2S,3S,7S; 2S,3R,7R; 2S,3R,7S) and that specific response profiles have been observed in the field. Synergistic or inhibitory effects of isomer mixtures could be important but can only be suggested by the present data. The availability of the more optically pure *threo* isomer samples KP-1 and KP-2 will help reduce the ambiguity of future field tests.

Electroantennogram

Electroantennogram (EAG) recordings were made for the jack pine-feeding sawflies; *N. rugifrons*, *N. dubiosus*, *N. swainei*, and *N. nigroscutum* (Figures 1 and 2). The propionate samples elicited stronger responses than the corresponding acetate forms of these isomers in all cases. This contrasted with the EAG responses of *N. lecontei*, *N. pinetum*, *N. nanulus*, *N. sertifer*, and *N. taedae linearis*, which were more sensitive to the acetate isomers, particularly 2S,3S,7S (Kraemer et al., 1979, 1981, unpublished data). The propionate-preferring species were also sensitive to the 2S,3S,7S isomer, but except for *N. swainei*, the *threo* isomer sample CP-1, 2S,3R,7(R/S)-P, elicited stronger responses than TP-4, 2S,3S,7S-P. *Neodiprion swainei* responded equally to these samples (Figure 1). The 2S,3R,7S-P isomer appeared to be most responsible for the strong response to the CP-1 sample. The TMP-2 sample, 2(R/S),3R,7S-P, elicited a greater EAG response than the TMP-1 sample, 2(R/S),3R,7R-P, in all cases. The *erythro* isomer components of these samples, 2R,3R,7R-P (TP-1) and 2R,3R,7S-P (TP-2), did not by themselves elicit significant EAG responses. The *N. swainei* and *N. nigroscutum* responses were most specific for the 2S,3R,7S-P isomer (TMP-2), whereas *N. dubiosus* and, to a lesser degree, *N. rugifrons* also responded to the 2S,3R,7R-P isomer (TMP-1). Because these samples contain two components, isomer interactions cannot be ruled out. However, several EAG tests of the optically more pure KP-1 and KP-2 samples indicated that this was not the

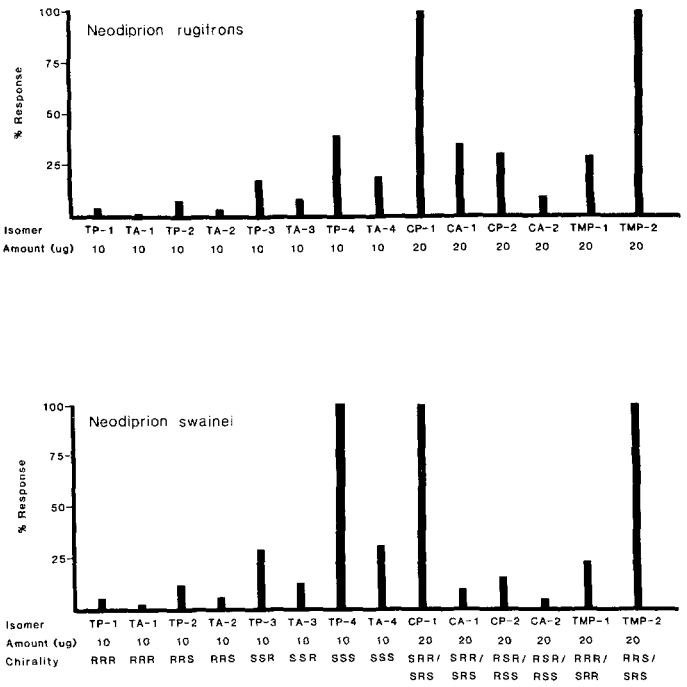

FIG. 1. Relative electroantennogram responses to optical isomers by males of *Neodiprion rugifrons* and *Neodiprion swainei*. The most active isomer is considered as 100% (2 mV) and all responses are the averages of 10 males. Chirality refers to the optical configuration at carbons 2, 3, and 7, respectively.

case. The KP-2 sample elicited a greater response than the KP-1 sample in all cases.

The strong EAG response to the 2S,3R,7S-P isomer was surprising, as it was not, by itself, strongly attractive in the field. It appeared to be attractive only in combination with the 2S,3R,7R-P isomer. Specific ratios of these isomers may play a role in the species-specific response of *N. rugifrons* and *N. dubiosus*. The response to the 2S,3R,7R-P isomer appeared stronger in *N. dubiosus* than *N. rugifrons* in both field and EAG tests, although the trap catch was greatest to a 1:1 mixture of the two isomers in both cases. The strong EAG response of *N. swainei* to TP-4 correlated positively with the field tests at St. Ambroise. The equally strong EAG response to the TMP-2 sample suggests that the 2S,3R,7S-P isomer may be an important component of the *N. swainei* sex pheromone. *Neodiprion nigroscutum* was not found in large numbers in the field, and therefore only the EAG response was determined. The TMP-2 sample elicited the strongest response, whereas the TMP-1 sample was relatively inactive (Figure 2). The 2S,3S,7S-P isomer may also be impor-

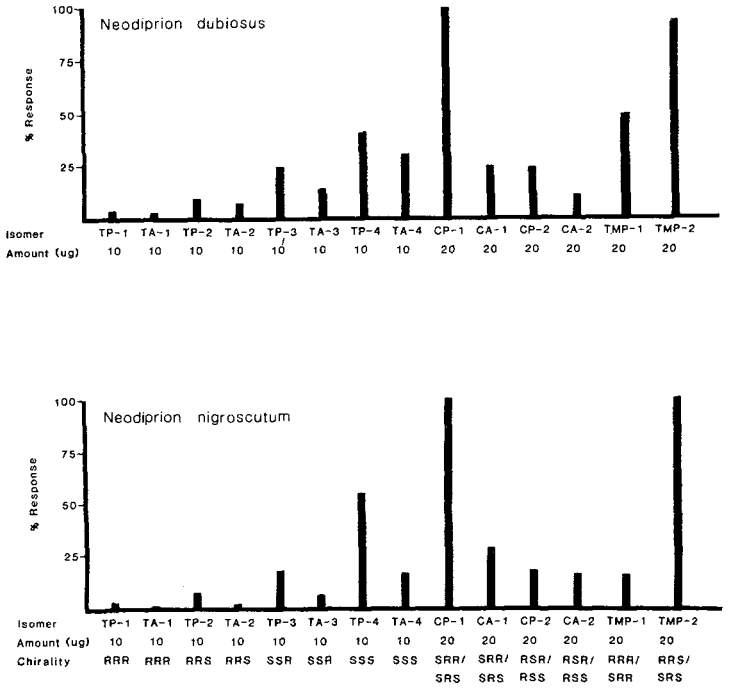

FIG. 2. Relative electroantennogram responses to optical isomers by males of *Neodiprion dubiosus* and *Neodiprion nigroscutum.* The most active isomer is considered as 100% (2 mV) and all responses are the averages of 10 males. Chirality refers to the optical configuration at carbons 2, 3, and 7, respectively.

tant with this species, as the EAG deflection produced by the TP-4 sample was about half that of TMP-2. The strong response to the 2S,3R,7S-P isomer by all four species and their preference for the propionate isomers suggest that these monophagous jack pine-feeding species are more closely related than implied by the evolutionary relationships proposed by Ross (1955). The larval coloration patterns and lancet characteristics used by Ross placed these four species in three of the four main branches of the *lecontei* species-group. Also, although apparently distinct species in the field, *N. rugifrons*, *N. dubiosus*, and *N. nigroscutum* have been shown to mate in the lab and produce viable hybrids (Kraemer and Coppel 1983).

Field and EAG tests have shown that three of the eight optical isomers of diprionol (2S,3S,7S; 2S,3R,7R; 2S,3R,7S) are most important in this group of jack pine-feeding sawflies. Most New World *Neodiprion* sawfly species are attracted to the 2S,3S,7S isomer, whereas the Old World genera *Diprion* and *Gilpinia* respond in the field or on the EAG to the 2S,3R,7R isomer. *Neodiprion pratti pratti* (unpublished data) and *N. dubiosus* also are attracted to

the 2S,3R,7R isomer in the field. The response to this isomer by species belonging to all three genera suggests that the 2S,3R,7R isomer was an important part of the sex pheromone of the diprionid stock from which the pine sawfly genera evolved. The divergence of the *Neodiprion* group probably corresponded with the development of the 2S,3S,7S isomer. The 2S,3R,7S isomer appears important only in the jack pine-feeding species discussed herein. The general lack of a strong field response to this isomer, alone, and the sensitivity to the 2S,3S,7S and 2S,3R,7R isomers in some of these species suggest that the 2S,3R,7S chiral configuration is a relatively recent evolutionary development. It is possible that the exceptional evolutionary diversity of the genus *Neodiprion* is related to the modifications of the basic isomer form. There are about 30 species or races of *Neodiprion* sawflies in North America. The taxonomic relationships and resolution of species complexes of this group need further study.

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THE ATTRACTIVITY OF THE FEMALE SEX PHEROMONE OF *Periplaneta americana* AND ITS COMPONENTS FOR CONSPECIFIC MALES AND MALES OF *Periplaneta australasiae* IN THE FIELD

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Abstract—The attractivity of virgin female odors of the American cockroach was examined in field experiments. Crude extracts of the female odor, the isolated sex pheromone fractions, periplanone-A and periplanone-B, and other compounds obtained during the isolation served as stimulants. An extract of male odors, obtained by identical collection methods, was used as a control. Males of *Periplaneta americana* were attracted by the crude extract and periplanone-B; males of the sympatric species, *P. Australasiae*, by periplanone-A. Experiments in which these components were presented subsequently and as mixtures indicate that, under certain conditions, periplanone-A may also function as an attracting substance for *P. americana* males and that periplanone-B and possibly some other components act as an inhibitor for males of *P. australasiae*.

Key Words—Sex pheromone components, *Periplaneta americana*, *Periplaneta australasiae*, Orthoptera, Blattidae, interspecific attractant, interspecific inhibitor, field experiments.

INTRODUCTION

The mating behavior of males of *Periplaneta americana* is induced by the pheromones of females (reviews by Persoons and Ritter, 1979; Bell, 1982). The structure of one component, periplanone-B, has been elucidated by Persoons (1977), Adams et al. (1979), and Still (1979). This pheromone attracts and sexually excites males of *Periplaneta americana*. Another component of the female extract, periplanone-A, and further not yet identified components emitted by females appear to function in the same manner

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(Persoons, 1977; Nishino et al., 1983). It remains unknown whether these components play different roles during mating. It is known only from recent work by Seelinger (1984b) that periplanone-A and -B have qualitatively different effects on males in the laboratory.

Isolated groups of males observed in the laboratory responded not only to female pheromones, but also to other odorants presented at relatively high concentrations (so-called sex pheromone mimics; see literature cited by Nishino and Kimura, 1981). For this reason, the extract fractions were used in our experiments in approximately the same amounts as they occur in individual female cockroaches. This dosage was based on the experiments of Sass (1983).

Furthermore, the attractivity of the components was to be examined under the most natural conditions possible. The behavioral experiments conducted in the laboratory were performed with males whose responsiveness was increased by an extended period of isolation. Chow et al. (1976) and Chow and Wang (1981) have already demonstrated that periplanone-B is attractive to *P. americana* males in the field also.

In the chosen research area, Jamaica, W.I., *P. americana* and *P. australasiae* occur sympatrically. Laboratory experiments with these insects have shown that the pheromones of *P. americana* females may have an interspecific function (Schafer, 1977b; Simon and Barth, 1977). Since males of *P. australasiae* also respond to the pheromones of *P. americana* females in the field, they were included in this study.

METHODS AND MATERIALS

Pheromone Bait Placements

The experiments were performed in a suburb of Montego Bay, Jamaica, W.I. The pheromone baits were set up in 12 locations at least 100 m from the nearest residential building and at least 40 m apart. They were attached at a height of approximately 1.30 m to freestanding trees or utility poles 20–50 cm in diameter, which were several meters away from low growth-covered walls or partially overgrown boulders or which were surrounded only by undergrowth. In a few experiments, the pheromone source was presented in a plate set on the ground. Such sources were as readily located by males as those attached to tree trunks. No data can be supplied on the population densities of the two species or on the encompassment of the area from which male cockroaches were attracted.

Experimental Conditions

The experiments were performed in the months of April and September 1982. They commenced 1–2 hr after sunset and were terminated after 2–3 hr. It

is known from laboratory experiments that the responsiveness to pheromone, at least in *Periplaneta americana* males, scarcely decreases during the first half of the night (Block and Bell, 1974; Chow et al., 1976; Hawkins and Rust, 1977; Seelinger, 1984a). According to our observations, the same appears to hold true for *Periplaneta australasiae.*

The temperature varied at all locations between 24 and 29°C during the length of the experiments. The wind velocity at the height of the scented paper was usually less than 1 m/sec.

Experimental Methods

Pieces of Kleenex tissue (10 × 13 cm) were used as substrates for the pheromone solutions. After evaporation of the solvent, the paper was attached to the trunk so as not to come directly in contact with its surface. The paper and its immediate vicinity were illuminated with a dim light.

Each test usually lasted 10 min. Afterward the scented paper was enclosed in an airtight container. The procedure was repeated with one or two additional fractions at the same location. The time interval between such tests was approximately 1 min.

During the course of the experiments, it was noted which fractions were attractive to the male cockroaches, and in order to avoid habituation, fractions which had proven less attractive were presented first. At least one-third of all tests (Table 2A) were carried out as the first test per evening for each of the pheromone components studied at each location. The crude female extract and periplanone-B were tested only twice on the same evening at each location.

Isolation and Fractionation of the Cockroach Pheromones

Five hundred virgin females and 500 males of *Periplaneta americana* were kept isolated in cages, in which filter-paper rolls served as hiding places. Two pheromone components, periplanone-A and periplanone-B, were extracted from the paper according to the same methods used by Persoons et al. (1974) and Sass (1983). A further fraction "D" has proven itself an effective stimulant in neurophysiological experiments (Selsam and Waldow, in press). The remaining odorants separated in the fractionation process were combined in a fraction "R." The fractions A, B, D, and R were diluted with hexane to the same concentrations at which they occur in the crude extract.

Purity of the Fractions and Stimulus Intensity

The above-mentioned fractions are odorant mixtures. We were able to confirm by means of analytic chromatography on HPLC columns in connection with electroantennograms (EAG) and behavioral experiments that the

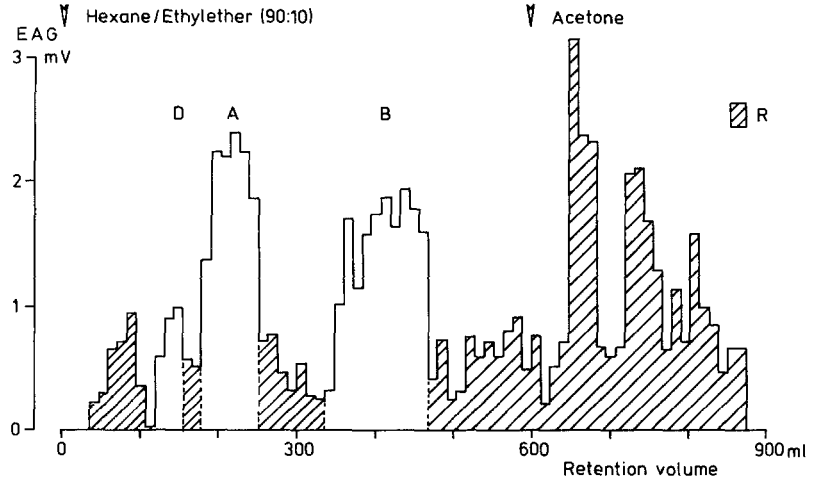

FIG. 1. Chromatographic fractionation of a pheromone extract. Chromatographic data: silica gel column, Lichoprep Si 60, 25–40 μm; diameter, 20 mm; length, 230 mm; flow rate, 8 ml/min; pressure, 6 bar. Abscissa, retention volume; ordinate, effectivity of the individual fractions according to the electroantennograms (EAG). The 12-ml fractions were combined in part to D, with unknown substances; A, containing periplanone-A; B, containing periplanone-B; and R, containing the remaining substances (hatched areas).

effective components of the individual fractions are not contained in any of the other fractions (Figure 1).

EAG measurements were performed to determine the pheromone content of the stimulant sources and the amounts of periplanone-A and -B emitted from them. For this purpose, the conditions in the experiments were simulated in the laboratory: filter papers with 100 μl of the test solution were set out for 10 min in an air current of 0.2 m/sec at 28°C (test paper, Table 1). Afterward, the remaining pheromone was extracted quantitatively with 10 ml hexane. Filter papers, which were loaded with pheromone in the same way and which were not exposed to the air current, were extracted quantitatively to serve as controls (loading of stimulant source, Table 1). The pheromone content of the resulting solution was determined by comparison with synthetic periplanone-B solutions (Sass, 1983).

Number of Approaching Flights and Running Activity of the Males

The insects' behavior at the pheromone source was quantified according to two methods: first, the minimum number of males known to have come in contact with the scented paper was determined. Newly arrived males ran especially rapidly toward the paper and demonstrated intense running be-

TABLE 1. CONTENT AND RELEASE OF PERIPLANONE-A AND -B FROM THE STIMULANT SOURCES[a]

	Periplanone-A						Periplanone-B		
	Concentrated			Diluted					
	ng	SD	N	ng	SD	N	ng	SD	N
Loading of stimulant source	2.45	0.43	16	0.19	0.02	5	1.69	0.4	19
Test paper after 10 min	1.64	0.27	17	0.13	0.02	6	1.33	0.18	10
Amount of pheromone emitted in 10 min	0.81			0.06			0.36		

[a]The amounts of the odorants (ng) were determined according to the comparison of their effectivity vs synthetic (±) periplanone-B (Sass, 1983). The amount of periplanone-A is given in periplanone-B equivalents. Periplanone-A was tested at two different concentrations. N, number of samples. Further explanation in text.

havior on the paper, making them easily distinguishable from males which had arrived earlier and which were usually moving about less excitedly. Only individuals exhibiting unambiguous behavior in this sense were counted as new arrivals. The number of arrivals may, therefore, in part be larger than the figures given in Tables 2 and 3. Whether an individual may have flown to the paper several times within the 10-min test period is not known.

A second set of data was obtained by determining the total number of contacts of all males with the scented paper. This figure is an expression of the running activity of the insects. The number of contacts of the heads of the cockroaches with the paper was counted each time a male coming from the trunk ran over or beneath it.

RESULTS

Behavior at Arrival and on the Pheromone Source

Two of the extract fractions tested attracted males of either *Periplaneta americana* or *P. australasiae* exclusively. The initial approach of the insects was in flight. Many of these males could be observed as they flew upwind, temporarily landing on objects blocking their paths. Afterward they flew to the correct tree within several meters above the pheromone source and ran hurriedly toward the paper. Males of *Periplaneta australasiae* succeeded more often than those of *P. americana* in flying directly to the pheromone impregnated paper.

Pheromone sources presented in plates on the ground (see methods) were approached by *Periplaneta americana* males only partially in flight. They

TABLE 2. APPROACHING FLIGHTS OF MALES TO DIFFERENT FEMALE PHEROMONE COMPONENTS OF *P. americana* AND *P. australasiae*[a]

		P. americana				*P. australasiae*			
Odor source	N	Total ♂	Median ♂/test		Confidence interval,* ♂/test	Total ♂	Median ♂/test		Confidence interval,* ♂/test
A									
Crude female	16	25	1	a	0–2	1	0	e	0
A	43	1	0	b	0	200	4	f	3–5
B	33	62	1	a	0–3	1	0	e	0
D	7	0	0	b	0	0	0	e	0
residue	15	1	0	b	0	1	0	e	0
B									
A + B	12	24	1–2	ac	0–4	20	1	c	0–3
A	12	9	0	bd	0–1	43	3–4	fd	1–5

[a](A) Two or three different tests were carried out in sequence per evening at each location. (B) The mixture periplanone-A + -B at a 1 : 1 ratio was tested as the first experiment each evening at each location. The same amount of A was tested afterward. (a,b, e, f) Median values of a (or e) are indistinguishable but are significantly different from b (or f) ($P \leq 0.05$, *U* test Mann–Whitney). (c, d) Median values differ significantly from one another (Wilcoxon rank test, $P \leq 0.01$).
*$P \leq 0.03$.

were often seen approaching the source by running the last several meters on the ground.

The males remained on the pheromone source for only 1 or 2 min, usually running across or beneath it several times and even momentarily completely off of the paper. Afterward the insects often ran up the trunk to a height of several meters and flew away. They rarely flew directly from the scented tissue paper. Other males, especially those of *Periplaneta australasiae*, remained on

TABLE 3. RUNNING ACTIVITY OF THE *P. australasiae* MALES FROM TABLE 2B ($N = 12$)[a]

Odor source	Total ♂	Median counts/♂		Confidence interval,* counts/♂
A + B	35	1–2	a	0–2
A	207	4	b	3–6

[a](a, b) Median values a and b differ significantly from one another (Wilcoxon rank test, $P = 0.01$).
*$P = 0.03$.

the trunk at some distance from the scented paper and either rested or groomed their antennae. Basically, the running activity of the males became less intensive the longer they stayed on the pheromone impregnated paper.

It could not be determined whether males which had left the trunk returned to the paper a second or further time. In any case, most arrivals occurred within 10 min after the paper had been presented. When the same pheromone was applied to the paper a second time, it was significantly less attractive than the first time.

Wing raising and fluttering displays by single males could be observed at the pheromone source. The meeting of two or more males on or near the paper sometimes resulted in copulation attempts. Especially in the experiments illustrated in Table 2B, homosexual behavior between males of both species was observed.

Experiments with Crude Extracts and Isolated Extract Components

The crude extract of the pheromones of *Periplaneta americana* females attracted conspecific males almost exclusively, except for one *P. australasiae* male (Table 2A). Up to six arrivals were counted per experiment. In six control experiments, employing the crude extract of male odors, not a single male approached the scented paper.

Fraction B (periplanone-B) attracted only males of *P. americana.* Fraction A (periplanone-A), however, attracted only one *P. americana* male for a short moment and a total of 200 *P. australasiae* males. In seven tests with periplanone-A, 10 times the usual concentration was applied.

Fraction D and the extract residue of the female pheromones were not attractive except for the approach of one male of each species.

Experiments with Mixtures and Sequence of Periplanone-A and -B

(*a*) P. australasiae. These experiments were supposed to be a first step toward an explanation of the fact that periplanone-A alone, but not in combination with the other components of the female crude extract, is attractive to *P. australasiae* males. The reaction to component B of the *P. australasiae* males could be observed when *P. australasiae* males had been lured with periplanone-A paper. Immediately after the addition of periplanone-B to the sample paper, *P. australasiae* males which had just arrived or which were still on the trunk turned away and ran or flew off. A newly presented paper containing periplanone-A alone attracted *P. australasiae* males again.

These observations were quantified in a series of 12 experiments (Tables 2B and 3), each being the first test carried out at each location on any one evening. A periplanone-A plus -B mixture at a 1 : 1 ratio was approached by *P. australasiae* males, although not as many came as when the same amount of

A was presented alone afterward. The insects usually came in contact with the paper only once, leaving the trunk immediately afterward. In a subsequent test, the same amount of periplanone-A was offered, but alone and not mixed with periplanone-B. It was observed that males of *P. australasiae* approached in flight with the same frequency (Table 2B) as is given for the tests for periplanone-A in Table 2A. In addition, the running activity of the insects was higher than for the mixture A plus B (Table 3).

(*b*) P. americana. The response of males of *P. americana* was not discernibly different from the response to the mixture of A plus B, to the female crude extract, or to periplanone-B alone. The following presentation of Fraction A alone attracted several males of this species with no significant difference from the test series with periplanone-A in Table 2A.

DISCUSSION

Stimulation Intensity and Attractivity of Periplanone-A and -B for P. americana

The fractions used in the field experiments were set to an intensity as similar as possible to those released by a female which is ready to mate. According to Seelinger (1983a), a female actively attracts males no more than 6 hr per night; nothing is yet known about the minimum duration of calling. Since, per night, about 0.6 ng periplanone-B and an equally effective amount of periplanone-A are emitted by a female (Sass, 1983), the amount of pheromone released per 10 min can at least be calculated at between 0.6 and 0.017 ng. The stimulant sources employed by us emitted periplanone-A and -B in amounts lying within these limits (Table 1).

Of the individual components of the female scent, periplanone-B was the only one able to attract *P. americana* males from a distance (Table 2A). Seelinger (1984b) also describes difference in the effectiveness of periplanone-B and -A. According to his work, males of *P. americana* in the laboratory are 30 times less sensitive to periplanone-A than to an amount of periplanone-B which is equally active in an electroantennogram. It may be possible that a 30- to 100-fold concentration of periplanone-A in our experiments would have also attracted males.

Seelinger also found that the effectiveness of periplanone-A is increased when tested simultaneously with periplanone-B. This may be the reason for the approaching flight of some males to periplanone-A after the mixture of A + B had been offered (Table 2B). Further functions of the components of the female pheromone in mixtures or in the close vicinity of the female during copulation, as have, for example, been observed with lepidopterans (Bradshaw et al., 1983), cannot be excluded at this time. In any case, a mixture

of periplanone-A and -B at a 1 : 1 ratio is just as effective for the approaching flight of males as the same amount of periplanone-B alone (Tables 2A and B).

Interspecific Activity of the Pheromones

The reactions of *P. australasiae* give an indication of the importance that the composition of the multicomponent pheromones of cockroach females may have. The species-specific pheromones of *P. australasiae* are not yet known. Since a similarity must be assumed between the female pheromones within the genus *Periplaneta* (Barth, 1970; Schafer, 1977a; Simon and Barth, 1977), it may be assumed that in this species, periplanone-A functions as a pheromone.

Individual components, however, may have very different effects on the behavior of various species: periplanone-B functions as an inhibitor for *P. australasiae* and as an attractant for *P. americana.* Other components of the crude extract of *P. americana* must also act as additional inhibitors, since the mixture of periplanone-A and -B was still able to attract few males, while the female crude extract was never attractive for *P. australasiae* males. Similar situations are, for example, also known for the multicomponent pheromones of several lepidopterans (Kaissling, 1979; Priesner, 1979). An attractive component for one species may function as an attraction inhibitor for another.

These results demonstrate that the female pheromone may be used to achieve the sexual isolation of related sympatric species. Seelinger (1984a) has reported also that calling females of *Periplaneta americana* attract only conspecific males in the same biotope. Previous laboratory experiments by Schafer (1977a) and Simon and Barth (1977) appear to contradict such an effect of cockroach pheromones.

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STRUCTURE-ACTIVITY RELATIONSHIP OF STRESS-INDUCING ODORANTS IN THE RAT

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Abstract—The stress for 12 sulfur-containing synthetized volatiles was evaluated in male Wistar rats and compared to that for fox-dropping extract concentrate. Stress behavior was analyzed by quantifying various stress responses in a standard open field and measuring the increase in plasma corticosterone concentration. Nine compounds induced stress—a dihydrothiazole, two cyclic polysulfides, five mercaptoketones, and a mercaptan. For the mercaptoketones, the following structure–activity relationships were observed. Size can vary considerably; the mercapto group can be either alpha or beta and either secondary or tertiary. The keto group is not essential, since a structurally related mercaptan remains active. The mercapto group is essential for activity in mercaptoketones, since conversion to a methyl sulfide resulted in a neutral response. This type of odorant could function as an allomone and may have potential in rat control as an area repellent.

Key Words—Rat, *Rattus norvegicus* sp., odorants, stress, behavior, open field, corticosterone, fox dropping, ketone, sulfur, compounds, tans, mercaptoketones, repellent, structure–activity.

INTRODUCTION

Structure–activity relationships (SAR) in airborne pheromones have been widely studied in a number of insect species on behavioral and physiological levels. In comparison, similar research on mammalian pheromones is virtually lacking, due to the difficulty of carrying on bridging experiments on several levels of olfactory activity in the same animal. Recent progress in

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this area has been reviewed by Wheeler (1976), Albone (1977) and Müller-Schwarze and Mozell (1976). Among the results is mentioned a well-known pheromone, dimethyl disulfide, found in the vaginal secretion of female hamsters (Singer, 1976). We, too, isolated this compound in fox feces and tested it. It was found to be attractive to rats. For this species and despite many early investigations on biological odors, especially sexual ones (Le Magnen, 1951; Carr and Caul, 1962), few active compounds have yet been identified.

Stevens (1972) found evidence for an alarm substance and Ludvigson (1967) pointed out the difference between frustration and success odors. Eslinger et al. (1980) are trying to identify these compounds. Many papers agree with the existence of a pheromone associated with dominance (Krames, 1968).

Rat responses to biological odors have been well described but little is known about responses to chemicals, because only a few of the active parts have been identified. Male rats are either repelled by or, at least, indifferent to most of the aliphatic acetates isolated in the preputial glands, which act as pheromones for the female (Stacewicz, 1977).

Gawienowski (1978) tested various sulfur-containing compounds on both male and female rats. Both sexes preferred dimethyl disulfide. Males were also attracted by dimethyl sulfide and hexyl mercaptan, while females preferred methyl isothiocyanate.

Laboratory-raised albino rats retain a hereditary stress response to predator odors, such as from the red fox (*Vulpes vulpes*), as measured by behavioral (Vernet-Maury, 1968), electrophysiological (Cattarelli, 1977), and biochemical (Vernet-Maury, 1970) assays. Thus, the rat presents a unique opportunity to investigate SAR for several types of activity with stress-inducing odorants.

The volatiles from fox droppings are particularly frightening to these rats. The volatiles were analyzed recently (Stoffelsma and Vernet-Maury, 1984) and the most active principle was identified as 2,4,5-trimethyl-Δ^3-thiazoline[4] (Vernet-Maury, 1980) among 70 characterized ingredients. In addition, a mercaptoketone (V) (Figure 1), suspected to be present in an active fraction but not identified, turned out to be indeed aversive.

The purpose of this study was to evaluate the fright response in Wistar rats for several of the synthetized fox-dropping volatiles, as well as for related compounds, and to search for eventual structure–activity relationships. The substances tested are listed in Figure 1.

The fear reaction was measured behaviorally and biochemically. Fright behavior was analyzed by quantifying various stress responses of the rat under laboratory conditions in a standard "open-field" box (Vernet-Maury,

[4]The term 2,5-Dihydro-2,4,5-trimethyl thiazole is used later in this text.

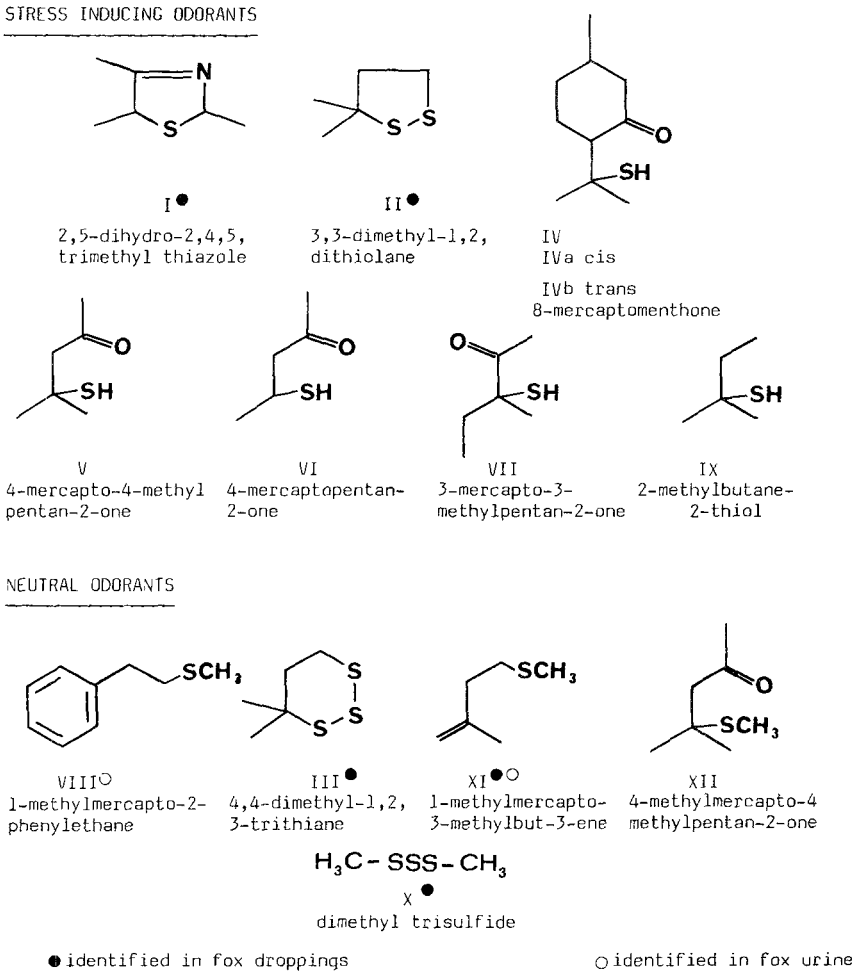

FIG. 1. Structural formulas of odorants.

1967). Physiological stress was analyzed by measuring the increase in corticosterone concentration in the blood plasma (Orlandi et al., 1973).

EXPERIMENTAL

Methods—Behavioral Assay

The "open-field" consists of a well-lighted (450-lux) white box, 120 × 90 × 40 cm, constructed of Plexiglas R with a loose transparent Plexiglas roof cover. The floor is devided into 15 cm squares to note horizontal dis-

placements. Otherwise, the enclosure is deprived of any topographical signposts. This type of device is suitable to measure motor behavior and, for rodents particularly, substitution behavior. Control air or odorized nitrogen is introduced through a nozzle in the roof. The air is odorized for 15 min and left to equilibrate for 5 min prior to each animal's entry. The box is washed with detergent, 20% ethanol, and water and then dried, and fresh air is blown for about 2 min until no odor is noticeable by the experimenters. Following this washing procedure we could not detect any volatiles by placing a mobile flame ionization detector at the top center of the area.

Twenty rats were used per product, and 20 controls per group of up to three or four products. Trials lasted 5 min in the enclosure. Two observers noted the rat's behavior according to criteria established previously. Total time including emergence time was 5–8 min. The sequence was as follows after a rat was placed in the entrance tunnel.

Measure of emergence time: Time needed to emerge from the entry tunnel into the enclosure, as the number of 15-sec intervals. This took only a few seconds for nontimid rats, longer in the opposite case. If the animal did not emerge spontaneously after 180 sec, he was gently pushed into the enclosure.

Exploratory horizontal movements: Number of squares crossed.

Exploratory vertical movements: Number of times rats rose on hind legs.

Substitution movements: Number of groomings and rubbings of snout with forelegs in a 10-sec interval. This behavior generally takes place only during the second stage of the test, when the animal began to become familiar with the surroundings. Aversive odors inhibited grooming.

Number of Defecations and Quantity of Urine Produced

Scoring.

- Emergence time: each 15-sec period is scored as +15.
- Horizontal movements: each square crossed is scored as −1.
- Vertical movements: each series of 10 is scored as −1.
- Visit to center of floor: each one is scored as −1.
- Grooming: each one is scored as −0.1.
- Defecation: each one is scored as +1.
- Urine is scored as +1 to +4, according to the surface wetted.

The final score for each rat is the sum of the above subscores.

Rating. In order to put scores between odorants on the same basis, the behavioral stress rating was calculated by deducting the score of the control rats from the score of the odor-exposed rats. The biochemical stress rating was defined as the ratio of the plasma corticosterone level of the odor-exposed rats to that of the control rats.

Biochemical Assay

This was the quantitative determination of corticoids, corticosterone being the main one for rats. The animals were decapitated on leaving the enclosure. The plasma was separated from the blood by centrifugation, frozen, and stored at −30°C until the assay could be carried out. A radioimmunological assay procedure was used (Murphy, 1967), using a liquid scintillation counter detector.

Statistical Analysis

Significance for the behavioral test was determined by the nonparametric. Mann–Whitney U test; for the biochemical assay, by the Student t test. Correlation between behavioral and biochemical results was done by least-squares estimates according to Fischer and Yates' statistical tables.

Materials

Odorants. See Figure 1 and Table 2. All single odorants were synthesized and purified when necessary by LLC on silica gel, from the Central Research Laboratory of PFW, B.V., Amersfoort, Holland, except for 4-methyl-4-methylmercapto-pentan-2-one, which was supplied by Oril, Paris, France, and *t*-amyl mercaptan (2-methylbutane-2-thiol), which came in a technical grade from Jansen Pharmaceutica, Belgium, and was purified by PFW.

Fox odor concentrate was prepared by concentrating the pentane extracts of fox droppings which had been stored frozen. Air was deodorized through an active charcoal filter. Nitrogen used was Air-Liquide, France, high purity. Odorants were stored neat at −30°C until used.

Stability: The sulfur-containing odorants used are unstable and prone to form less odorous byproducts including polymers in the presence of air, heat, light, moisture, and trace metals. The β-mercaptoketones tend to eliminate H_2S and revert to the unsaturated ketones. The α-mercapto ketone (VII) tends to dimerize to either the disulfide or the cyclic thiohemiacetal.

Purity: Odorant purity is listed in Table 2. Analyses were done by gas–liquid chromatography on a Carbowax column from PFW, B.V., or, if decomposition could not be avoided, by thin-layer chromatography on silica plates from F. Chastrette, University of Lyon I.

Natural occurrence: The materials marked with a filled circle in Figure 1 had been identified in fox-dropping extract (Stoffelsma and Vernet-Maury, 1983; Wilson, 1978). 4-Mercapto-4-methylpentan-2-one (*V*) (Figure 1) is the addition product of mesityl oxide and hydrogen sulfide (Pearce, 1967). It was suspected to occur (Vernet-Maury and Dubois, 1977) in a GLC fraction of fox-dropping extract. Since this fraction was active, it was synthe-

sized and tested. When its activity was confirmed, a number of homologues and isomeric mercaptoketones made available to us were also tested. V has an odor reminiscent of mature tom-cat urine and of heated asphalt tar. The odor was described in 1889 (Fromm and Baumann) as extremely powerful and highly objectionable to humans. It has been identified as an off-odor in canned foods as a result of the addition of food-generated hydrogen sulfide to traces of mesityl oxide in the acetone solvent base used to lacquer the inside of cans (Aylward, 1967). For similar reasons, V has been blamed as an off-odor in cheese (Steinsholz, 1979) in beer (Cosser, 1980), and in polluted river water (Maarse, 1974).

3,3-Dimethyl-1-2 dithiolane (II), 4,4-dimethyl-1,2,3-trithiane (III), and 1-methylmercapto-3-methylbut-3-ene (XI) were also identified in fox droppings. Since all three have the basic mercaptoisopentane skeleton of mercaptokeptone (V), they were tested. Compound II had been identified previously in the anal gland of mink and polecat (Schildnecht, 1976). XI had been previously identified with phenylethyl methyl sulfide (VIII) and six nonthio compounds in fox urine (Wilson, 1978). The author attributed the "skunky" odor of fox urine to XI and VIII. A synthetic mixture of the eight odorants found induced characteristic marking behavior in wild red foxes.

8-Mercaptomethone IV occurs in the volatile oil of distilled Buchu leaves (Lamparsky, 1971). Both the oil and the ketone are used as a black-currant flavorant.

Concentration: Saturated odor vapor above the neat odorant at 20°C was flushed with nitrogen at 640 cm^3/min. This flow was further diluted with deodorized air at 6.5 liters/min until reaching a final dilution in the enclosure of 1/15,000 of the saturated vapor. The concentration was monitored by a flame ionization detector calibrated on the vapor from the neat odorant (Packard 901 modified for higher sensitivity).

However, odorants II and III were tested from *n*-pentane solutions, due to lack of material, at unknown vapor concentrations. The rats' reaction to pentane alone was tested. Pentane by itself induced emotional reactivity that was not statistically different from that of deodorized air (−1.5 and −0.3, $U = 182$, NS).

The molarity of odor concentrations entering the enclosure was not determined. The trend of relative concentration differences is listed in Table 3 in three ways: by molecular weight (MW), by boiling point (BP), and by estimated vapor pressures calculated according to the method of Dravnieks (1977) for nonsulfur compounds by Mr. B. Eminet (Department of Organic Chemistry, University of Lyon). Only the vapor pressure of mercaptan IX was known (Boublik, 1973) and was used as a reference point to estimate the *relative* vapor pressures of the other mercapto compounds. Dose–response relationships were not investigated.

Rats. We used only male rats to prevent variations of emotional reactivity. Wistar rats (SPF) 8–10 weeks old, weighing 185–200 g, were housed four to a cage away from the experimental area and had access to food and water ad libidum. Rats were tested in the open field without any odor except their own. As far as odor integration in the CNS is concerned, we can state that only a new odor is an active stimulus for the rat's brain.

TABLE I. BEHAVIORAL TEST AND BIOCHEMICAL ASSAY RESULTS[a]

	N	BEH	*P*	Bio	*P*	Stress
Mercaptoketones						
IVa. *Cis*-8-mercaptomenthone	19	6–9	0.05	1–2.5×	0.05	+
IVb. *Trans*-8-mercaptomenthone	10	10–14	0.05	1.5–3×	0.05	+
IV. Mix of *cis–trans*-8-mercaptomenthones	20	8–12	0.02	1–2.2×	0.001	+
V. 4-Mercapto-4-methylpentan-2-one	10	9–14	0.02	1–2×	0.01	+
VI. 4-Mercaptopentan-2-one	14	6	0.02	1.6×	0.1	+
VII. 3-Mercapto-3-methylpentan-2-one	20	8	0.02	1.5×	0.05	+
Related mercapto (thiol) compounds						
II. 3,3-Dimethyl-1,2-dithiolane	20	9	0.02	1.5×	0.001	+
III. 4,4-Dimethyl-1,2,3-trithiane	10	2.5	0.02	1.2×	0.05	+
IX. 2-Methylbutane-2-thiol (*t*-amyl mercap.)	20	9	0.02	Not analyzed		+
XI. 1-Methylmercapto-3-methyl-but-3-ene	15	1.5	NS	0.8×	NS	0
XII. 4-Methylmercapto-4-methyl pentane-2-one	9	1	NS	1.5×	0.05	0
Other odorants						
VIII. 1-Methylmercapto-2-phenylethane	15	1	NS	1.3×	NS	0
Buchu oil	9	1	NS	1×	NS	0
Concentrate of fox-dropping solvent extract	20	12	0.002	3×	0.01	+
I. 2,5-Dihydro-2,4,5-trimethylthiazole	30	10.5	0.002	4–5×	0.001	+
X. Dimethyl trisulfide	20	1.3	NS	1×	NS	0
n-Pentane	20	1.5	NS	Not analyzed		0

[a] *N*, number of rats tested per product; BEH, behavioral stress rating relative to controls = 0 calculated from (original) raw data; Bio, biochemical stress rating as corticosterone level in plasma relative to no-odor controls = 1; *P*, probability of the result being the same as for the no-odor control group; Stress, stress response positive (+) or neutral (0); NS, not significant.

RESULTS

Behavioral

Nonstressed rats always obtained a negative behavioral score, while stressed rats obtained scores ranging from weakly negative to positive. Thus, most odorants could be classified into neutral (non-stress-inducing) and stress-inducing categories at equal saturated vapor dilutions. About half of the substances tested produced stress. From Table 1, the following three groupings are suggested.

Greatest stress response: fox extract and D.H. thiazole (I).

Medium stress response: dithiolane (II), mercaptoketones (IV, IV_a, IV_b, V, VI, VII), and mercaptan (IX).

No or little stress response: trithiane (III) and sulfides (VIII, X, XI, XII).

Biochemical

The corticosterone levels essentially confirm behavioral results. For the regression line ($a = 1.69$; $b = 13.55$) a positive correlation ($r = 0.66$; $P < 0.01$) was observed between the actual behavioral and the biochemical values.

TABLE 2. ANALYSES BY GAS–LIQUID CHROMATOGRAPHY (GLC) ON CARBOWAX AND THIN-LAYER CHROMATOGRAPHY (TLC) ON SILICA PLATES

Code	Name	GLC	TLC
I	2,5-Dihydro-2,4,5-trimethylthiazole	98% cis + trans, 1:1	
II	3,3-Dimethyl-1,2-dithiolane	"Pure"	
III	4,4-Dimethyl-1,2,3-trithiane	"Pure"	
IVa	*Cis*-8-mercaptomenthone (α^{D}_{20}, +40°C)	98%	
IVb	*Trans*-8-mercaptomenthone (α^{D}_{20}, −32°C)	99%	
IV	Mix of *cis* + *trans*-8-mercaptomenthone	66% trans 30% cis 2% pulegone	
V	4-Mercapto-4-methylpentan-2-one	94% 6% mesityl-oxide	One spot
VI	4-Mercaptopentan-2-one	>99% decomp.	One main spot, two small impurities
VII	3-Mercapto-3-methylpentan-2-one	98%	One spot
VIII	1-Methylmercapto-2-phenylethane	99%	
XII	4-Methylmercapto-4-methylpentan-2-one	97%	One spot
IX	2-Methylbutane-2-thiol	99.5%	
XI	1-Methylmercapto-3-methylbut-3-ene	98.5%	
X	Dimethyl trisulfide	Not analyzed	

TABLE 3. RELATIVE TREND OF VOLATILITY AMONG PRODUCTS

	MW	BP (°C/mm Hg)	Vapor pressure trend at 25°C in arbitrary units relative to *t*-AmSH (IX = 100)
Mercaptoketones			
IV	186	88–100/2	0.07
V	132	53–58/12	2
VI	118	56–58/12	7
VII	132	55–57/12	2
Mercaptans			
IX	104	99–105/760	100
Sulfides			
I	129	55–56/12	5
II	134	No data available	
III	166	No data available	
VIII	152	66/1	0.4
X	126	54/12	
XI	116	80–81/90	21
XII	146	50–55/0.06	
		78–80/12	0.7

DISCUSSION

Fox-dropping extract (FDE) is a complex mixture of volatile components (70 identified). A number of these have also been found in nonpredator dropping such as swine (Yasuhara, 1980). One of the sulfur-containing components occurring in FDE (as in swine) is dimethyl disulfide; it is attractive to the female rat (Gawienowski, 1978). Since the most active GLC fractions of FDE contained sulfur, it was decided to test the other thio compounds identified (I, II, III, X, XI), as well as suspected to occur, in these fractions (V, VIII). Of these products, VIII, XI, and X were found to produce no or little stress. II, III, V, and I evoked stress approaching that of FDE. This suggests that I at least is a predator-specific allomone for the rat. The term is as defined by Albone (1977). The stress-inducing odorants belong to four chemical structural classes: dihydrothiazole, cyclic sulfides (II, III), mercaptoketones (IV, V, VI, VII), and mercaptan (IX).

To humans, the odor qualities seem quite different, except between the last two classes, which are very similar. This suggests that, by the rat also, they could be qualitatively discriminated but that each class is perhaps reminiscent of some alarming feature of the complex FDE odor. Dose–response relationships were not investigated further, as our objective was to make a comparison among the various odorants at equal fractions of saturated vapor

pressure. The rat's behavior is based on an "all or none" response, depending on whether it detects the odor or not. To the experimenters, intensity levels resembled those of fox droppings or extract.

Structure–Activity Relations

Insertion of a third sulfur atom in disulfide (II) suggests a weakening of the stress response (III) ($U = 49$; $P < 0.05$).

Figure 1 reveals that among the mercaptoketones and mercaptan (IX) evaluated, stress behavior may be associated with the following features:

(a) The mercaptoketones can be aliphatic (V, VI, VII) or alicyclic (IV).

(b) The thiol (mercapto) group is essential. Conversion of V to the methylsulfide derivate (XII) leads to the disappearance of stress.

A similar decrease in activity had been noted when mercaptans were extracted with mercuric chloride from FDE pentane extract (Vernet-Maury and Dubois, 1977).

(c) The keto group is not essential. Its removal (IX) from V did not lead to the disappearance of stress.

It should be noted that menthone and isobutylmethylketone, while not tested here, have entirely different odors to humans than the corresponding mercaptoketones IV and V. Moreover, Buchu oil, which contains l-pulegone, an unsaturated version of menthone, as well as related non-sulfur-containing terpenes, was inactive, despite the natural presence of traces (0.1%) of IV.

(d) The thiol group can be tertiary (IV, V, VII, IX) or it can be secondary (VI).

(e) The thiol group can be beta to the keto group (IV, V, VI) or alpha (VII).

(f) Molecular size can vary considerably. V and VI can be considered as fragments of larger-sized IV, yet they contain the essential structural features to signal stress.

The phenomena of size tolerance has been noted previously (Polak et al., 1978). They found that *cis-cis*-2,6-dimethylcyclohexanol ($C_8H_{16}O$) was confused at low concentrations by human objects with the larger-sized, earthy-smelling, bicyclic alcohol "geosmin" ($C_{12}H_{22}O$), which contains the same structural fragment. Both cis (IVa) and trans (IVb) forms of IV are active and not very different in the response they produce. This indicates that the spatial position of the methyl group relative to the isopropyl group in IV is not activity determining.

The absence of a strong geometric isomer effect in IV is not surprising. It does not mean that rats would not be able to differentiate between IVa and IVb in an odor discrimination test. For humans, cis and trans isomers are usually qualitatively very similar in odor, e.g., the anetholes, the men-

thones, and geraniol-nerol. The cis–trans mix (IV) appeared behaviorally more active ($U = 109$, $P < 0.05$) than the single isomers. This would require verification with a reconstructed mixture and more accurate concentration control.

Müller-Schwarze et al. (1976) had observed that black-tailed deer responded more strongly to the cis (Z) isomer of a lactonic pheromone than to the trans (E) isomer.

Fear-producing odorants may have potential as area repellents in pest control (Bullard, 1977), provided habituation does not interfere. Field tests on our most active compounds (I and V) are still to be carried out on wild rats.

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SOURCES OF FALL ARMYWORM, *Spodoptera frugiperda* (LEPIDOPTERA: NOCTUIDAE), KAIROMONES ELICITING HOST-FINDING BEHAVIOR IN *Cotesia* (=*Apanteles*) *marginiventris* (HYMENOPTERA: BRACONIDAE)

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Abstract—Bioassay responses in *Cotesia marginiventris* (Cresson) females to materials derived from fall armyworm (FAW) larvae, *Spodoptera frugiperda* (J. E. Smith), were most intense for frass and somewhat less intense for larval and pupal cutical materials, scales, exuviae, silk, and oral secretion, with FAW larval hemolymph eliciting only a slight response. The highest percentage of ovipositor probing was caused by frass (100%) and moth scales (90%). Various types of corn-leaf damage when assayed alone did not produce responses as intense as when assayed in combination with frass, cuticle material, and oral secretion. Parasitoid response was somewhat better to frass derived from FAW larvae feeding on corn and peanut leaves than from larvae feeding on the foliage of soybeans, Bermuda grass, cowpeas, or laboratory diet. Hexane and chloroform were better than methanol and water for extracting active material from FAW frass, and chloroform was the best of these solvents for extracting corn leaves. Serial dilutions of frass extracts resulted in a reduction in parasitoid response.

Key Words—Fall armyworm, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, kairomone, host-finding behavior, *Cotsia* (=*Apanteles*) *marginiventris*, Hymenoptera, Braconidae, frass attraction, scales attraction, pest management.

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INTRODUCTION

The mechanism by which parasitoids find a host is a critical aspect of their behavior. Thorpe and Jones (1937) and Laing (1937) report host odor as a factor in the host-finding process. Additional research supports and enlarges their findings (Jones *et al.*, 1973; Leonard *et al.*, 1975; Lewis and Jones, 1971; Vinson and Lewis, 1965; Vinson, 1968; Weseloh, 1981) by indicating that parasitoids respond to chemicals known as kairomones that are derived from the host as well as chemicals originating in the plant (synomones). Kairomones and synomones act as orientation stimuli in host finding. These semiochemicals vary in composition and can be present at more than one location in the host insect (Vinson, 1977). More than 10 kairomones mediating host finding and host acceptance of several parasitoids are known. These chemicals do not harm nontarget biota in the environment and are potentially useful for parasitoid manipulation in pest management programs (Lewis *et al.*, 1976).

Loke *et al.* (1983) defined the "find and attack" cycle comprising the host-finding behavior of *Cotesia marginiventris* (Cresson), a larval parasitoid of the fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith). In addition, Loke and Ashley (unpublished data) demonstrated that corn leaves damaged by fall armyworm larvae affected the searching behavior of this parasitoid. In the present study we extend the above research by examining various materials associated with host presence to determine which of these serve as possible sources of kairomones or synomones mediating the host-finding response in *C. marginiventris.*

METHODS AND MATERIALS

Bioassays were conducted under ambient laboratory conditions (ca. 25°C, 60% RH) and ca. 30 cm beneath two 20-W fluorescent bulbs. Female parasitoids were 2 to 4 days old and had been exposed to males since eclosion. FAW larvae and *C. marginiventris* adults were reared as described by Loke *et al.* (1983). Bioassays were conducted in 1.5 × 10-cm-diameter plastic petri dishes and fresh setups (petri dish plus chemical) were used for each female tested. Stippled corn-leaf sections (1.2 cm^2) were prepared by piercing the leaf with a pin and puncturing it with a hole puncher.

Parasitoid responses were quantified using a modification of a four-point system developed by Lewis and Jones (1971). A response consisted of any interruption of the parasitoid's movement for the purpose of investigating the chemical, regardless of the intensity of the investigation. Responses were additionally classified as intense if rapid antennal palpatation and/or ovipositor probing occurred. Intense responses were further divided into those that resulted in ovipositor probing. The occurrence of a response by the parasitoid

when the semiochemical was contacted the first time was scored as a three. If the response occurred on the second or third contact, scores of two and one were given, respectively. A zero was recorded if no response was elicited after three contacts.

Host Sources. Materials for bioassays were obtained from second- or third-instar larvae, female FAW pupae, or female moths and applied as a spot onto 9-cm Whatman No. 1 filter paper. Frass (0.5 mg), ca. 10 μl of oral secretion (obtained by holding the larva behind the head and at the posterior end and gently forcing the larva to regurgitate onto the filter paper), ca. 10 μl of hemolymph (obtained by pin-pricking the larva), 25 rolls of larval silk around a minuten pin, five transfers of larval cuticle material (a single transfer consisted of rubbing fifth- or sixth-instar larvae onto the filter paper), a complete exuvium, five transfers of pupal cuticle, and wing scales were bioassayed. Responses from 30 parasitoids for each material were scored using the four-point system.

Frass from Different Foods. Frass was obtained from FAW larvae reared on laboratory diet (King and Leppla, 1983), other FAW larvae, and the leaves of corn, peanuts, cowpeas, soybeans, and Bermuda grass, *Cynondon dactylon* (L.). Plants were grown in a greenhouse in 14.5 × 15-cm-diameter pots. Each type of frass (0.5 mg) was bioassayed using 30 *C. marginiventris* females.

Plant-Host Materials. Stippled leaf sections (1.2 cm^2) with oral secretion, frass, and cuticle material and a multiple combination of stippled leaf plus oral secretion, frass, and cuticle material were assayed. For comparison, parasitoid responses to leaf sections with edges taped and exposed, and stippled, as well as crushed corn leaf (0.5 mg) and corn-leaf juice (10 μl) also were included. Frass (0.5 mg) from corn-reared FAW larvae served as a standard. Twenty parasitoid females were assayed for each treatment.

Kairomone Extraction. Frass from corn-reared FAW larvae was collected and stored at −10°C in tightly capped glass vials. Extracts were prepared from 500 mg of frass in 10 ml of deionized-distilled water, nanograde methanol, chloroform, and *n*-hexane. Extraction consisted of grinding the frass in the solvent with a mortar and pestle for 3 min and then filtering the mixture through a Whatman No. 1 filter paper. Bioassays were conducted immediately after filtration by spotting 5 μl of extract onto filter paper and allowing sufficient time for the solvent to evaporate before placing the material into a petri dish. Extracts (except the water extract, which was kept in a refrigerator) were stored at −10°C in tightly stoppered vials. Equivalent amounts (500 mg) of fresh corn leaves were similarly extracted and bioassayed. Extracts of scales were made by soaking five adult females with 10 ml of each solvent for 60 min, followed by filtration. Responses from 20 parasitoids were recorded for each extract, and frass (0.25 mg) was used as a standard.

RESULTS AND DISCUSSION

Frass elicited the strongest responses in *C. marginiventris* females compared to the other host materials (Table 1). The statistical comparisons must be interpreted cautiously since the quantities of each material were arbitrarily selected. Nevertheless, frass, materials from larval and pupal cuticle, exuviae, and scales elicited the most positive responses, with frass and scales causing the highest proportion of ovipositor probing. Parasitoids showed no response to FAW diet. Oral secretion from starved, diet-reared, fifth instars (not shown in Table 1) elicited a mean response of 2.2. A substantially higher response was recorded for oral secretion from nonstarved, corn-fed, FAW larvae. Although hemolymph was the least stimulatory material, it elicited responses from over 80% of the parasitoids. No significant differences existed among frass, larval cuticle materials, pupal cuticle material, scales, and exuviae. The last four materials are of similar embryonic origin and contain long-chain hydrocarbons associated with lipids (Baker and Nelson, 1981; Richards, 1978). Long-chain, methyl-branched hydrocarbons have been identified as important kairomones for some parasitoids (Jones *et al.*, 1971, 1973; Vinson *et al.*, 1975). Strong responses to moth scales were not considered unusual because *C. marginiventris* will parasitize newly hatched larvae that are still in proximity to a FAW egg mass covered with scales. The response of *C. marginiventris* to a variety of kairomones from the same host is consistent with results of kairomone research on other parasitoids (Jones *et al.*, 1976; Lewis *et al.*, 1976; Sato, 1979; Vinson, 1977).

Stippled and crushed corn leaves elicited higher responses from para-

TABLE 1. RESPONSE OF *C. marginiventris* TO SUBSTANCES DERIVED FROM CORN-REARED FALL ARMYWORM (FAW) LARVAE[a]

FAW substances	Mean response (±SE)	Percentage		
		Responding	Responding strongly	Probing with ovipositor
Frass	3.00 ± 0.00 a	100	100	100
Larval cuticle material	2.87 ± 0.06 ab	100	87	43
Pupal cuticle material	2.83 ± 0.07 ab	100	83	33
Scales	2.83 ± 0.07 ab	100	80	90
Exuviae	2.80 ± 0.07 ab	100	80	70
Silk	2.67 ± 0.11 b	100	70	47
Oral secretion	2.63 ± 0.11 b	100	70	53
Hemolymph	1.26 ± 0.14 c	83	3	3

[a]Means in the same column followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple range test.

TABLE 2. RESPONSE OF *C. marginiventris* TO CORN LEAVES AND COMBINATIONS OF STIPPLED CORN LEAVES AND SUBSTANCES FROM DIET-REARED FALL ARMYWORM LARVAE[a]

Samples bioassayed	Mean response (±SE)	Percentage		
		Responding	Responding strongly	Probing with ovipositor
Lab diet	0.00	0	0	0
Intact leaf	0.05 ± 0.05 a	5	0	0
Cut leaf	1.25 ± 0.10 b	100	0	5
Leaf juice	1.80 ± 0.16 c	100	15	0
Stippled leaf (SL)	2.15 ± 0.17 d	100	35	30
Crushed leaf	2.80 ± 0.09 e	100	80	70
SL, oral secretion (OS)	2.90 ± 0.07 e	100	90	85
SL, fras (F)	2.90 ± 0.07 e	100	90	95
SL, cuticle material (CM)	2.95 ± 0.05 e	100	95	100
SL, OS, F, CM	3.00 ± 0.00 e	100	100	100

[a]Means in the same column followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple range test.

sitoids than did undamaged leaves (Table 2). Parasitoids favored the untaped cut edges of leaves and reacted to crushed leaves much as they did to materials derived directly from the host. Adding host-derived substances to plant materials increased the response compared to plant materials used alone. A

TABLE 3. RESPONSE OF *C. marginiventris* TO FRASS OF FALL ARMYWORM LARVAE REARED ON DIFFERENT FOOD SOURCES[a]

Food source	Mean response (±SE)	Percentage		
		Responding	Responding strongly	Probing with ovipositor
Cowpea	2.70 ± 0.09 a	100	70	83
Lab diet	2.73 ± 0.08 ab	100	70	43
Bermuda grass	2.77 ± 0.08 abc	100	77	90
FAW	2.80 ± 0.07 abc	100	80	53
Soybean	2.83 ± 0.07 abc	100	80	90
Peanut	2.93 ± 0.05 bc	100	93	100
Corn	2.97 ± 0.03 bc	100	97	100

[a]Means in the same column followed by the same letter are not significantly different (5%) level as determined by Duncan's multiple range test.

similar situation was reported by Sauls *et al.* (1979) for responses of *Microplitis croceipes* (Cresson) to homogenized cowpea cotyledons in combination with frass of diet-reared *Heliothis zea* (Boddie).

High responses were elicited from parasitoids to frass, regardless of the FAW larval food source (Table 3). Frass derived from plant materials consistently produced stronger responses from the parasitoids, particularly in the percentage probing with the ovipositor. This situation may be due to the presence of natural host-seeking kairomones or their precursors in plants and the absence of these chemicals in the ingredients of the laboratory diet. Since *C. marginiventris* has been reported from many ecosystems (Ashley, 1979; Boling and Pitre, 1970; Mueller and Kunnalaca, 1979; Nickle, 1977), it is not surprising that this parasitoid responds to frass from larvae having fed on a variety of host plants.

Hexane, chloroform, and methanol extracts of frass elicited significantly greater responses from *C. marginiventris* than did a water extract (Table 4). The solvents themselves produced no response when contacted by the parasitoid. The most pronounced differences between the frass standard and the frass extracts occurred in the percentage parasitoids probing with their ovipositors. The chloroform extract of corn leaves was significantly better than corn-leaf extracts made with other solvents. Serial dilutions of these extracts resulted in a substantial decrease in parasitoid response (Figure 1). Hexane and chloroform were the best of the solvents tested.

TABLE 4. RESPONSE OF *C. marginiventris* TO EXTRACTS OF FRASS FROM CORN-REARED FALL ARMYWORM LARVAE[a]

Material bioassayed	Mean response (±SE)	Percentage		
		Responding	Responding strongly	Probing with ovipositor
Frass extracts				
Frass standard	3.00 ± 0.00a	100	100	100
Hexane	2.90 ± 0.06a	100	90	35
Chloroform	2.85 ± 0.08a	100	85	35
Methanol	2.85 ± 0.08a	100	85	25
Water	2.45 ± 0.14b	100	50	15
Corn-leaf extracts				
Water	0.95 ± 0.20a	65	5	0
Hexane	1.45 ± 0.20ab	85	10	5
Methanol	1.70 ± 0.20b	90	15	5
Chloroform	2.60 ± 0.11c	100	60	15

[a]Means in the same column within each extract group followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple range test.

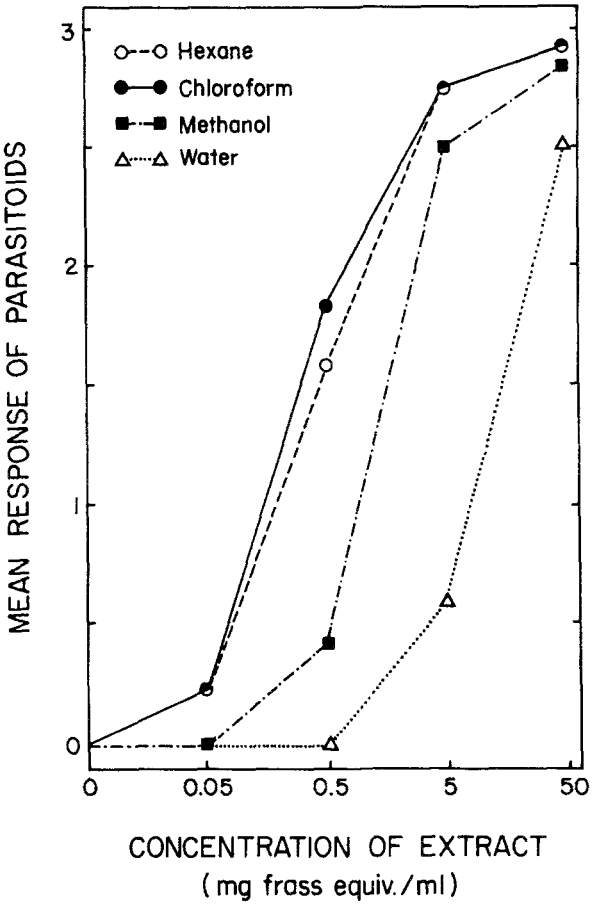

FIG. 1. Mean responses of *C. marginiventris* females to serial dilutions of hexane, chloroform, methanol, and water extracts of frass from corn-reared fall armyworm larvae.

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QUANTITATIVE VARIATION OF PHEROMONE COMPONENTS IN THE SPRUCE BARK BEETLE *Ips typographus*[1] FROM DIFFERENT ATTACK PHASES[2]

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Abstract—*Ips typographus* beetles were collected in the field, separated into eight attack phases (from beetles walking on the trunk of a tree under attack to those excavating gallery systems with a mother gallery longer than 4 cm), and immediately frozen in liquid nitrogen. 2-Methyl-3-buten-2-ol, *cis*- and *trans*-verbenol, verbenone, myrtenol, *trans*-myrtanol, ipsenol, ipsdienol, and 2-phenylethanol were quantified from excised hindguts against an internal standard, heptyl acetate, in the extraction solvent. Methylbutenol, the pinene alcohols, and 2-phenylethanol showed the same pattern of variation between attack phases in males, with the largest amounts present before accepting females and then a fast decline. Ipsenol and ipsdienol were not detected in males before the females were accepted, and the amounts increased when the females start their egg laying. Verbenone occurred only in trace amounts. The beetles were sampled from five Norway spruce trees (*Picea abies*) of differing resin flow. The correlations between the nine pheromone components and five major host monoterpenes in the gut showed that the variation in the amount of methylbutenol, ipsenol, and ipsdienol could not be explained by the variation in the amounts of host monoterpenes. In contrast over 80% of the quantitative variation of *cis*-verbenol, *trans*-verbenol, and myrtenol was explained by the amount of α-pinene. The nine pheromone components from 36 individual males were also quantified. Both methylbutenol and *cis*-verbenol showed a large variation in both amounts and proportions. Females contained *trans*-verbenol and traces of most other components found in males. When accepted by the male, they also contained a female-specific compound, β-isophorone. Behavioral and biosynthetic implications of the results are discussed.

[1]Coleoptera: Scolytidae.
[2]This study was made within the Swedish project "Odour Signals for Control of Pest Insects."

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Key Words—*Ips typographus*, spruce bark beetle, Coleoptera, Scolytidae, 2-methyl-3-buten-2-ol, ipsenol, *cis*-verbenol, ipsdienol, *trans*-verbenol, verbenone, myrtenol, *trans*-myrtanol, 2-phenylethanol, β-isophorone.

I. INTRODUCTION

The colonization of a spruce tree by *Ips typographus* (L.) is initiated by the males. During epidemics, they attack living trees, whose resistance by resin flow is overcome by a mass attack. Females are accepted by a male after he has excavated a nuptial chamber in the phloem under the bark (Postner 1974).

The aggregation of bark beetles to a specific tree is directed by pheromones. Pheromones in bark beetles were first investigated in *I. paraconfusus*, which was shown to use a combination of ipsdienol, ipsenol, and *cis*-verbenol (Silverstein et al., 1966; Wood et al., 1966) as an aggregation pheromone. All three substances were later shown to be oxygenation products of host resin monoterpene hydrocarbons (see Wood, 1982, for a review). These substances were shown to be characteristic for the genus, and one or more are present in the hindgut of all species investigated (Vité et al., 1972; Wood, 1982), although the behavioral roles of the compounds are not clarified in all species.

The presence of male-produced attractants in *I. typographus* for synchronization of the mass attack was first shown in biological experiments by Bakke (1970) and Rudinsky (1971). From gas chromatographic analyses, Vité et al. (1972) reported the monoterpene alcohols *cis*-verbenol, *trans*-verbenol, ipsdienol, and ipsenol from male hindguts. Ipsdienol together with the two verbenols and α-pinene was found in field experiments to be the most attractive blend for trapping beetles (Bakke 1976). In a GC-MS study the isoprene alcohol 2-methyl-3-buten-2-ol was identified as an additional component (Bakke et al., 1977) which, combined with *cis*-verbenol and ipsdienol, showed the highest trap catches and was suggested to be the aggregation pheromone.

The aggregation pheromone of *I. typographus* as used today, including (4S)-*cis*-verbenol, is a potent one for attracting beetles into pipetraps (Lie and Bakke, 1981). However, many steps in the colonization of a spruce tree by *I. typographus* are still unknown, e.g., how the rapid initial aggregation of males takes place in response to release/production of pheromone by the few pioneering males. Also, the biosynthesis of different compounds needed in the different phases of initial aggregation, mass attack, and termination have not been studied in the field. Mechanisms terminating the attack and causing a switching of swarming beetles to adjacent trees (Coulson, 1979; Hoff, unpublished) are, in this species, almost unknown, although recently Byers (1983) indicated a pheromonal mechanism in *I. paraconfusus*. A tree would easily be overpopulated without such density-regulating mechanisms

so that food shortage for the developing brood would result (Eidmann and Nourteva, 1968; Ogibin, 1972).

Biological tests showed a decrease in the attraction to males after they have been joined by females (Rudinsky et al., 1971). Chemical analyses revealed that unmated males in logs have no ipsdienol (Bakke, 1976; Hackstein and Vité, 1978) and very small amounts of *cis*-verbenol but large amounts of methylbutenol (Hackstein and Vité, 1978). Also, analyses in our laboratory of small batches of males boring in logs under controlled conditions showed a large individual variation in pheromone content.

Several hypotheses can be proposed to account for these variations in pheromone content. Pheromone production/release should be rapid after contact with the tree. The reduction of attraction could be due to either qualitative differences in the production of compounds from either or both sexes or a decrease in the quantity of male production of attractive substances. The low amounts of *cis*-verbenol and lack of ipsdienol could be due to insufficient amounts of certain host monoterpene precursors in logs.

To test these hypotheses we have qualitatively and quantitatively analyzed, by GC-MS, hindguts of batches of field-collected beetles from different attack phases and host trees and, in addition, 36 individual beetles.

2. METHODS

2.1 Collection and Preparation

Spruce bark beetles were collected in May 1981 close to Lysvik (60°N, 13°E), province of Värmland, Sweden, in the center of a large outbreak area of *I. typographus* in Scandinavia (Löyttyniemi et al., 1979).

Beetles were collected from Norway spruce [*Picea abies* (L.) Karst.] trees in an area with a large amount of expanding infestations in 1980/1981 (J. Regnander, personal communication). Three of the sampled trees, (A, B, G) stood on a well-drained, gentle north slope at 125 m above sea level, and the remaining two (D, F) on a well-drained, steeper east slope at 200-m altitude 1 km further north. Two logs were sampled from a log pile 20 km further north at 200-m altitude.

Trees D and B were selected as "weak" since they showed only small signs of resin exudation. We designated trees A and F as "normal," as they had resin flowing from beetle entrance holes. We were able only to find one "resistant" tree, G, which had resin flow from all entrance holes and in which beetles were killed by resin flow (Table 1).

The long chain of behavioral steps from landing to continued egg laying (Borden 1974) was divided into eight phases (Figure 1, Table 2). Beetles from galleries with two females were analyzed in phases 4 to 7, as two females per male was the most common ratio. In addition, males with one female were

TABLE 1. CHARACTERISTICS OF SAMPLED *Picea abies* TREES AND LOGS ATTACKED BY *Ips typographus* IN MAY 1981, LYSVIK, PROVINCE OF VÄRMLAND, CENTRAL SWEDEN

	DBH (dm)[a]	Exposure[b]	Resin flow[c]	Response to attack	Attack density[d] (males/dm^2)	Date of first attack	Date of sampling	Note
Tree G	3	E, 10	Very rich	Not overcome	1–5	21?	23	Baited[e]
Tree B	4	E, 5	Poor	Taken over	10–15	11	16–18	Baited[e]
Tree F	4	SE, 20	Rich	Taken over	5–10	21?	21–22	Baited[e]
Tree A	3	S, 10	Rich	Taken over	5–10	11	16–23	Baited[e]
Tree D	5	N, 0	Poor	Taken over	10–15	18 (19?)	20–24	Unbaited
Log C	4	Log pile	None	—	1–5	11?	19	Unbaited
Log H	6	Log pile	None	—	1–5	11?	24	Unbaited

[a]DBH, diameter at breast height, or largest diameter of logs.
[b]Direction and distance in meters to nearest forest edge.
[c]Very rich, resin flow from every boring; rich, strings of resin from borings and frass wet from resin; poor, no strings of resin and most frass dry; none, all frass dry.
[d]As estimated from photographs of sampled bark.
[e]Baited with IPSLURE (0.4 m) to initiate attack.

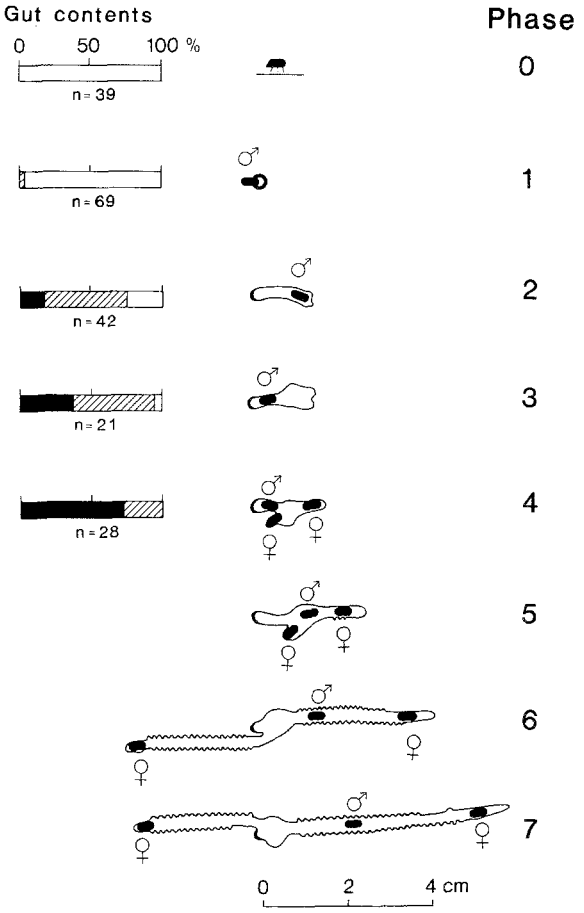


FIG. 1. The different phases of gallery construction as defined in this study. Gut content of bark material in male guts: open, empty or only old material in hindgut; hatched, fresh phloem material in mid- and hindgut; filled, whole gut well filled with phloem. Gallery outlines were drawn from photos of sampled galleries.

analyzed in phases 4 and 5 from trees D and F. As a rule, both of the females were in the same phase or in adjacent phases. The male was assigned the same phase as the highest phase of the females.

Beetles were collected from the trunks of trees in the interval from 0.5 to 3.0 m above ground, although at least two-thirds of the trunk was infested on all trees. Beetles of both sexes also were collected from irregular galleries on stumps cut the year before, close to tree F.

When necessary, as in phases 0, 4, and 5, sexes were tentatively separated by pronotal bristle density with a ×22 lens (Schlyter and Cederholm, 1981).

TABLE 2. THE ATTACK PHASES OF *Ips typographus* AS USED FOR ANALYSIS IN THIS STUDY

Phase	Time[a]	Definition	Analyses performed			
			Sex	Trees	Number of extracts	Sum of individuals in extracts
0	—	Beetles walking on the trunk of a tree under attack	Males	F	2	48
			Females			40
1	0–1 hr	Boring in the outer bark (cortex) with only the head under the surface	Males	F	3	70
2	4–24 hr	Boring in the phloem with the whole body under the surface	Males	ABDF	12	161
3	1–2 days	Nuptial chamber completed; no female admitted	Males	ABCDFGH	18	210
4	2–3 days	Two females admitted; mother galleries initiated, but no eggs laid	Males	ABCDFGH	10[b]	109[b]
			Females	ABD	9	138
5	2–4 days	At least one egg laid, but mother gallery not longer than 2 cm	Males	ABDH	8[b]	98[b]
			Females	ABD	6	108
6	4–6 days	Mother gallery length between 2 and 4 cm	Males	ABD	5	54
			Females	ABD	6	87
7	>>7 days	Mother gallery longer than 4 cm (up to approx. 8 cm)	Males	A	2	31
			Females	A	3	41
				7	86[b,c]	1195[b,c]

[a]Approximate cumulative time after the initiation of boring.
[b]Extracts from males, with only one female from phase 4 (three extracts) and phase 5 (two extracts) from trees D and F not included.
[c]Four extracts from each of males and females feeding in old stumps not included.

Excised beetles were placed in glass jars (20 ml) in a dark cooling box at 0 to +4°C, where they soon were immobilized. Less than 1 hr later they were inserted in groups of five to eight into paper-lined, cotton-plugged glass tubes (45 mm long, 3.5-mm ID) and immediately immersed in liquid nitrogen (−196°C).

The tubes were later separated into categories of trees and phases at −20°C in a few hours and again stored in liquid nitrogen.

Prior to dissection the glass tubes were placed on ice. The hind- and midguts were dissected and frozen on a block of aluminum at −20°C. The sex was carefully checked in all beetles during dissection, as 0–10% were incorrectly assigned to sex during sampling. The frozen guts were transferred to 60 μl of redistilled pentane with 5% ethyl acetate, kept at −20°C. Heptyl acetate, 1 ng/μl solvent, was used as internal standard. Each sample was crushed at room temperature, and after 10 min the extract was transferred to an ampoule, which was sealed under nitrogen and stored at −18°C. The gut content was noted in males from tree F. Guts of individual beetles were dissected into 10 μl of pentane with heptyl acetate as internal standard, cooled on dry ice (−79°C), crushed and extracted as above, and immediately analyzed. The dry weight of the body was taken after 24 hr at 105°C.

2.2 Chemical Analysis

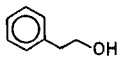
A Finnigan 4021 GC-MS (quadrupole) and a Hewlett-Packard 5830A GC were used for the analyses. Glass capillary columns were made in our laboratory in Göteborg, coated with Superox FA (Alltech). For GC-MS a 50-m × 0.25-mm-ID column [d_f = 0.54 μm, 2500 theoretical plates/m, k' = 3.1 (octanol)] was used. A second column, 25 m × 0.25-mm ID (d_f = 0.4 μm, 2500 theoretical plates/m), was used for GC only. To minimize GC contaminants, each day was started by a temperature-programmed run with only pentane injected, before the column was connected to the ion source. This was followed by a reference run with synthetic compounds: 2-methyl-3-buten-2-ol, ipsenol (2-methyl-6-methylene-7-octen-4-ol), ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol), *cis*-verbenol (*cis*-4,6,6-trimethylbicyclo[3.1.1]hept-3-en-2-ol), *trans*-verbenol (*trans*-4,6,6-trimethylbicyclo [3.1.1]hept-3-en-2-ol), myrtenol (6,6-dimethylbicyclo[3.1.1]hept-2-ene-2-methanol), *trans*-myrtanol (*trans*-6,6-dimethylbicyclo[3.1.1]heptane-2-methanol), and 2-phenylethanol, in equal amounts (CA names according to Swigar and Silverstein, 1981).

Nine different concentrations of the reference mixture were used, one each day. The concentration series covered the range 0.1–1000 ng/2.5 μl pentane, always including a constant amount of internal standard (60 ng heptyl acetate/2.5 μl pentane). The separation was checked, especially the critical one between ipsdienol and *trans*-verbenol, which is strongly de-

pendent on a sufficient film thickness of the Superox FA column. Ipsdienol and *trans*-verbenol separate with only seven relative retention value units, but baseline separation was achieved up to at least 300 ng for both compounds. The reference run also enabled us to quantify the amounts of volatile components from the hindguts.

By extracting selected ion current profiles (EICP) from continuous scanning operation and registration of the total mass spectrum over the

TABLE 3. COMPOUNDS IDENTIFIED FROM HINDGUTS OF *Ips typographus* MALES FROM NATURAL ATTACKS ON *Picea abies*

Abbreviation	Formula	Compound (MW)	Retention value	Characteristic MS peaks, m/z[a]
MB		2-Methyl-3-buten-2-ol (85)	1033	71 (bp)[b] 43 (80) 59 (35) 31 (20)
Ie		Ipsenol (154)	1545	68 (bp) 69 (65) 67 (35)
cV		*cis*-Verbenol (152)	1667	94 (bp) 59 (85) 109 (70)
Id		Ipsdienol (152)	1685	85 (bp) 41 (35) 67 (11)
tV		*trans*-Verbenol (152)	1692	109 (bp) 94 (70) 59 (40)
Vn		Verbenone (150)	1735	107 (bp) 135 (70) 80 (70) 150 (40)
Mt		Myrtenol (152)	1806	79 (bp) 91 (35) 108 (25)
tM		*trans*-Myrtanol (154)	1878	123 (70) 69 (bp) 81 (65) 93 (50)
PE		2-Phenylethanol (122)	1930	91 (bp) 92 (60) 65 (25)

[a]Percentage of base peak in parentheses.
[b]MS fragments used in quantification.

region m/z 29–300, with the exception of 32, it was possible to detect and quantify the compounds linearly down to 0.1 ng/injection. The selected mass spectra peaks (Table 3) were prominent and characteristic for each compound.

One-third of the content of an ampoule, containing 10–15 μl of extract, was injected (splitless) into the GC-MS. In total, 93 analyses were made (65 male and 28 female extracts (Table 2). In 59 of the analyses (49 extracts of males and 10 of females), another third of the volume was also used on a separate GC in confirmation of the relative retention values and the critical separations.

The GC oven temperature was kept at +60°C for 5 min, then programmed at 5°/min to 200°C and kept isothermal for 30 min. The injector temperature was 200°C. Mass spectra were obtained at 60 eV (maximal sensitivity). Identifications were made by comparing mass spectra and gas chromatographic retention values (in relation to straight-chain alkanes, $N_c \times 100$) with those of reference compounds. In addition, the identity of 2-methyl-3-buten-2-ol was also confirmed through coinjection.

For both GC-MS and GC the accuracy of the retention values was ± 1 unit ($1/N_c \times 100$).

3. RESULTS

3.1 Males

3.1.1 Bark Material Content of Guts. Beetles walking on the trunk (phase 0) contained no fresh host material in their guts, and only 3% of those penetrating the bark (phase 1) had some fresh phloem material. In contrast, 76% of the males beginning to excavate in the phloem (phase 2) had fresh material in the dissected part of their guts.

Of the established males, phases 3 and 4, almost all had fresh material and many, 38 and 75%, respectively, had a well-filled gut (Figure 1).

3.1.2 Chemical Identification. Nine volatile compounds of beetle origin, found in extracts from male guts, have been identified and quantified (Table 3). Representative capillary gas chromatograms from phases 3 and 6 in males are given in Figure 2. Only small amounts of monoterpene hydrocarbons from the host tree were found by EICP monitoring (ions m/z 93 and 68 were used). α-Pinene, β-pinene, myrcene, limonene, and β-phellandrene were detected in almost all extracts. Traces of some sesquiterpene hydrocarbons were found, especially in the later phases. At the nanogram level, some other compounds were identified: α-terpineol, borneol, *cis*-myrtanol, perilla alcohol, and 2-phenylethyl acetate. In the isothermal region of the gas chromatogram (after C_{20}), only straight-chain hydrocarbons, probably of cuticular origin, were found. Trace amounts of short, straight-chain hydro-

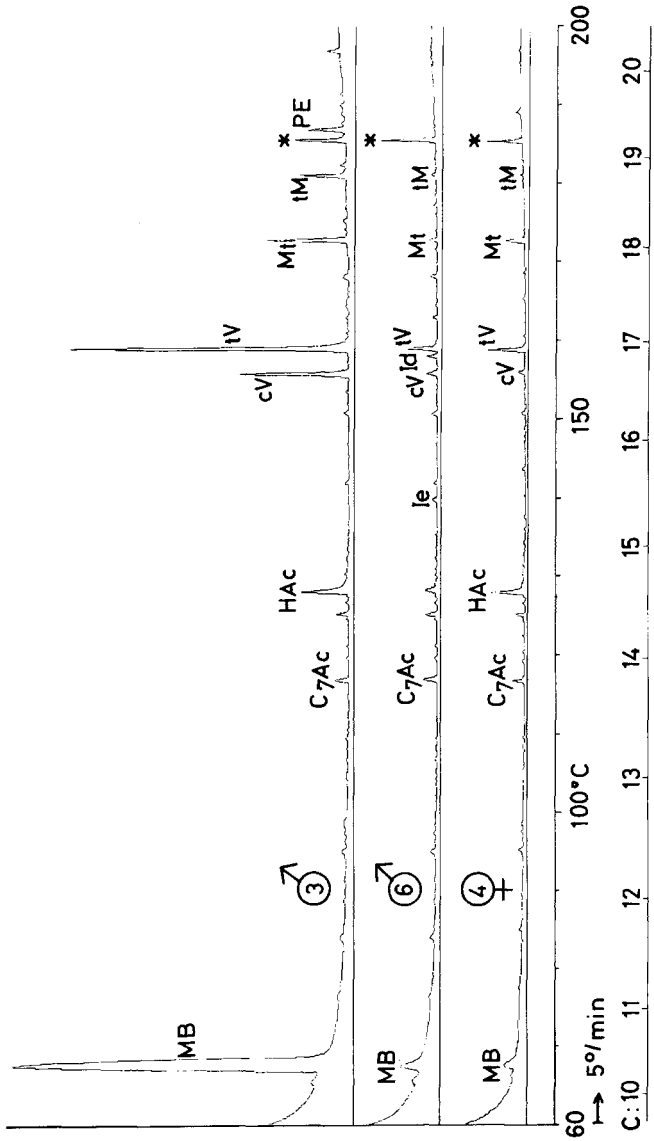


FIG. 2. Total ion chromatograms, m/z 29–31, 33–300, from gut extracts of males from phases 3 and 6 and of females from phase 4. For a description of phases, see Figure 1 and Table 2. The chromatograms of male extracts represent 14 and 12 individuals, respectively. The internal standard, heptyl acetate, corresponds to 60 ng in all chromatograms. For abbreviations of compounds, see Table 3. Asterisks mark a contamination which is a stabilizer of diethyl ether (2,6-di-*tert.*-butyl-4-methyl phenol) probably present in laboratory air. The female chromatogram represents 17 individuals.

carbons were contaminants from the alkane solvents, even in freshly distilled pentane. Acetic acid was present in most extracts. We are inclined to regard this as a contaminant emanating from the addition (5%) of ethyl acetate to the extraction solvent.

One volatile component gave a mass spectrum indicating a monoterpene alcohol of mass 154, by m/z 139 and 136, which corresponds to $(M-15)^+$ and $(M-18)^+$, respectively, and the base peak on m/z 69. One fragment, m/z 123, is particularly distinct compared with other monoterpenes. The compound was identified as *trans*-myrtanol (*trans*-6,6-dimethylbicyclo[3.1.1]heptane-2-methanol) by comparison with the hydrogenation products from myrtenol. Coinjection of the natural material with these, i.e., *cis*- and *trans*-myrtanol, and synthetic *cis*- and *trans*-myrtanol from other sources confirmed the identity. The two isomers separate fully on the column used, and they possess characteristic proportions of the MS fragments m/z 81 and 93. (For *trans*-myrtanol, m/z 81 $>$ 93, whereas for the *cis*-isomer, 93 $>$ 81.)

The only nonisoprenoid compound of those quantified was identified as 2-phenylethanol.

3.1.3 Production of Volatiles by the Beetles During the Different Phases. As shown in the gas chromatogram from phase 3, methylbutenol is clearly the dominating substance in this phase, followed by *trans*- and *cis*-verbenols (Figure 2). The amount of methylbutenol is of the order of 500 ng/hindgut or 6×10^{-9} mol/beetle, compared to *cis*-verbenol, with 40 ng/beetle or 0.3×10^{-9} mol/beetle. 2-Phenylethanol is present in the smallest amount of the components quantified (about 10 ng/beetle). Other compounds quantified in this phase are myrtenol and *trans*-myrtanol. The average amounts per male beetle of these volatiles, given in Figure 3, all maximize during the preparation of the nuptial chamber (phases 2–3). As shown by Figure 3, the amounts of all compounds are low at the start of boring. When the bettles have penetrated the bark and started boring in the phloem, after phase 1, the amounts increase dramatically. Later, they also decrease rapidly and reach the initial amounts when the females have started elongation of their galleries (phases 5–6).

In phase 6, the absolute amounts of the compounds quantified from phase 3 have decreased drastically (Figure 2). Ipsdienol and ipsenol, which were not detected in phase 3, are now present in appreciable amounts (each about 10 ng/beetle). Traces of these compounds appear first in phase 4. During phases 5 and 6, they reach amounts comparable to those of the pinene alcohols in these phases; see Figure 4B.

Methylbutenol stands out in the blend, not only because of the large amounts but also because of its very sharp decline after phase 4, reaching the same level as the other compounds in the last phase (Figure 3).

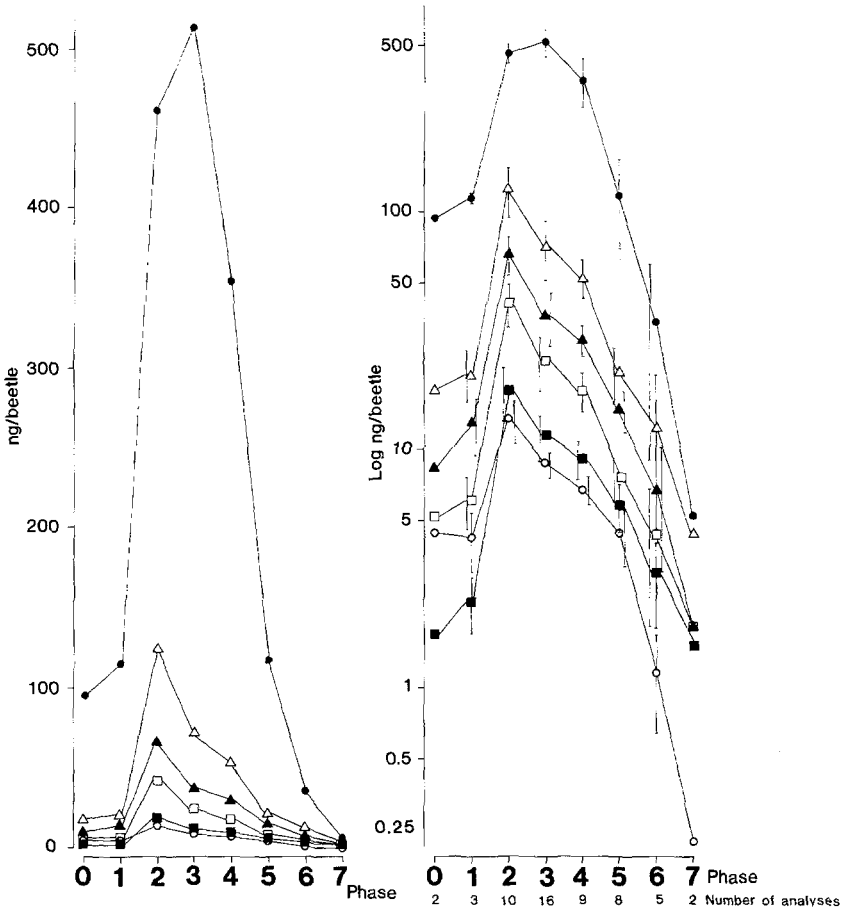


FIG. 3. Mean content of components in males from different phases. (●) Methylbutenol; (△) *trans*-verbenol; (▲) *cis*-verbenol; (□) myrtenol; (■) *trans*-myrtanol; (○) 2-phenylethanol. Brackets represent ±SE.

A second group of substances is *trans*-verbenol, *cis*-verbenol, verbenone, myrtenol, *trans*-myrtanol, and 2-phenylethanol. They are present in amounts from 2 to 25% of that of methylbutenol in phases 2 to 4. All these monoterpene alcohols show the same pattern, with a maximum amount in phase 2 followed by a steady decline. Compared with the pinene alcohols, 2-phenylethanol is relatively larger when the males are landing on the tree and start their boring activity. After reaching the maximum, 2-phenylethanol declines along a convex curve. Verbenone has a distribution curve similar to that of the pinene alcohols but with an average maximum not exceeding 1 ng/beetle in phases 3–4 (Figure 4A). The mean ratio of *trans*-verbenol to *cis*-

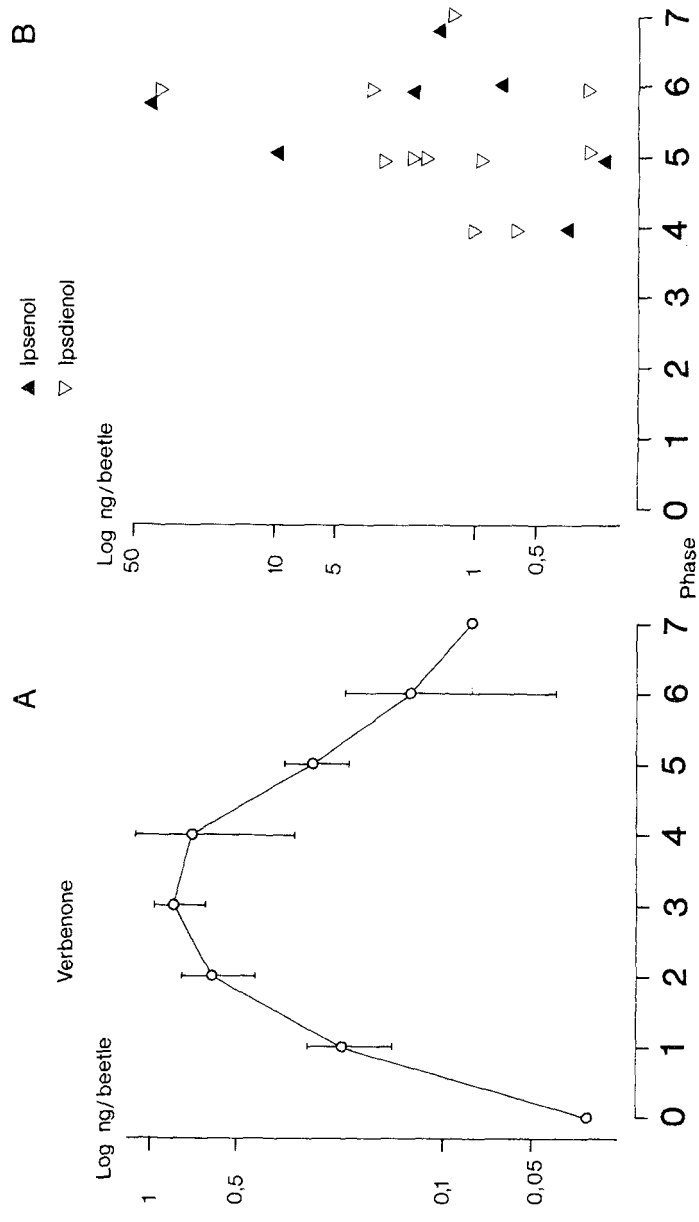


FIG. 4. (A) Mean content of verbenone in male guts ±SE. (B) Content of ipsenol and ipsdienol in extracts from male guts. The figure shows all extracts where either substance has been detected. Zero values are thus not plotted.

verbenol was rather constant in phases 1 to 4, with a mean of 1.60 ± 0.05 ($\bar{X} \pm$ SE). The ratio of methylbutenol to *cis*-verbenol in phases 1 to 4 was more variable, with a mean of 9.18 ± 1.32.

Ipsenol and ipsdienol form a third group of substances. Neither of them can be detected when the other compounds are at a maximum. However, in phases 4–6 at least ipsdienol was found in 11 of 24 extracts. Thus they were identified in only about half of the samples from the later phases, in spite of a detection limit of 0.03 ng/beetle. When present, they were found in amounts from about 0.5 to 40 ng/beetle. The correlation between ipsdienol and ipsenol was very high, $r = 0.975$ ($P < 0.1\%$, $N = 11$), with a mean ratio of 1:0.9, respectively. Both of them seem to increase in the later phases (Figure 4B), although their variations are too large to show any clear pattern.

Males with only one female, analyzed from phases 4 and 5 in trees D and F, did not differ in pheromone content from males with two females in the same phases. Beetles found in old stumps contained only trace amounts (less than 0.5 ng/beetle) of the normally largest components.

3.1.4 Pheromone Components Produced in Relation to Monoterpenes in the Trees. The amount of visible resin flow caused by the boring males was estimated in the field. It showed a considerable variation among different trees and logs (Table 1). The host-plant monoterpene hydrocarbons were quantified in the GC-MS analyses of male hindguts from phases 2–4.

When the amounts of the monoterpenes are summed for each tree and the trees are compared, they fall into three groups (Table 4). Beetles from tree G, which had an unusually rich resin flow and was not killed by the beetles, have 5 to 10 times higher amounts of the monoterpene hydrocarbons (except myrcene) in their gut than beetles from any other tree. Trees A, B, and F form a second and homogeneous group, which we believe to represent "normal" trees. Tree D and the two logs, which had virtually no resin flow, compose a third group having only about 60% of the amount of the terpenes found in normal trees. The proportions of the monoterpene hydrocarbons among the different trees and logs varied, however, without any obvious pattern (Table 4). It may also be noticed that the amount of resin flow visible is not aways directly related to the monoterpene content of the hindgut. This is shown by tree B, which had a poor resin flow but the highest terpene content of the normal trees.

The pheromone component content in male beetles from different trees is also shown in Table 4. The distribution of the group 2 alcohols in the trees and logs corresponded to the same three groups formed by the sum of the host monoterpenes. Thus, beetles from tree G produced the highest amounts of the pinene alcohols; the normal trees A, B, and F, intermediate amounts; and tree D and the two logs, the lowest amounts. The proportions of all the alcohols in beetles from the normal trees A, B, and F are also similar. The

TABLE 4. HOST-PLANT MONOTERPENE HYDROCARBONS AND BEETLE-PRODUCED ALCOHOLS FOUND IN MALE HINDGUTS FROM PHASES 2, 3, AND 4 FROM DIFFERENT TREES AND LOGS

	Tree					Log	
	G[a]	B	F	A	D	C[b]	H[b]
Hydrocarbon[c]							
α-Pinene (93)	38.6	4.7	4.2	3.6	0.9	1.1	0.9
β-Pinene (93)	13.2	3.1	0.7	2.8	1.7	0.4	0.3
Myrcene (93)	4.6	2.4	6.1	3.2	3.0	6.3	5.4
Limonene (68)	1.8	1.0	0.7	0.3	0.3	0.8	1.1
β-Phellandrene (93)	2.7	1.2	0.2	1.1	0.6	0.2	0.2
	60.9	12.4	11.8	11.0	6.4	8.8	7.9
Alcohol[d]							
MB group 1	200	579	407	672	178	600	686
cV group 2	97.4	65.4	51.0	44.2	29.7	18.8	11.1
tV group 2	215	137	134	95.2	11.0	13.3	14.1
Mt group 2	74.8	38.7	40.4	31.9	9.4	6.7	4.9
tM group 2	19.1	23.3	7.6	18.5	12.3	2.9	2.1
PE group 2	14.7	11.3	9.8	12.2	6.7	7.4	9.4
Sum, group 2	421	276	243	202	69.1	49.1	41.6
Id[e] group 3		1.0	0.2	0	0	0	0.6

[a]Data available only from phases 2 and 3.
[b]Data available only from phases 3 and 4.
[c]Amounts are given as nanograms per male, quantified on the MS base peaks (shown within parentheses) of the hydrocarbons.
[d]For abbreviations of alcohols, see Table 3.
[e]Data presented only from phase 4, Ipsenol was identified only in later phases.

rather constant proportions may be of biosynthetic and/or behavioral significance.

In order to quantify whether the host monoterpene variation is a source of variation in pheromone content, the amounts of eight pheromone components have been tested for linear correlation with the five major monoterpene hydrocarbons using the SPSS program SCATTERGRAM (Nie et al., 1975). Of these 40 correlations, 20 were significant ($P < 2\%$ or less) and are listed in Table 5, while the other correlations were not significant ($P > 20\%$). No correlations had P values between 2 and 20%.

The variation in methylbutenol, ipsenol, and ipsdienol could not be explained by host variation as none of these was significantly correlated to any monoterpene. However, methylbutenol was negatively, but insignificantly,

TABLE 5. CORRELATION BETWEEN HOST MONOTERPENE HYDROCARBONS AND BEETLE-PRODUCED PHEROMONE COMPONENT ALCOHOLS AS QUANTIFIED FROM HINDGUTS

Y	log X	r	r^2	$P(\%)^a$
MB[b]	None significantly correlated			
Ie	None significantly correlated			
cV	αP[c]	0.899	0.808	<<1
	βP	0.827	0.684	<<1
	βPh	0.797	0.635	<<1
	L	0.644	0.415	<1
Id	None significantly correlated			
tV	αP	0.930	0.865	<<1
	βP	0.746	0.557	<<1
	βPh	0.701	0.491	<<1
	L	0.611	0.373	<1
Mt	αP	0.950	0.903	<<1
	βP	0.799	0.638	<<1
	βPh	0.756	0.572	<<1
	L	0.620	0.384	<1
tM	βPh	0.820	0.672	<<1
	βP	0.776	0.602	<<1
	αp	0.598	0.358	<1
	M	−0.584	0.341	<2
PE	αP	0.728	0.530	<<1
	βPh	0.673	0.453	<1
	βP	0.630	0.397	<1
	L	0.546	0.298	<2

[a]Test of significance of the correlation coefficient in a two-sided test by SPSS subprogram SCATTERGRAM ($N = 18$).
[b]For abbreviations of alcohols, see Table 3.
[c]αP, α-pinene; βP, β-pinene; βPh, β-phellandrene; L, limonene; M, myrcene.

correlated with four terpenes (r between −0.39 and −0.01). Between 39 and 53% of the variation in 2-phenylethanol was explained by α-pinene, β-phellandrene, or β-pinene. In contrast, over 80% of the variation in *cis*-verbenol, *trans*-verbenol, and myrtenol was explained by α-pinene. Also β-pinene or β-phellandrene explained 50% or more of the variation in *cis*-verbenol, *trans*-verbenol, and myrtenol, as well as more than 60% in *trans*-myrtanol. Limonene variation explained less than 50% of the variation in any alcohol.

3.1.5 Individual Variation in the Pheromone Content of Male Beetles. Individual variation in pheromone content and dry weight was studied in 36 males in phase 3 from tree D (Figures 2 and 5). The frequency distributions were analyzed using the subprogram FREQUENCIES of the SPSS (Nie et al., 1975). The dry weights of the analyzed beetles had a normal dis-

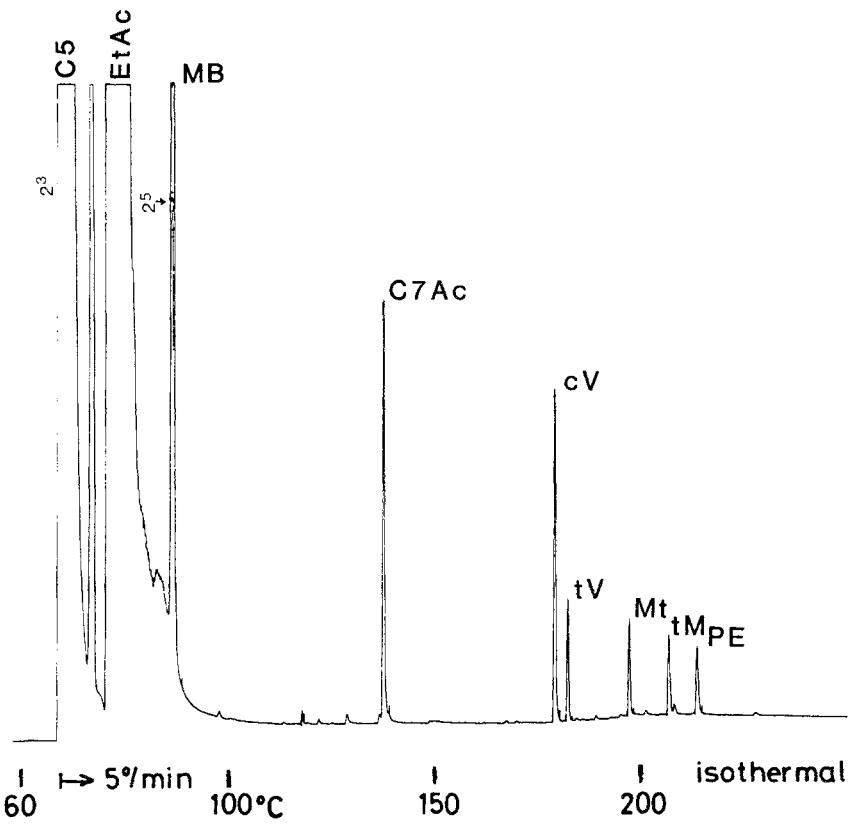


FIG. 5. Gas chromatogram of one-third of an extract of single male gut in phase 3 from tree D. The internal standard, heptylacetate (C7Ac), was 60 ng. The content of methylbutenol was 430 ng, and *cis*-verbenol was 51 ng in this beetle, as quantified from GC-MS analysis of the same extract. This is a chromatogram of a strong extract, but the six major compounds in phase 3 (Figure 2) could always be quantified by GC-MS and usually also by GC alone from individual beetles. For abbreviations of alcohols, see Table 3.

tribution (skewness = 0.299, $P > 10\%$; kurtosis = 0.541, $P > 10\%$, Sokal and Rohlf, 1969, p. 112), with a coefficient of variation (CV = SD/mean) of 17% (Figure 6).

The amounts of pheromone components had nonnormal distributions, with high variances and with many individuals having a low content of both methylbutenol and pinene alcohols (Figure 6). The frequency distribution of methylbutenol had a slight skewness ($P > 10\%$) and was platycurtic (less "peaked" than a normal distribution; $P < 10\%$), with a CV of 85%. The distribution of *cis*-verbenol was highly skewed ($P < 0.1\%$) and leptokurtic

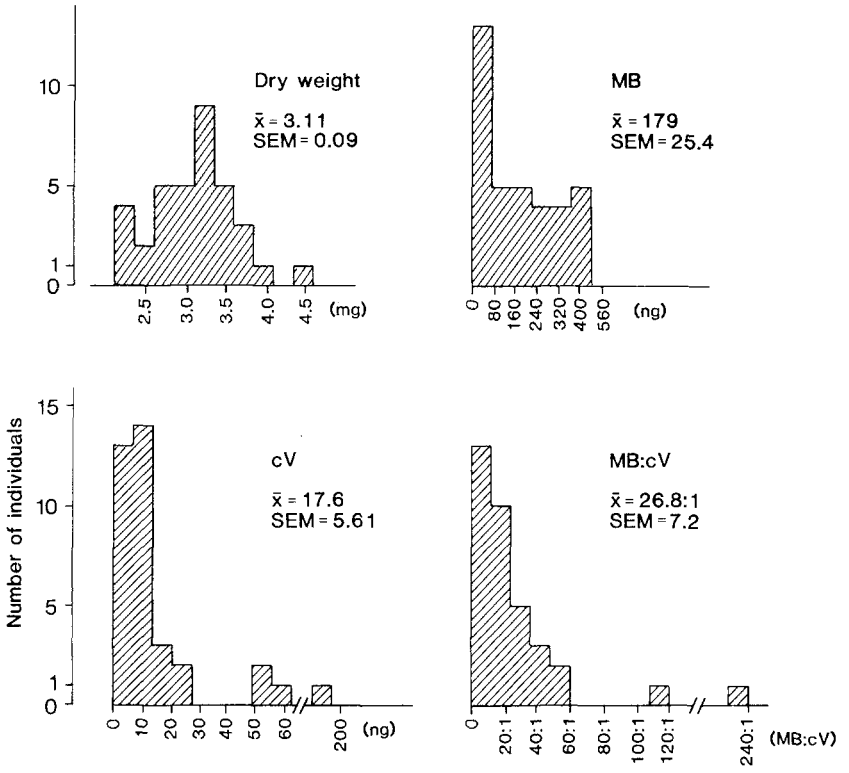


FIG. 6. Frequency distributions of dry weight, methylbutenol content, *cis*-verbenol content, and ratio of methylbutenol to *cis*-verbenol from the individual analysis of 36 males from phase 3, tree D. Class widths are proportional to the standard deviation of each parameter.

(more peaked than normal; $P < 0.1\%$), with a CV as high as 191%. However, a logarithmic transformation showed that the distribution was very close to a log-normal (skewness and kurtosis less than 0.2, $P >> 10\%$, CV = 61%).

The ratio between methylbutenol and *cis*-verbenol (Figure 6) was highly skewed ($P < 0.1\%$) and leptokurtic ($P < 0.1\%$), with a high variance (CV = 159%). The distribution of the methylbutenol:*cis*-verbenol conforms approximately to a log-series. The 2-phenylethanol:*cis*-verbenol ratio showed the same pattern, with a CV of 104%. In contrast, the ratio between the sum of *trans*-verbenol, myrtenol, and *trans*-myrtanol and the amount of *cis*-verbenol was normally distributed (skewness and kurtosis < 0.3, $P >> 10\%$), with a mean of 1.37:1 and a CV of only 16%.

The dry weight showed no significant correlation to either methylbutenol ($r = -0.3$) or *cis*-verbenol ($r = 0.0$).

3.2 Females

Females walking on the trunk (phase 0) contained only trace amounts of methylbutenol (3 ng/beetle), *trans*-verbenol (0.9 ng/beetle), and myrtenol (0.3 ng/beetle). Females in phases 4 to 7 all had fresh material throughout their hindguts.

The gas chromatogram of females in phase 4 (Figure 2) represents the maximal amounts of volatiles present in the gut. Methylbutenol, *cis*- and *trans*-verbenol, myrtenol, *trans*-myrtanol, and α-terpineol were identified and quantified. *trans*-Verbenol was the largest, with an average of 12 ng/female. Methylbutenol reaches an amount of only about 9 ng. Thus, of methylbutenol and *cis*-verbenol, the females contained only 3 and 7%, respectively, of the amounts present in males of the same phase (Figure 3). Furthermore, neither ipsenol, ipsdienol, nor 2-phenylethanol was detected in any female extract.

The ratio of *trans*-verbenol to *cis*-verbenol was much higher than in males. It was 3.5:1 in phase 4 and increased to 4.6:1 and 16.9:1 in phases 5 and 6, respectively, compared to 1.6:1 in males.

Small amounts (less than 1 ng/beetle) of a female-specific compound were found in beetles from phase 4 and later. It had a retention value (Superox FA column) of 1425. The mass spectrum shows characteristic peaks at m/z 96 (base peak), 81, 123, and 138 (molecular ion). The substance was tentatively identified as β-isophorone (3,5,6-trimethyl-3-cyclohexene-1-one) by its mass spectrum. The identity was confirmed by coinjecting female extracts with synthetic β-isophorone, isomerized from α-isophorone (3,5,5-trimethyl-2-cyclohexene-1-one) according to Widmer (1977).

4. DISCUSSION

4.1 Pheromone Compounds and Behavior During Attack Phases

Our quantifications clearly show a maximum of volatiles present in guts when the male is preparing his nuptial chamber, before accepting females (phases 2 and 3), but also rather large amounts in the first phases. A decline in pheromone attraction after mating has been shown in the field for other *Ips* species (Byers, 1981a; Švihra, 1982) and in the lab for *I. typographus* (Schlyter and Löfqvist, unpublished). Byers (1981a) also showed in the field that ipsenol and ipsdienol in *I. paraconfusus* declined after mating.

The early production of pheromone components found in our study, one-fifth of the maximum value before even entering the phloem, is in contrast to the findings in *Ips paraconfusus*. In this species frass is not attractive until several hours of boring (Wood and Bushing, 1963; Bordon, 1967), and hindguts do not contain pheromone components until exposed to terpenes or after sustained feeding (Byers et al., 1979; Byers, 1981b). It should be

noticed that the males of *I. typographus* containing these appreciable amounts of pheromone had not fed, as shown by their empty hindguts. It is, however, congruent with laboratory studies of *I. typographus* showing their attraction to logs after only 0.5–2.5 hr of male infestation (Schlyter and Löfqvist, unpublished) and the production of methylbutenol without feeding but enhanced by the application of juvenile hormone (Hackstein and Vité, 1978). Indeed, the aggregation pheromone in *I. typographus* may act more like a "contact pheromone" as in *Dendroctonus* than as a "frass pheromone" as in most *Ips* (Vité et al., 1972). This would agree well with the aggressive, tree-killing potential of *I. typographus* compared to most *Ips* species (Postner 1974).

Our analyses of the potential pheromone components from the hindgut of male *I. typographus* clearly show that the compounds fall into three groups: methylbutenol, the pinene alcohols, and ipsdienol–ipsenol.

Since the pioneer work by Silverstein and Wood on pheromones in *Ips paraconfusus* (cf. Wood 1970), both the biology and the chemistry of pheromones from at least 12 *Ips* species from both North America and Europe have been studied (cf. reviews by Renwick and Vité, 1981; Wood, 1982). Ipsdienol, ipsenol, and *cis*-verbenol, which were the monoterpene alcohols first identified by Silverstein et al. (1966), together with *trans*-verbenol have been shown to be common in the genus. Some species reportedly use mainly one substance; others, two or more in a blend. An isoprene alcohol has, however, been found in only two species: 2-methyl-3-buten-2-ol in males of *I. typographus* (Bakke et al., 1977) and 3-methyl-3-buten-1-ol in males of *I. cembrae* (Stoakley et al., 1978). Both these closely related European species differ from most of their congeners by attacking healthy standing trees during epidemics (Postner (1974). Their relatives are often less aggressive, colonizing mainly decaying trees. At least in *I. typographus*, the isoprene alcohol is known to be an essential compound in the attractive lure (Bakke et al., 1977). Dickens (1981) points out that 2-methyl-3-buten-2-ol is probably a short-range orientation and/or arrestment substance. This assumption was based on the very high response threshold and narrow response width to saturation in EAG measurements. The adaptive value of a short-range orientation substance for an aggressive species attacking healthy trees is clear. Such a substance can orient and concentrate the beetles to a tree in the critical initial aggregation phase, when the pioneering males have to fight a heavy resin flow from a resistant tree.

The second group of potential pheromone compounds consists of *trans*-verbenol, *cis*-verbenol, myrtenol, *trans*-myrtanol, and 2-phenylethanol. All of them are present in the highest amounts in phase 2, when the male excavates the nuptial chamber. It should be noted that these substances, including *cis*-myrtanol and verbenone, with the exception of 2-phenylethanol, are all derived from a pinane skeleton.

trans-Verbenol and *cis*-verbenol were identified from males of *I. typographus* by Vité et al. (1972), but the rest of the substances were unknown from this species.

trans-Myrtanol has, to our knowledge, not been identified from any bark beetle earlier, while the isomer *cis*-myrtanol has been identified from *D. frontalis* (Silverstein and West in Thatcher et al., 1981).

(4S)-*cis*-Verbenol combined with methylbutenol has been shown to attract males and females of *I. typographus* into traps (Bakke et al., 1977; Krawielitzki et al., 1977).

The almost parallel increase and decrease in the amounts of *trans*-verbenol, *cis*-verbenol, myrtenol, *trans*-myrtanol, and *cis*-myrtanol in the gut of male *I. typographus* in the various phases indicate that they originate in the same biochemical pathway (White et al., 1980).

Verbenone is a common pheromone compound in *Dendroctonus*- species (Wood, 1982). It has, in *Ips*, been identified only from males of *I. confusus* (sensu Lanier et al., 1973), *I. pini* (Lanier et al., 1980), and *I. amitinus* (Francke et al., 1980). Although present in small amounts, only 1% of the *cis*-verbenol content, verbenone differs somewhat from the pinene alcohol group of potential pheromone substances by showing a peak amount one phase later and by decreasing in amount more slowly. It is produced in many *Dendroctonus* species and has a termination effect on the attraction (cf. Wood, 1982). Verbenone also inhibits the response of *I. paraconfusus* to its natural pheromone (Byers and Wood, 1980, 1981). When presented in the same amounts as *cis*-verbenol, both enantiomers of verbenone reduced significantly the attraction of *I. typographus* to traps baited with methylbutenol, (4S)-*cis*-verbenol, and ipsdienol (Bakke, 1981).

Ipsdienol and ipsenol form the third group of potential pheromone substances. They are characteristic of the genus (cf. Wood, 1982) and were identified early from *I. typographus* (Vité et al., 1972). Ipsdienol in *I. typographus* is produced as (R)-ipsdienol (Francke et al., 1980). Both substances in this species are produced by the males first when the females have entered the nuptial chamber and mainly when they have started egg laying. Bakke et al. (1977) found that racemic ipsdienol increased the trap catch about 50% when combined with methylbutenol and *cis*-verbenol. Dickens (1981) was not able to verify any synergistic attraction effect to sleeve olfactometers by either the (S)- or the (R)-enantiomer of ipsdienol or both together with *cis*-verbenol and methylbutenol, compared with such baited with only *cis*-verbenol and methylbutenol.

Ipsenol, presented in the same amount as ipsdienol, significantly reduced the number of beetles attracted to traps baited with methylbutenol, (4S)-*cis*-verbenol, and ipsdienol and even more so when combined with verbenone (Bakke, 1981).

The production of ipsdienol and ipsenol by the males when the females

have entered the nuptial chamber indicates that neither of these substances is active in the initial aggregation.

4.2 Pheromone Biosynthesis

Many bark beetle pheromone compounds are oxygenation products of resin monoterpene hydrocarbons from the host tree. Quantification of the major host monoterpenes from the hindgut of the bark beetles may therefore elucidate the biosynthesis of the potential pheromone compounds and also account for the large variation in pheromone content in beetles from different trees. The high number of significant correlations rules out random effects (Nie et al., 1975, p. 3), and the negative correlation between the most volatile alcohol, methylbutenol, and the volatile monoterpenes makes evaporative losses during sample preparation as causes of correlations less likely.

In the laboratory several authors have shown that bark beetles convert α-pinene to *cis*-verbenol, *trans*-verbenol, and myrtenol (Vité et al., 1972; Hughes, 1973, 1975; Renwick et al., 1976a; Klimetzek and Francke, 1980; Byers, 1981b). It has also been shown that the enantiomers of α-pinene cause the selective production of *cis*-verbenol from (−)-α-pinene and *trans*-verbenol from (+)-α-pinene in the laboratory by *I. paraconfusus* (Renwick et al., 1976a) and *I. typographus* (Klimetzek and Francke, 1980), while myrtenol is always produced. As the ratios of (+)- and (−)-α-pinene probably varied among our sampled trees, it is not surprising that myrtenol showed the best correlation to racemic α-pinene ($r = 0.950$), as it is produced from both enantiomers. Indeed, the sum of *cis*- and *trans*-verbenol was very well correlated to α-pinene ($r = 0.933$), second only to myrtenol.

The good correlations between β-pinene, β-phellandrene, or limonene and the pinene alcohols may be an effect of multicollinearity (Nie et al., 1975, p. 340), i.e., the independent variables (monoterpenes) are highly intercorrelated and the effects of the major monoterpene α-pinene might produce all the correlations observed. However, the minor terpenes may also be substitute precursors, and in the case of *trans*-myrtanol, α-pinene is only the third-best correlate.

It has been found that biosynthesis of a bark beetle pheromone compound, or more accurately the amount in which it is present in the hindgut, not only depends on the presence of a specific amount of a host resin compound, but also is influenced by the hormonal system in the bark beetle, especially by juvenile hormone (cf. Renwick and Vité, 1981). It is now interesting to note that beetles from tree D, showing small amounts of resin compounds, also produce small amounts of both methylbutenol and the tree-related verbenols, which could be explained as a hormone-controlled weak signal from beetles which do not need any help to overcome a resin flow, resulting in a weak attraction of further beetles to that tree.

The well-documented biochemical pathway from myrcene to ipsdienol and ipsenol (Hughes, 1974; Byers et al., 1979; Fish et al., 1979; Hendry et al., 1980; Byers, 1982) does not show up in the correlations. This is in agreement with the fact that ipsdienol in this species, in contrast to other investigated *Ips* species (Hughes, 1974; Vité et al., 1974; Renwick and Dickens, 1979), is not produced after exposure to myrcene in the laboratory (Bakke, 1976; our own unpublished results). Nor is ipsdienol produced in the initial aggregation phases, which indicates a different behavioral role and maybe also a hormonal control of its production in this species.

4.3 Individual Variation

The amounts of the potential pheromone substances quantified in 36 males from phase 3 showed a high individual variation in the amounts of the substances and skewed distributions. This large variation could be an experimental artifact or reflect a true biological pattern. Quantification of synthetic substances (cf. Methods) clearly showed that the quantification method used was adequate. Also, the dry weights had a normal distribution and a small variance. A single sample from a resin-poor tree (D) and many other factors could, however, be regarded as the reason for the observed distributions and their skewness. But comprehensive studies of corresponding field material collected in 1982 have shown the same magnitude of variation (Schlyter et al., unpublished). There is still, however, a possibility that the high variation and the skewness of the sample could depend on our sampling technique. The limits we have set up for phase 3 could be too wide for maximum pheromone production. There could also be a diurnal rhythm in pheromone production as shown for the bark beetle *Polygraphus poligraphus* (Lenze in Renwick and Vité, 1981), which could partly explain our results, because we collected the beetles from the morning to the late evening.

The log normal frequency distributions of the ratios of methylbutenol: *cis*-verbenol and of 2-phenylethanol:*cis*-verbenol show two pairs of variables with independent variation (Southwood, 1978, p. 423). This indicates that the biosyntheses of methylbutenol and 2-phenylethanol are quite separate from that of *cis*-verbenol.

In a comprehensive study of individual pheromone content from females of *Argyrotaenia velutinana* (Lepidoptera: Tortricidae), Miller and Roelofs (1980) showed a smaller magnitude of variation. The coefficient of variation (CV) for Δ-11-tetradecenyl acetate was 58% in their study, while it was 191% for *cis*-verbenol in our study. It is likely that female moths have a smaller variation because they are not dependent on host colonization and host compounds of pheromone production. They also showed that the proportion of the E isomer of 11-tetradecenyl acetate had a small variance (CV = 9.7%). This should be compared to our data on the proportion of *cis*-verbenol of

the total pinene alcohols, which also had a small variance (CV = 16%). In both cases the smaller variance in proportions could be an effect of small differences in biosynthesis from common precursors (insect and plant derived, respectively).

4.4 Females

The presence in the females of all the major substances produced by males in the first phases is somewhat surprising, as females have not been found to cause attraction in the genus *Ips* (Wood, 1982). It should, however, be noted that only *trans*-verbenol, myrtenol, and *trans*-myrtanol are found in more than 10% of the amount present in males. Methylbutenol and *cis*-verbenol were also positively identified in females from this field study, but in very small amounts. However, neither substance could be identified from females exposed to racemic α-pinene separately from males in the laboratory, while *trans*-verbenol and myrtenol were always produced (our own unpublished data). One possible explanation is that the amounts of methylbutenol and *cis*-verbenol found in the female are the result of a contamination in the gallery from the high-producing male.

The chain of behavioral steps (Borden, 1974) and the role of different substances in releasing them is not known for any bark beetle. We think that a phenomenon as complex as the colonization and killing of a large tree by thousands of bark beetles can never be understood before the whole chain of behaviors and the releasers of its various steps have been described.

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PHEROMONE BIOSYNTHETIC PATHWAYS: Conversion of Ipsdienone to (−)-Ipsdienol, a Mechanism for Enantioselective Reduction in the Male Bark Beetle, *Ips paraconfusus*

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Abstract—The enantiomeric composition of the pheromone components (+)-ipsdienol, e.e. 87.6%, and (−)-ipsenol, e.e. 93.8%, produced by the male bark beetle *Ips paraconfusus* (Scolytidae) under natural conditions was determined by HPLC separation of their diastereomeric ester derivatives. Males confined in an atmosphere of ipsdienone produced (−)-ipsdienol, e.e. 28%, and (−)-ipsenol, e.e. 86%, indicating an enantiomeric selectivity in the conversion of the ketone to the alcohols. These findings demonstrate an enantioselective conversion mechanism in the biosynthetic pathway to the pheromones from myrcene, a host-plant terpene.

Key Words—*Ips paraconfusus*, bark beetle, Coleoptera, Scolytidae, ipsdienone, ipsenol, ipsdienol, enantiomers, diastereomers.

INTRODUCTION

The aggregation pheromone of the bark beetle *Ips paraconfusus* Lanier (Coleoptera: Scolytidae) [then included under *I. confusus* (Le Conte)] was reported by Silverstein et al. (1966a) to be a combination of three terpene alcohols isolated from frass [(S)-(−)-2-methyl-6-methylene-7-octen-4-ol (ipsenol), (S)-(+)-2-methyl-6-methylene-2,7-octadien-4-ol (ipsdienol), and (S)-(+)-*cis*-verbenol]. The enantiomeric excess (e.e.) of these three compounds is of special interest as Birch et al. (1980) reported pure (−)-ipsdienol as the aggregating pheromone for a competitive species, *I. pini*, in California.

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However, males of *I. pini* in New York populations produced a 65:35 blend of (+),(−)-ipsdienol.

Hughes (1974) and Hughes and Renwick (1977) found that male *I. paraconfusus* produced ipsenol and ipsdienol in the hindgut when exposed to the vapors of myrcene, a terpene component of the host tree. Byers et al. (1979) reported a direct quantitative relation between the myrcene vapor concentration and the amount of ipsdienol and ipsenol produced by the male.

Hendry et al. (1980), using D_2-labeled myrcene, demonstrated that the beetle converted the terpene almost directly into the pheromone compounds. Fish et al. (1979) exposed beetles to racemic ipsdienol, 64% of which was labeled with deuterium at the carbon atom bearing the hydroxy group. Subsequently, they recovered ipsdienol and ipsenol from the exposed males that was 59 and 25% deuterium labeled, respectivley. They postulated that ipsdienone was being formed oxidatively causing the deuterium loss. They also showed that the male converted (−)-ipsdienol to ipsenol in much greater quantities than with the (+) enantiomer and, in addition, could convert ipsdienone to both ipsdienol and ipsenol. Thus, the pathway for the conversion of myrcene to ipsdienol and ipsenol and the subsequent interconversion of (−)-ipsdienol and ipsdienone, as an enantiomerically selective process, have been indicated by these studies. However, the enantiomeric selectivity of the conversion of ipsdienone to ipsdienol is unknown.

In this paper, we wish to verify the enantioselectivity of this ketone reduction mechanism by directly measuring the relative amounts of each enantiomer of the two pheromone alcohols produced by the male bark beetle when exposed to ipsdienone vapors. For comparative purposes, we also measured the enantiomer ratios of the two pheromone alcohols isolated from the atmosphere directly above beetles boring into host logs. While these ratios have been reported (Plummer et al., 1976) for ipsdienol from *I. paraconfusus* males boring dust as (+) 80% e.e., determined by chiral shift reagents and NMR spectroscopy, we believe that such a technique may lack the desired accuracy and should be corroborated by an alternate method. Silverstein et al. (1966b) reported a rotational value of $[\alpha]_D^{20} = -17.5°$ for ipsenol isolated from *I. paraconfusus* males boring dust, but no optical purity was assigned.

METHODS AND MATERIALS

Synthesis of Ipsdienone

Pyridinium chlorochromate (2.9 g ≅ 13.4 mmol) and sodium acetate (2.28 mg ≅ 2.7 mmol) were dissolved in 10 ml of methylene chloride at room temperature and stirred for 15 min. Racemic ipsdienol (1.0 g ≅ 6.6 mmol) was added directly into the mixture, which immediately formed a black

solution. This material was stirred for an additional 15 min. Diethyl ether (75 ml) was added, then stirred, and the solution was filtered through a short Florisil column. The ether was then removed with a rotary evaporator to yield 0.6 g (61%) of crude ipsdienone. GC analysis using a 2-mm-ID × 3-m glass column, 4% Carbowax 20M on Chromosorb G, 60/80 mesh, indicated the crude as 90% ipsdienone with no detectable ipsdienol. The ipsdienone was purified by preparative GC, first with a Carbowax 20M (coated as above) column [10 mm × 4.5-m glass column], then with a 3% Apiezon L on Gas Chrom Q, 100–120 mesh [2 mm × 2-m glass column]. GC-CIMS (isobutane) analysis of the purified material yielded the pseudomolecular M+1 ion m/z 151 (100% base peak) and the NMR spectrum was consistent with that reported by Silverstein et al. (1966b).

Synthesis of Ipsenone

Ipsenone was prepared from 0.5 g of racemic ipsenol by oxidation with 1.04 g of pyridinium chlorochromate and 75 mg of anhydrous sodium acetate in 20 ml of methylene chloride as described for ipsdienone. However, as ipsenol appeared to react more slowly, the mixture was stirred for 15 hr at room temperature prior to purification. A GC analysis showed that the mixture contained approximately 30% of the starting ipsenol. The ketone was isolated by preparative GC (Carbowax 20M) and gave the following spectroscopic data:

^{1}H NMR (90 MHz, $CDCl_3$, TMS) 6.42 ppm (quartet), $\underline{H}\rangle\langle\begin{matrix}\underline{H}\\H\end{matrix}$,

(J_{cis} = 11 Hz; J_{trans} = 18 Hz); 5.14 ppm (doublet), $\underline{H}\rangle\langle\begin{matrix}H\\H\end{matrix}$,

(J_{trans} = 18 Hz); 5.07 ppm (doublet), $H\rangle\langle\begin{matrix}H\\\underline{H}\end{matrix}$,

(J_{cis} = 11 Hz); 5.11–5.16 $\underline{H}\rangle\langle H$; 3.26 ppm (singlet), $C{=}C(-\underline{C}H_2-C{=}O)$;

2.30 ppm (multiplet), $C(=O)-\underline{C}H_2-CH$;

2.13 ppm (multiplet), $-CH\langle$;

0.93 ppm and 0.88 ppm $CH\langle\begin{matrix}\underline{C}H_3\\\underline{C}H_3\end{matrix}$ in the correct ratios.

The chemical ionization mass spectrum (isobutane) showed an ion at m/z 153 (M + 1), 100% relative abundance, with no other fragments present.

Exposure of Beetles to Precursors and Collection of Natural Pheromones

Ipsdienone Precursor. Two batches (150 and 200 each) of male *I. paraconfusus* were exposed to ipsdienone vapors in 400-ml dark glass bottles for 40 hr (Fish et al., 1979; Byers, et al., 1979). These beetles were reared from ponderosa pine, *Pinus ponderosa* Lawson, slash collected in Madera County, California. Silane-treated glass wool was used as a substrate in the bottles to reduce injury caused by crowded beetles biting each other. Ipsdienone was allowed to evaporate into the bottle head space from 20 μl of liquid spotted on a small piece of glass filter paper. After exposure, the abdomens were removed from the beetles of both batches, combined, and crushed in CS_2. The liquid phase was filtered through a short Florisil column. The solid residue was then washed twice with diethyl ether, and each wash was poured through the filter and combined with the CS_2. The combined solution was concentrated under a stream of nitrogen. The ipsdienol and ipsenol in the concentrate were isolated using a 10 mm × 4.5-m glass preparative GC column (Carbowax 20M).

Ipsenone and Ipsenol Precursors. Five individuals of a given sex were enclosed in a flame-sealed 20-ml glass ampoule along with glass wool and 1 ml of precursor on glass paper as before and exposed for 24 hr in atmospheres containing either ipsenone or ipsenol. The workup was similar to that described above for analysis of products.

Natural Ipsenol and Ipsdienol. The enantiomeric composition of naturally produced ipsenol and ipsdienol was determined from volatiles produced over a 48-hr period by 50 male beetles as they bored into a fresh-cut ponderosa pine. Such an infested bolt is highly attractive to both sexes when presented to wild populations and is believed to represent the initial host colonization phenomena (Wood et al., 1966). Beetles were placed into preformed holes in the bark 10 cm apart.

After the beetles began excavation of their nuptial chambers, the bolt was wrapped near each end with a 5 × 5-cm polyurethane foam strip of sufficient length to form rings. Then a length of transparent stiff plastic sheet somewhat longer than the bolt was wrapped around the bolt, forming a cylinder held away from the bolt surface by the foam rings at each end. The porous foam allowed almost free passage of outside atmosphere into the space above the bark to replace air withdrawn from the midpoint of the bolt into a cryogenic extractor, (Browne et al., 1974). Organic vapors were separated from the large volumes of water collected by first salting (NaCl) the aqueous sample and then extracting it with three different ether washes, each of which was equal to one and one-half times the volume of the sample. These ether

extracts were combined, and most of the ether was removed by vacuum distillation at −75° C. Ipsdienol and ipsenol fractions were isolated from the concentrate by preparative GC (Carbowax 20M, 4.5 × 10 mm). The collected fractions were rechromatographed on Apiezon L (2 m × 2 mm).

Determination of the Enantiomeric Composition of Ipsenol and Ipsdienol

The enantiomeric composition of each alcohol was determined by high-pressure liquid chromatography (HPLC) separation of their respective diasteromeric esters. Ipsdienol was treated with (−)-camphanoyl chloride (Fluka, >99.5% optical purity) in pyridine containing a catalytic amount of *p*-*N*,*N*-dimethylaminopyridine to form the diastereomeric camphanic esters, which were subsequently separated by HPLC on a modular unit as previously described (Bergot et al., 1978), using a Zorbax-SIL (22 cm × 0.46-mm) column (DuPont) eluted with 4% ethyl acetate in pentane (50% water saturated), with uv detection at 254 nm. These separations were repeated using 20% pentane in dichloromethane (50% water saturated). (+)-α-Methoxy-α-trifluoromethylphenylacetic acid (MTPA; Aldrich Chemical) was converted into its acid chloride upon stirring with thionyl chloride. The product was distilled (90–5° at 10 mm, greater than 99.5% optical purity) and was used to convert ipsenol to its MTP esters. These esters were separated on the above HPLC column using 0.1% ethyl acetate in pentane (50% water saturated). In each case, ester pairs were separated with baseline resolution and quantification accomplished by peak area determination. For a general discussion of these procedures, see Bergot et al. (1978).

RESULTS AND DISCUSSION

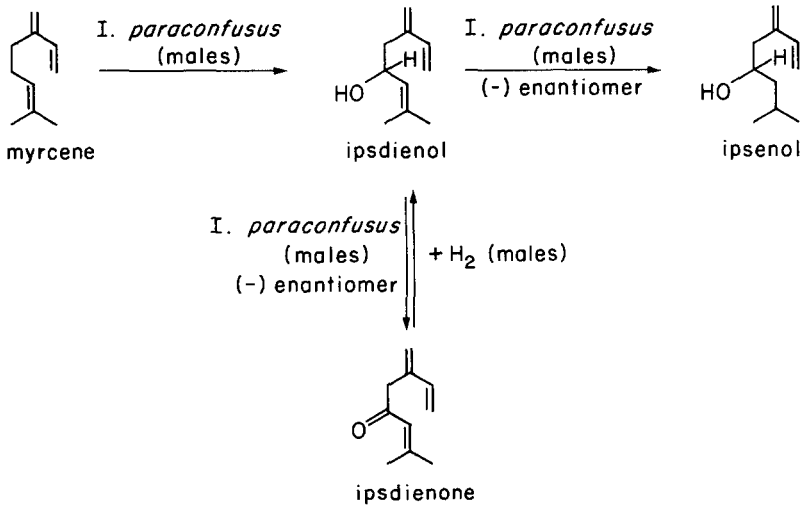
The isolation of pheromones, by passing air over *I. paraconfusus* males boring in host material, should yield reliable data on the naturally occurring enantiomeric compositions of ipsenol and ipsdienol. It is necessary to know what the beetle produces naturally in order to compare the enantiomeric compositions produced when they are exposed to ipsdienone.

The 50 male beetles boring into the ponderosa bolt produced (+)-ipsdienol , e.e. 87.6% [6.2% (−) and 93.8% (+)], and (−)-ipsenol, e.e. 93.8% [96.9% (−) and 3.1% (+)]. The 90% (+) : 10% (−) enantiomeric composition of ipsdienol that had been isolated from frass reported by Plummer et al. (1976) appears to be in close agreement with our results. Their estimates were based on NMR experiments and optical rotation data. Mori (1979) reported a value of $[\alpha]_D^{20}$ of −18.4° for synthetic (−)-ipsenol. By comparison, Silverstein et al. (1966b) obtained a rotational value for ipsenol, isolated from *I. paraconfusus* male-produced frass, of $[\alpha]_D^{20}$ −17.5 ± 0.7°, which indicates that a small amount of the (+) enantiomer may have been present in the

natural material. We found 3.1% (+)-ipsenol present in the air condensate, which is consistent with the previous results.

However, when males were confined in an atmosphere rich in ipsdienone, and no other precursor, they produced and sequestered a very different enantiomeric composition of ipsdienol in their bodies. Yet under these conditions, the composition of ipsenol varied only slightly from the natural ratio. The 350 males in the ipsdienone atmosphere produced (−)-ipsdienol, e.e. 28% [64% (−) and 36% (+)], and (−)-ipsenol, e.e. 86% [93% (−) and 7% (+)]. More importantly, the actual e.e. of (−)-ipsdienol produced was probably higher than these findings indicate, since the (−)-ipsdienol was converted to ipsenol as the (+) enantiomer accumulated.

These results allow the proposed pathway shown in Scheme 1 for conversion of mrycene to (+)- and (−)-ipsdienol and, subsequently, to (−)-ipsenol and have (−)-ipsdienol being both in equilibrium with the ketone and converted to (−)-ipsenol. In addition, the bioconversion of (−)-ipsdienol to (−)-ipsenol appears to be irreversible, as males confined in atmospheres containing ipsenol did not produce detectable amounts of ipsdienol. Furthermore, any alcohol–ketone interconvertibility of the reduced product could not be shown, as no ipsenol was detected in males exposed to an atmosphere containing ipsenone. Also, ipsenol vapors appear to be quite toxic, since we found 100% mortality of both males and females at the end of a 24-hr exposure. The guts of both sexes of beetles exposed to clean air contain few or no compounds within the polarity and volatility range of the compounds that would be detected by the chromatographic techniques used in these



SCHEME 1. Biosynthetic pathways to ipsdienol and ipsenol from myrcene, a host-plant terpene hydrocarbon.

studies. However, guts of both sexes contain several detectable compounds following exposure to each of the precursors.

These dramatic results confirm our earlier findings (Fish et al., 1979) of enantioselectivity in the conversion of (−)-ipsdienol to its ketone and to (−)-ipsenol. In addition, the present study clearly shows the (−)-enantiomer's role in the biological reduction of ipsdienone. Thus, a mechanism for the biological resolution of the active (+)-ipsdienol occurs by the selective conversion of (−)-ipsdienol. This may, in fact, be a general phenomenon in the enantioselective accumulations and transformations of pheromone alcohols in other insect species.

Byers (1981) discussed beetle assimilation of precursor terpenes and demonstrated variations in the amount of pheromone alcohols sequestered by the beetle when it feeds normally in the host (where it presumably ingests and inhales a large number host-produced compounds) versus the quantities of these pheromones produced when the beetle is exposed to vapors of a single precursor. We, however, chose the inhalation route to administer precursor compounds, because of the precision that could be maintained over the experiments, as well as the fact that inhalation of precursors materials is expected to occur in nature. However, when an isolated precursor is administered via a single pathway, there always remains a danger that significant qualitative as well as the demonstrated quantitative variations from the "real" or "normal" biosynthetic processes can occur. For these reasons we attempted to estimate the enantiomeric composition of the "natural" pheromone alcohols produced by beetles under what we considered to be near-natural conditions. We believe that the enantioselective pathway described in Scheme 1 yields pheromones that are consistent with the natural products. These findings greatly increase the likelihood that myrcene is the primary precursor used by the male *I. paraconfusus* to produce ipsdienol and ipsenol in nature.

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ASSOCIATION OF PARTICULAR SYSTEMS WITH THE RELEASE OF NEUTRAL LIPIDS IN *Echinostoma revolutum* (TREMATODA) ADULTS

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Abstract—Since free sterol excretory-secretory (E-S) products are involved in pheromonal communication in adult *Echinostoma revolutum* (Trematoda), attempts were made to associate specific systems with the release of lipids from this organism. A micropipet design was used to isolate neutral lipids from the excretory system versus those obtained from both the alimentary and the reproductive systems. Tegumentary lipids were obtained by rubbing the surface of worms with gauze. As determined by thin-layer chromatography, the major neutral lipid obtained from all systems was free sterol. Additional minor neutral lipid fractions were obtained from the excretory, alimentary, and reproductive systems. Histochemical oil red O studies showed neutral lipids only in the excretory system. Neutral lipids released from all of the above-mentioned systems may play a role in pheromonal communication in this species.

Key Words—Trematoda, *Echinostoma revolutum*, neutral lipids, thin-layer chromatography, histochemistry, excretory system, alimentary system, reproductive system, tegument, pheromonal communication.

INTRODUCTION

Neutral lipid excretory-secretory (E-S) products are involved in pheromonal communication in several hermaphroditic digenetic trematodes. The free sterol, cholesterol, has been tentatively identified as a chemoattractant in *Leucochloridiomorpha constantiae* by Fried and Giosca (1976) and in *Echinostoma revolutum* by Fried et al. (1980), and Fried and Robinson (1981) found that sterol esters are involved in chemoattraction in *Amblosoma suwaense*.

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As discussed by von Brand (1973), it is difficult to associate particular trematode systems with the release of E-S products. Although attempts to associate a particular structure with the release of amino acids in trematodes have been made (Lutz and Siddiqi, 1971; Bailey and Fried, 1977), similar studies on neutral lipids are not available.

The purpose of this study was to associate a particular system with the release of neutral lipid E-S products in *E. revolutum*. The systems of interest were the tegumentary, alimentary, reproductive, and excretory systems.

METHODS AND MATERIALS

E. revolutum adults, 2 weeks old, were obtained from experimentally infected domestic chickens (Fried and Weaver, 1969) and washed rapidly in three changes of sterile Locke's solution prior to use.

To obtain E-S products released from the excretory pore, the posterior third of the worm along with minimal Locke's solution was introduced into a 10-μl disposable micropipet (Microcaps, A.H. Thomas Co., Philadelphia, Pa.) using mouth suction. To obtain E-S products from the mouth and gonopore, the anterior third of the same worm was introduced similarly into another 10-μl micropipet. The middle third of the worm was covered with gauze (previously determined by TLC to be lipid free) moistened in Locke's to prevent drying.

This design was used in two experiments: (1) 20 worms were used immediately and allowed to release E-S products into the micropipets for 30 min; and (2) 20 worms were maintained in Locke's solution for 1 hr at 38° C to accumulate lipids before being placed in the micropipets for 30 min. Both experiments were done at 38 and at 22° C. After 30 min of incubation the worms were removed and discarded, and micropipets with their contents were extracted in 2 ml of chloroform/methanol (2/1) according to Folch et al. (1957). To determine differences in the anterior versus posterior worm products, the micropipets containing E-S products from the mouth and gonopore were extracted separately from those containing products from the excretory pore. As a control, 10-μl micropipets without worms and with minimal Locke's solution were extracted in 2 ml of chloroform/methanol (2/1). To determine the presence of tegumentary lipids, 20 worms were held by the mouth with fine forceps and gently rubbed on the dorsal side (to avoid the gonopore and excretory pore) with filter paper that was subsequently extracted in 2 ml of chloroform/methanol (2/1). All samples were dried under nitrogen gas and reconstituted with 100 μl of chloroform/methanol (2/1). Reconstituted samples were spotted with 1-, 2-, and 5-μl disposable micropipets (Microcaps) on the origins of precleaned (chloroform/methanol, 1/1) 20 × 20-cm silica gel sheets (Baker-flex IB2, J.T. Baker Chemical Co., Phillipsburg, N.J.), 2.5 cm from the bottom of the sheet. Sheets were also

spotted with 0.1 to 1 $\mu g/\mu l$ of a neutral lipid standard (18-4A, Nu-Chek-Prep, Inc., Elysian, Minn.) containing equal amounts of cholesterol, cholesterol oleate, triolein, oleic acid, and methyl oleate. Chromatograms were developed 10 cm from the origin in a glass rectangular tank (Chromaflex, Kontes Glass Co., Vineland, N.J.) lined on three sides with filter paper. The tank was allowed to saturate for 20 to 40 min with petroleum ether/diethyl ether/acetic acid (70/30/1) prior to development in this solvent system. Neutral lipids were visualized by spraying chromatograms with a 5% ethanolic solution of phosphomolybdic acid and then drying the sheets in an oven at 110°C for 3 min. Densitometric quantitation of the free sterol fraction was done on a representative chromatogram as described by Sonenshine et al. (1981), using a Kontes fiber optics densitometer (K-495000) with baseline corrector and strip-chart recorder.

Histochemical oil red O (ORO) studies were done to localize specific areas associated with neutral lipid activity. Worms fixed in cold neutral-buffered formalin were prepared as cryostat sections (Butler and Fried, 1977) and stained with ORO (Lillie, 1944). Sections through the mouth, uterus, and excretory bladder were examined.

RESULTS

Observations of the contents in the micropipets showed the presence of refractile lipophilic granules in posterior- but not anterior-worm products. For all experiments TLC analyses of both anterior and posterior E-S products showed that free sterol ($R_f = 0.11$) was the major neutral lipid fraction, with lesser amounts of free fatty acids ($R_f = 0.46$), triglycerides ($R_f = 0.55$), and sterol esters ($R_f = 0.70$). Some variation existed in the minor neutral lipid fractions, and in some trials either triglycerides, free fatty acids, or sterol esters were absent. The free sterol fraction was present in anterior- and posterior-worm products in 10 of 11 trials. In one trial the free sterol fraction was absent in the posterior products. TLC differences in worms incubated at 38 or 22°C were negligible. Qualitative TLC showed that worms allowed to accumulate lipids for 1 hr released slightly more neutral lipids than worms used immediately. Control micropipets were always lipid negative. TLC analysis of tegument E-S products showed only free sterols.

Densitometric TLC analysis showed 100 ng of free sterol from the anterior-worm products of 20 worms used immediately after necropsy of the host and incubated at 38°C and 400 ng of free sterol from the posterior-worm products of the same 20 worms. These values equal 5 ng of free sterol/worm released anteriorly and 20 ng of free sterol/worm released posteriorly.

Histochemical ORO studies showed neutral lipid droplets in the lumen and epithelium of the excretory bladder, but the mouth and uterus were negative. Sections treated with chloroform/methanol (2/1) prior to staining were lipid negative.

DISCUSSION

TLC and histochemical analyses indicated that *E. revolutum* adults released neutral lipids mainly from the excretory system. Because of the small size of *E. revolutum* (about 1 cm in length) and the proximity of the gonopore to the mouth (see Beaver, 1937), we were unable to collect separately the neutral lipids emitted from the mouth and gonopore. Attempts to intubate neutral lipids from pores or to collect them on filter paper (Siddiqi, 1971) were unsuccessful. The neutral lipid fractions identified in the anterior and posterior E-S products are similar to the neutral lipids of whole-worm incubate as described by Fried and Appel (1977). The quantities of free sterol released per worm are in accord with data on whole-worm incubates (Bennett and Fried, 1983). They reported a mean release of 53 to 330 ng/whole adult incubated for 1 hr at 38° C. Lutz and Siddiqi (1971) found that prior incubation of *Fasciola gigantica* in Tyrode's solution increased the output of protonephridial fluid. The maintenance of *E. revloutum* in Locke's solution prior to incubation in micropipets resulted in a slight increase in neutral lipid release. Bailey and Fried (1977) could not establish an unequivocal difference in amino acid E-S products in anterior- versus posterior-worm products in *E. revolutum*. The presence of qualitatively similar neutral lipids in anterior-, posterior-, and tegument-worm products suggests the same conclusion for neutral lipids. Our histochemical study could not confirm the presence of neutral lipids in the tegumentary, alimentary, or reproductive system. Because histochemical lipid studies are considered less reliable than TLC analyses (Fried and Butler, 1977), we assume that neutral lipids present in these systems were not detected histochemically.

While we did not find major differences in the general types of lipid present, the individual constituents in the lipid fraction in each part of the worm may be different. Further studies are needed using chromatographic procedures other than thin layer to analyze individual lipid constituents from each part of the worm.

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THE CHEMICAL FEEDING ECOLOGY OF *Neodiprion dubiosus* SCHEDL, *N. rugifrons* MIDD., AND *N. lecontei* (FITCH) ON JACK PINE (*Pinus banksiana* LAMB.)

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Abstract—Two monophagous sawflies, *Neodiprion dubiosus* and *N. rugifrons*, actively avoid feeding on the current season's needles of jack pine, *Pinus banksiana*, while the polyphagous *N. lecontei* feeds indiscriminately on foliage of all age classes. Bioassay studies with needle extracts indicate that *N. dubiosus* and *N. rugifrons* larval feeding preference is governed by a group of tricyclic diterpene resin acids. Resin acids commonly occur in needles of all ages; however, levopimaric, palustric, dehydroabietic, and neoabietic acids were at higher concentrations in the new needles. *N. dubiosus* was significantly deterred from feeding on 1-year-old foliage when treated with the pure resin acids, palustric and levopimaric, at 0.5 and 1.0 mg/ml MeOH. *N. rugifrons* larvae were deterred from feeding by pure dehydroabietic, neoabietic, and palustric acids at 1.0 mg/ml MeOH.

Key Words—*Neodiprion dubiosus*, *N. rugifrons*, *N. lecontei*, Hymenoptera, Diprionidae, feeding behavior, resin acids, palustric, levopimaric, neoabietic, dehydroabietic, jack pine, *Pinus banksiana*.

INTRODUCTION

Among the confier-associated sawflies, at least 15 diprionid species (Hymenoptera: Diprionidae) prefer to feed on mature needles rather than the current season's needles of their respective hosts (Baker, 1972; Hard et al., 1976; Wilson, 1977). *Pristiphora erichsonii* (Htg.) and *Pikonema alaskensis* (Roh.) (Hym.: Tenthredinidae) exhibit a similar feeding preference on their hosts, *Larix* spp. and *Picea* spp., respectively (Wagner et al., 1979; Wilson, 1971). Knowledge of the mechanism(s) that governs this feeding behavior would allow us to better understand the ecology of these species and possibly provide us with a natural control mechanism.

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Within the Diprionidae, two closely related monophagous species, *Neodiprion swainei* Midd. and *N. rugifrons* Midd., prefer mature needles to the current season's needles of their host, jack pine, *Pinus banksiana* Lamb. (All and Benjamin, 1975a). Antifeedants present in the current season's jack pine foliage were responsible for this preferential feeding behavior (All and Benjamin, 1975b). Ikeda et al. (1977) identified these antifeedants as two diterpene resin acids, 13-keto-8(14)-podocarpen-18-oic and dehydroabietic, which deterred larval feeding of *N. rugifrons* by 63.5 and 24.6% and of *N. swainei* by 46.5 and 36.1%, respectively.

P. erichsonii larvae feed exclusively on the tufts of needles on old wood of tamarack, *L. laricina* (DuRoi) K. Koch., early in the growing season and reject the single needles on the new shoots later in the season. Ohigashi et al. (1981) demonstrated that the chemical mechanism governing the larval differentiation between single needles and tufts of needles lies primarily in a group of five resin acids that occur in higher content in single needles than in tufts of needles. These resin acids were identified as abietic, dehydroabietic, 12-methoxyabietic, sandaracopimaric, and isopimaric.

N. dubiosus Schedl is monophagous on jack pine and has a feeding pattern similar to that of *N. swainei* and *N. rugifrons* (Becker, 1965). Our studies were designed to determine if similar resin acids are responsible for its feeding behavior. Studies on *N. rugifrons* were conducted for comparative purposes.

A polyphagous multivoltine species, *N. lecontei*, also occurs on jack pine. *N. lecontei* larvae feed on foliage of all age classes from mid-June through several generations into October. However, it is not known whether this is an indiscriminate behavior or if larvae exhibit a preference for a particular age class. We report on the degree of preference for jack pine foliage of different ages by *N. lecontei*.

METHODS AND MATERIALS

Source of Insects and Rearing Techniques

Jack pine foliage for rearing sawflies was collected from Dane, Jackson, Marquette, and Richland Counties, Wisconsin. *N. lecontei* and *N. rugifrons* larvae were field collected from Oneida and Sauk Counties, Wisconsin. *N. dubiosus* larvae were collected near Chapleau, Ontario, Canada. Larvae were reared in 9.5 × 19 × 26.5-cm ventilated plastic boxes at room temperature (20–25° C) on an 18L:6D photoperiod. Foliage was changed every other day to ensure a fresh supply of food.

Foliage Collection and Extraction

Jack pine foliage for extraction and bioassay was collected from Sauk County Forest, Sauk County, Wisconsin. For extraction, five 20-g units of

current, 1-year-old and 2-year-old needles were randomly picked from 15- to 25-year-old trees in the field throughout July, 1982. Samples were frozen at −25° C until analysis. Ten grams of each needle type were oven dried at 100° C to determine moisture content. The remaining 10 g were cut in half and extracted five times with 50-ml portions of diethyl ether in an Omnimixer (Ivan Sorvall, Inc.). The crude ether extract was brought to dryness in a Rotovap rotary vacuum distiller at 20° C and weighed.

Crude extracts were separated into neutral and acid fractions using a diethylaminoethyl (DEAE)-Sephadex column following the Sephadex preparation and elution procedure of Zinkel and Rowe (1964). The DEAE-Sephadex was loaded into a 16-cm, 5-mm-i.d. column. A 50-ml portion of ether:methanol:water (89:10:1) was washed through the column to settle the Sephadex. The crude extract was dissolved in 10 ml of ether:methanol:water (89:10:1) and added to the column. After the solution passed into the bed, neutrals were washed through the column using 250 ml of the same solvent. Weak acids were then eluted with 250 ml of ether:methanol (90:10) saturated with CO_2. To remove more acidic materials, 100 ml of 1% acetic acid:ether was washed through the column. Nitrogen pressure was applied when necessary to maintain a satisfactory flow rate. Eluted fractions were concentrated in a rotary vacuum distiller and stored at −25° C under nitrogen.

The weak acid fractions were methylated with diazomethane (Nestler and Zinkel, 1963) for analysis by gas–liquid chromatography. Gas–liquid chromatography was done with BDS or SE-30 glass capillary columns at an oven temperature of 190° C and a helium flow of u = 30 cm/sec using a Hewlett–Packard Model 5840A gas chromatograph equipped with a flame-ionization detector and a 18835B inlet splitter (Foster and Zinkel, 1982). Resin acids were identified by gas chromatographic retention behavior in comparison with known samples. Percentage composition of resin acids was based on normalized resin acid peak areas.

Bioassay Method

The bioassay procedure is a modification of the technique described by Wagner et al. (1979). A standard bioassay consisted of a 7- to 10-cm twig of 1-year-old jack pine foliage stripped of needles until 10 pairs remained at each end. The twigs were rinsed in distilled water and allowed to air dry for 30 min. Feeding preference bioassay twigs consisted of different-aged foliage on opposite ends of the twig. Bioassay twigs used to test extracts and commercial resin acids consisted of 1-year-old foliage at both ends.

Crude ether extracts of current season's, 1-year-old, and 2-year-old needles were dissolved in methanol (MeOH) to an approximate concentration of 10 mg extract/ml MeOH for bioassay. The commercial resin acids, dehydroabietic, levopimaric, neoabietic, and palustric, were obtained from B.C. Research (Vancouver, B.C., Canada) and dissolved in methanol to obtain concentrations of 0.5 and 1.0 mg/ml.

Needles at one end of each twig were covered with an extract or acid: methanol solution by pipetting a few drops at the base of the needle and allowing it to flow to the tip. Needles at the other end were treated similarly with solvent (methanol) only. A similar bioassay twig received the solvent at one end and nothing at the other end to serve as an additional control. The treated needles were allowed to air dry for 30 min.

Each twig was suspended horizontally by an insect pin that passed through the center of the twig; the pin was then inserted through a 2.5-in. square of moss-green paper and into a No. 11.5 rubber stopper. Five third- or fourth-instar larvae were placed on the needles at each end of the twig. The twig was placed in an environmental chamber under continuous light at 20° C. The environmental chamber was lined with moss-green paper to simulate natural conditions (Brelje, 1970). Four bioassay twig replicates were used for each experiment.

At 1-hr intervals for 4 hr, the number of larvae at each end of the twig was recorded. The percentage of larvae on the solvent end vs total larvae was employed to determine biological activity. The test material was recognized to have deterrent activity if more than 70% of the larvae had settled on the solvent-only end after 4 hr. Raw data were analyzed using the Student *t* test (Sokal and Rohlf, 1969).

RESULTS AND DISCUSSION

Feeding preference bioassays indicate that *N. dubiosus* and *N. rugifrons* larvae prefer to feed on 1-year-old needles and actively avoid the current season's foliage (Table 1). *N. lecontei* fed with equal intensity on needles of both age classes. These results are in accord with feeding preferences observed in the field and cited in the literature.

TABLE 1. RESPONSE OF *N. dubiosus, N. rugifrons,* AND *N. lecontei* LARVAE TO THE FEEDING PREFERENCE BIOASSAY

Species	No. of larvae feeding during 4 hr: Current season's foliage $\bar{X}$	Current season's foliage %	One-year-old foliage $\bar{X}$	One-year-old foliage %	No. of expts.
N. dubiosus	1.5	16	8.4	84*[b]	4
N. rugifrons	1.0	10	8.9	90*	3
N. lecontei	4.8	52	4.1	48	3

[a]Ratio of mean larvae feeding at a position/mean of total larvae × 100.
[b]Significance level; (*) 0.001 (Student's *t* test).

TABLE 2. FEEDING RESPONSE OF *N. dubiosus, N. rugifrons,* AND *N. lecontei* TO CRUDE NEEDLE EXTRACTS AND FRACTIONS OF CURRENT SEASON'S FOLIAGE FROM DEAE-SEPHADEX COLUMNS

Extract	Concentration (mg/ml MeOH)	% feeding deterrency[a]			No. of expts.
		N. dubiosus	*N. rugifrons*	*N. lecontei*	
Crude extract					
Current season's foliage	11.25	74**[b]	76*	62	4
One-year-old foliage	10.25	61	53	60	4
Two-year-old foliage	12.20	62	43	38	4
Fractions of current season's foliage					
Weak acid	7.87	84***	72**	NT[c]	6
Strong acid	9.58	48	53	NT	5
Neutral	10.32	23	35	NT	4

[a]Expressed as the percentage of larvae found on the solvent-treated end of the twig.
[b]Significance levels (Student's *t* test): (*) 0.05, (**) 0.01, and (***) 0.001.
[c]Not tested.

Results of bioassays using crude ether extracts of current season's, 1-year-old, and 2-year-old needles are similar to those in the feeding preference studies (Table 2). *N. dubiosus*, *N. lecontei*, and *N. rugifrons* larvae were not inhibited from feeding on needles treated with 1- or 2-year-old crude needle extracts. Crude extracts of current season's needles deterred larval feeding of *N. dubiosus* and *N. rugifrons* but not of *N. lecontei.* The ability of *N. lecontei* to feed on current season's foliage indicates that this species can tolerate the antifeedants present.

The crude extract of current season's needles was fractionated to obtain neutral, weak acid, and strong acid fractions. Bioassay studies with each of these fractions were conducted with *N. dubiosus* and *N. rugifrons* larvae. Of the three fractions tested, only the weak acid fraction was biologically active in deterring larval feeding (Table 2). *N. dubiosus* demonstrated a higher degree of sensitivity than *N. rugifrons* to this active fraction. Strong acid and neutral fractions did not significantly influence larval feeding.

Gas chromatographic analysis of the methylated weak acid fraction showed that the fraction consisted primarily of several tricyclic diterpene resin acids (Figure 1). Since these are common resin acids, it was important to establish if a significant difference existed in the levels of these chemicals between current season's and 1-year-old needles. An initial difference was noted in the dry weight of the weak acid (resin acid) fractions. The fraction

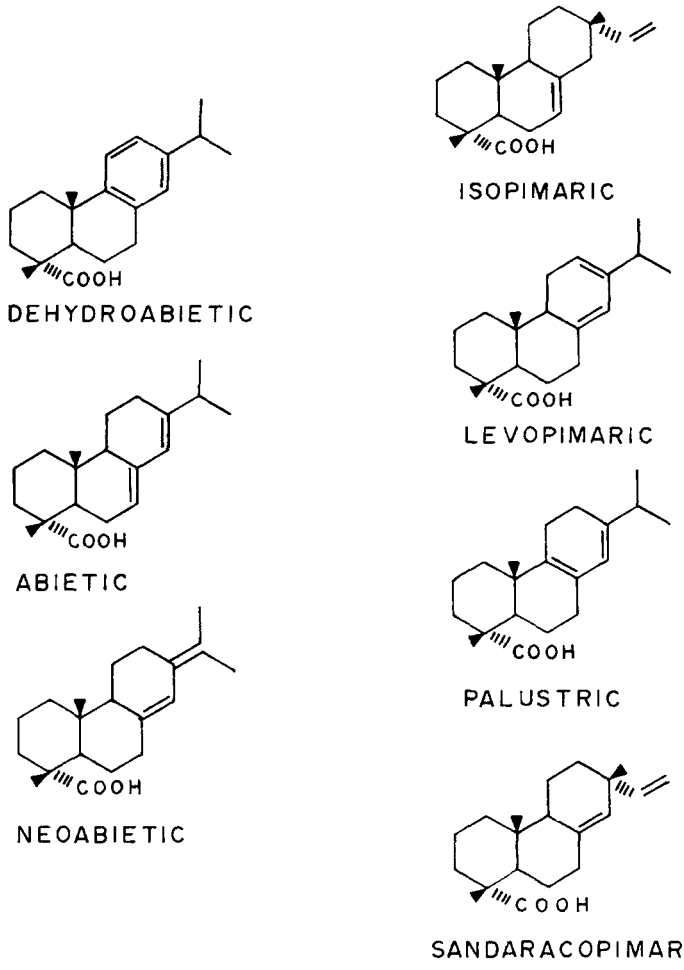

FIG. 1. Chemical structures of resin acids isolated from jack pine needles.

isolated from the current season's foliage weighed twice as much, 142 mg/10 g needles, as the fraction from 1-year-old needles, 69 mg/10 g needles. Analyses of the resin acid composition for both needle types are presented (Table 3). The levels of the compounds were found to be different for the two types of foliage. Four resin acids, neoabietic, dehydroabietic, and a levopimaric/palustric mixture, were at higher concentrations in current season's needles.

To determine the biological activity of candidate feeding deterrents, pure neoabietic, dehydroabietic, levopimaric, and palustric were bioassayed at 0.5 and 1.0 mg/ml MeOH. *N. rugifrons* was inhibited from feeding by dehydroabietic, neoabietic, and palustric resin acids at 1 mg/ml (Table 4).

TABLE 3. RESIN ACID COMPOSITION OF CURRENT SEASON'S AND ONE-YEAR-OLD JACK PINE FOLIAGE

Resin acid methyl ester	Normalized % of fraction	
	Current season's foliage	One-year foliage
Abietate	2.1	2.5
Dehydroabietate	13.6[a]	12.2[a]
Isopimavate	0.5	1.8
Levopimarate/palustrate	31.8	19.6
Neoabietate	43.0	41.0
Sandaracopimarate	2.3	3.3
Other	6.7	19.6

[a]Since dehydroabietate is a common by-product of unstable resin acids, these values may vary slightly (±0.5).

Palustric and levopimaric acids actively deterred *N. dubiosus* larval feeding at 0.5 and 1.0 mg/ml.

Ikeda et al. (1977) isolated and identified the resin acid, 13-keto-8(14)-podocarpen-18-oic acid, from current season's jack pine foliage and demonstrated that it significantly deterred larval feeding of *N. rugifrons* and *N. swainei*. To explain the absence of this compound in our investigation, we considered the possibility that the compound was not eluted from the DEAE-Sephadex column with the solvent system used. To investigate this possibility, pure 13-keto-8(14)-podocarpen-18-oic acid was produced by hydrolysis of the methyl ester following a modification of the technique

TABLE 4. FEEDING RESPONSE OF *N. dubiosus* AND *N. rugifrons* LARVAE TO MATURE FOLIAGE TREATED WITH COMMERCIAL RESIN ACIDS

Resin acid (% purity)	Activity (%)[a] (No. of expts.)			
	N. dubiosus		*N. rugifrons*	
	Acid concentration (mg/ml MeOH)			
	0.5	1.0	0.5	1.0
Dehydroabietic (99+)	51 (4)	57 (4)	40 (4)	81 (5)**
Levopimaric (99+)	71 (4)*[b]	83 (4)***	45 (3)	67 (4)
Neoabietic (99+)	50 (4)	67 (5)	59 (4)	83 (4)***
Palustric (90–95)	70 (5)**	78 (4)**	67 (6)	99 (4)***

[a]Expressed as the percentage of larvae found on the solvent-treated end of the twig.
[b]Significance levels (Student's *t* test): (*) 0.05, (**) 0.01, and (***) 0.001.

described by Miles and Parish (1972). The compound was eluted through the DEAE-Sephadex column following the same procedure used with the jack pine needle extracts. Gas chromatographic analysis of all three fractions indicated that 13-keto-8(14)-podocarpen-18-oic acid eluted from the CO_2-saturated ether:methanol (90:10) solvent, as did the resin acid fraction from the jack pine needle extracts.

From this experiment, we concluded that if 13-keto-8(14)-podocarpen-18-oic acid was not complexed with another compound it would have eluted through the DEAE-Sephadex column with the other resin acids from the jack pine needle extracts. It is possible that the initial extraction of the needles with ether was not as efficient in extracting this compound as methanol, which was used by Ikeda et al. (1977). However, further investigation into this discrepancy was beyond the scope of this study. Therefore, the presence or absence of this compound in the jack pine needles used in our investigation remains unresolved.

Dehydroabietic, neoabietic, and palustric acids, when topically applied to 1-year-old jack pine foliage deter *N. rugifrons* larval feeding, and levopimaric and palustric acid deter *N. dubiosus* larval feeding. These resin acids are also present at higher concentrations in the current season's jack pine foliage than in 1-year-old foliage. We conclude that these resin acids may contribute significantly to the natural deterrence of the current season's foliage against *N. rugifrons* and *N. dubiosus*.

In addition to deterring larval feeding of *N. rugifrons* and *N. dubiosus*, resin acids also deter larval feeding of *N. swainei* and *P. erichsonii* (Ikeda et al., 1977; Ohigashi et al., 1981, respectively). These acids are natural products of metabolism in the host plants. Their exploitation as control agents may provide an alternative to the use of broad-spectrum pesticides in controlling several sawfly species.

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EVALUATION OF TIME-AVERAGE DISPERSION MODELS FOR ESTIMATING PHEROMONE CONCENTRATION IN A DECIDUOUS FOREST[1]

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Abstract—The Sutton and more recent Gaussian plume models of atmospheric dispersion were used to estimate downwind concentrations of pheromone in a deciduous forest. Wind measurements from two bivane anemometers were recorded every 12 sec and the pheromone was emitted from a point source 1.6 m above ground level at known rates. The wing-fanning response of individually caged male gypsy moths (*Lymantria dispar*) at 15 sites situated 20 to 80 m downwind was used to monitor when pheromone levels were above threshold over a 15-min interval. Predicted concentrations from these Gaussian-type models at locations where wing fanning occurred were often several orders of magnitude below the known behavioral thresholds determined from wind tunnel tests. Probit analyses of dose–response relationships with these models showed no relationship between predicted dose and actual response. The disparity between the predictions of concentration from these models and the actual response patterns of the male gypsy moth in the field was not unexpected. These time-average models predict concentrations for a fixed position over 3-min or longer intervals, based upon the dispersion coefficients. Thus the models estimate pheromone concentrations for time intervals appreciably longer than required for behavioral response.

Key Words—Pheromone dispersion, active space, Sutton model, Gaussian plume model, gypsy moth, *Lymantria dispar*, Lepidoptera, Lymantriidae.

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INTRODUCTION

Central to understanding how an organism reacts to airborne pheromone is a delimitation of the pheromone concentrations impinging on the responding individual. In a wind field, concentrations are dependent principally upon the rate of pheromone emission and turbulent dispersion. Wright (1958) and Bossert and Wilson (1963) formulated a conceptual framework, now widely accepted, of pheromone dispersal in the wind, generation of "active spaces," and subsequent response in terms of "threshold." Their pioneering efforts were based upon the Sutton (1953) time-average model for dispersal of gases by atmospheric turbulence and the use of this model to calculate an average concentration of an airborne material emanating from a point source at any location downwind. The Sutton model continues to be widely used in pheromone research, despite theoretical objections raised by Wright (1958) and elaborated upon by Mason (1973), Aylor et al. (1976), Miksad and Kittredge (1979), and Murlis and Jones (1981) of applying time-average models to behavioral reactions that occur over short time intervals. Alternate approaches suggested by Aylor et al. (1976) and Miksad and Kittredge (1979) are, at present, difficult to apply because these models consider dispersion relative to a meandering plume centerline and thus do not estimate the concentration at any point fixed in space. A recent review of dispersion models as they apply to pheromones is given by Elkinton and Cardé (1984).

Pheromone biologists generally have ignored the extensive meteorological literature that has accumulated over the last 25 years on the dispersal of atmospheric pollutants. In this literature the Sutton equations have been supplanted by more recent "time-average" models. Fares et al. (1980) were the first to apply a more general Gaussian plume model to pheromone dispersion. In this study we have mapped the dimensions of a pheromone plume in the field and evaluated the applicability of the Sutton model and the more modern Gaussian plume models using dispersion coefficients utilized by Pasquill (1961), Gifford (1968), and Fares et al. (1980) to describe pheromone dispersion in the wind.

We have used the male gypsy moth, *Lymantria dispar* L., and the synthetic female pheromone as an exemplar chemical communication system. This choice was justified as follows. (1) The male's diel response periodicity in the field indicates maximal activity in the early afternoon (Cardé et al., 1974), an ideal time for direct behavioral observations. (2) The relatively large size of the male gypsy moth also simplifies monitoring of behavior. (3) The female, as far as is known, uses a single-component attractant. For species with multiple components, Roelofs (1978) proposed that the threshold of pheromone responsiveness differs with the proportion of components in certain pheromone blends. By choosing an organism that evidently releases a single-

component attractant, we eliminate such potential confounding factors. The synthetic attractant, (+)-disparlure, is available (Cardé et al., 1978). (4) Conceptual models of the male's sequences of response to various concentrations of pheromone are available (Cardé and Hagaman, 1983; Hagaman and Cardé, 1984). (5) No native population of this species near (<30 km) the test site is known.

METHODS AND MATERIALS

Selection of a Behavioral Reaction. The size and shape of the active space of a plume of synthetic (+)-disparlure in the field were measured by observing the wing-fanning behavior of caged male gypsy moths positioned at various points downwind from the source. In nature, the most important change in behavior that correlates with the male gypsy moth's perception of the "active space" is the change from appetitive (non-pheromone-mediated) flight to upwind anemotaxis. A simple field assay to measure this particular change in behavior has not been devised. However, a change from quiescence to wing fanning while walking is an "earlier" reaction in the sequence of response to pheromone (Hagaman and Cardé, 1984). The *proportion* and the *latency* of wing fanning while walking can be related to either concentration or flux of stimulus. The pheromone threshold for this behavior has been determined under the controlled conditions of the wind tunnel milieu (Cardé and Hagaman, 1983). The concentration of (+)-disparlure that elicits a 50% wing-fanning response is ca. 1×10^{-18} g/cm^3.

Experimental Design. The assay for the active space consisted of 15 separate racks positioned simultaneously at various locations downwind of a pheromone source. Each rack had 30 moths held individually in cylindrical wire-mesh cages (0.45-cm mesh, 7-cm long, 6-cm diameter) with removable tops. The cages were anchored to the 61 × 61-cm rectangular wire-mesh rack mounted vertically on a steel post. All racks were positioned 1.6 m above ground (the same height as the source), with the moth cages facing upwind toward the pheromone source and the observer standing behind (downwind). Observations on the occurrence of wing-fanning behavior of each of the 30 moths on all racks were recorded with an audio tape recorder once every 15 sec for the duration of the experiment. Experiments were run for 45 min and consisted of a 15-min observation period before releasing pheromone to establish the background rate of spontaneous wing-fanning activity, 15 min of pheromone release, and a final 15 min of observation after removal of the pheromone source.

Fifteen racks, each with one observer and 30 moths, were spaced every 10 or 20° along three arcs (five racks/arc) at 20, 40, and 60 or 80 m downwind of

the pheromone source (Figure 1). Racks were put in place at least 30 min prior to the start of the experiment. The experiments were conducted in July and August 1979, beginning at ca. 15:00. A total of 3.4 million observations of individual male wing-fanning behavior at 15-sec intervals was obtained.

Experimental Site. The experiments were conducted in a mature oak–maple woodlot measuring ca. 200 × 300 m on the campus of Michigan State University. The canopy was closed and ca. 20 m high. The terrain in the woodlot was fairly level (±ca. 2 m) and there was very little understory vegetation. The pheromone release site was located at the center of the woodlot at least 10 m from the nearest tree. Strings radiating from the release site were placed every 20° at ground level and they extended at least 80 m in all directions to facilitate positioning of the racks of moths in relation to the pheromone source.

Meteorological Data. Wind speed and direction were monitored by two bivane anemometers (stall speed = 0.3 m/sec; R. M. Young Co., Traverse

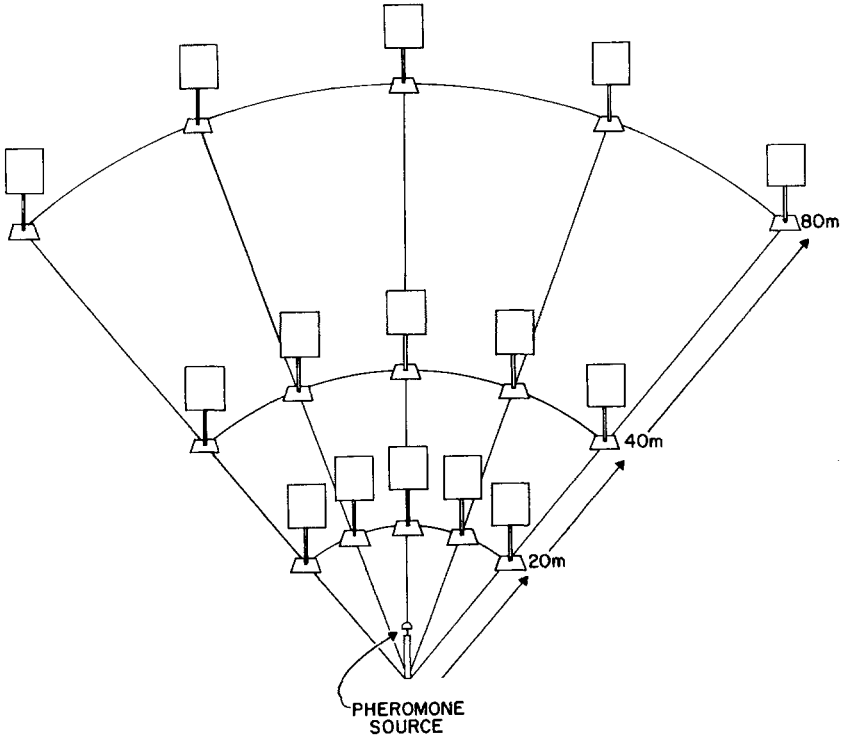


FIG. 1. Spatial layout of racks, each containing 30 male gypsy moths over a 40° arc at three distances from a pheromone source.

City, Mich.) positioned 2 m above ground 10 m east and 15 m north of the pheromone source. Air temperature was measured by a Yellow Springs thermistor probe (Yellow Springs, Ohio) in a standard instrument shelter at a site ca. 15 m northwest of the source. Temperature, wind speed, and both vertical and horizontal wind directions from the two anemometers were recorded and digitized every 12 sec on an Esterline Angus PD 2064 data logger and stored on tape with a Techtran 8000 data cassette for subsequent computer storage and analysis. The acquisition of meteorological data was synchronized within 3 sec of the 15-sec sample interval of the behavioral observations.

Pheromone Source. The pheromone source was 100 μg or 10 μg of (+)-disparlure (Cardé et al., 1978) dispensed from a cotton wick, 0.8-cm diameter $\times$ 1.0-cm long, positioned 1.6 m above the ground at the center of the woodlot. The pheromone was pipetted onto the wick in 100 μl of petroleum ether ca. 16 hr prior to the experiment and placed overnight in a fume hood to allow the pheromone release rate to stabilize. The cotton-wick dispenser was held on an insect pin affixed to a small cork, which in turn was attached to the top of the 1.6-m stake. A small piece of aluminum foil between the stake and the cork was replaced daily to prevent contamination of the stake. The pheromone dispenser was placed inside a glass test tube, which in turn was sealed in a glass jar and taken to the field. At the start of the pheromone release period, the jar was opened and the dispenser was removed from the test tube and placed on top of the stake. At the end of the 15-min period of pheromone release, the wick was placed back inside the test tube, sealed in the jar, and removed immediately to a position downwind of the racks of moths.

The release rate from a cotton wick loaded with 100 μg of disparlure after 16 hr was ca. 296 and 159 pg/sec, respectively, in wind speeds of 132 and 81 cm/sec at 23° C, as calculated by residue analysis of the dispenser using GLC and *cis*-9,10-epoxyeicosane as an internal standard. The release rate for each experimental run was calculated by linear interpolation from these two values using the mean wind speed measured by the anemometer.

Rearing of Moths. Male gypsy moths were reared in mass culture (Bell et al., 1981) at the Otis Methods Development Laboratory at Otis Air Base, Massachusetts, and shipped as pupae to Michigan. The pupae were held in paper cups inside of wire-mesh emergence cages in a walk-in screen cage at the northwest corner of the experimental woodlot. Pheromone was not released when the walk-in cage was downwind of the pheromone prior to the experiment. The males remained in the wire-mesh emergence cages for 1–5 days (usually 1–3 days) prior to use in the experiments. On the morning before each afternoon bioassay, the old moths were removed from each rack of 30 cages, the rack was rinsed with acetone to remove possible pheromone residue, and new moths were placed in each cage.

Calculation of Percentage Wing Fanning and Data Analysis. The moth response data for each grid over 45 min were transcribed from audio tape (Figure 2). To compare the observed response with the predictions of the various atmospheric dispersion models, we computed the average wind speed and direction for the appropriate time interval beginning at the moment of pheromone release. The estimated concentrations from the Sutton model and the Gaussian plume models were based on a mean wind speed and a direction unique for each sample interval. We also estimated average pheromone flux by multiplying the estimated concentration by the mean wind speed. The estimated average concentration at each location was plotted against the proportion of the 30 moths that initiated wing fanning during the sample period (3, 10, 15 min) appropriate for each model. The behavioral sample interval began at each distance at the estimated time of arrival of the plume. This time was determined from the average wind speed starting at the time of pheromone release.

The percentage wing-fanning response at each location for each sample interval (3, 10, 15 min) was calculated in two ways. The first method focused on the percentage of males that initiated wing fanning at any time during the

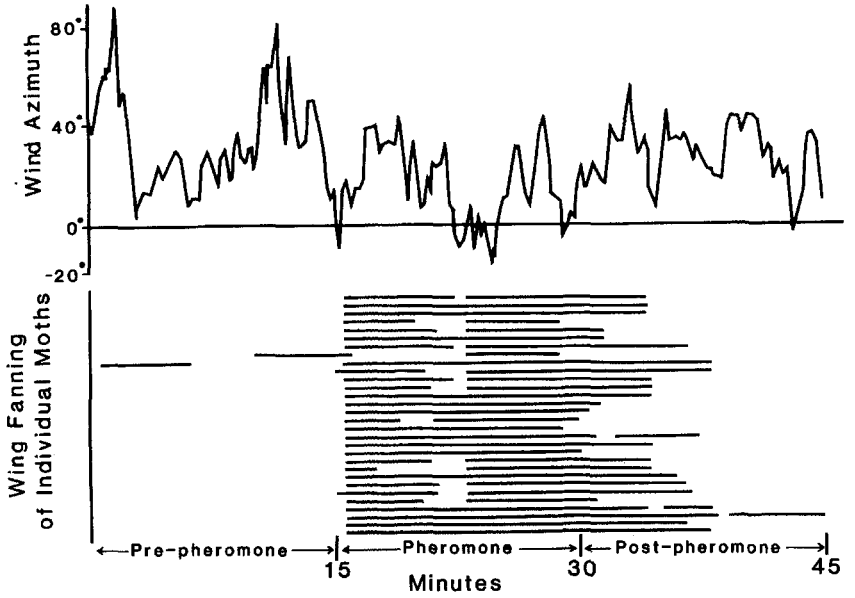


FIG. 2. Wing-fanning behavior of 30 moths at one rack position in the field. The behavior of each moth was recorded every 15 sec during the 45 min of prepheromone, pheromone, and postpheromone observations. The wind azimuth equals 0° whenever the wind was blowing directly from the pheromone source toward the moths.

interval. The estimate of percentage wing fanning was corrected for spontaneous background wing-fanning activity as follows:

$$\left[\frac{R-B}{N-B}\right] \cdot 100, \tag{1}$$

where R is the number wing fanning during the interval, B is the number wing fanning during an equal interval prior to pheromone release, and N is the number of moths in the rack minus any that were already fanning immediately prior to the estimated time of plume arrival.

The second way to estimate percentage wing fanning was to compute the total proportion of time spent wing fanning by all 30 moths during the interval. This estimate was corrected for spontaneous wing-fanning activity in the same manner as above [Eq. (1)], except that N is now the total number of seconds in the interval, and R and B are the number of seconds spent wing fanning during and prior to pheromone exposure, respectively.

To compare observed behavior with the model prediction, probit analyses (Finney 1971) were used to generate regression lines of the percentage wing-fanning response transformed to probits against the log concentrations predicted at each location by the Sutton and Gaussian plume models. Regression lines were calculated separately for each distance. For each regression line a χ^2 statistic was calculated as well as an ED_{50}, which is the concentration of pheromone estimated to produce a 50% wing-fanning response. A large, statistically significant χ^2 occurs when the observed responses depart widely from the values predicted by the regression lines.

Sutton Model. The most widely used pheromone dispersion model is that of Sutton (1947, 1953), which was first applied to pheromones by Wright (1958) and Bossert and Wilson (1963). It estimates the concentration (C_{xyz}) of an airborne material at any point (xyz) downwind of a point source:

$$C_{xyz} = \frac{2Q}{\pi C_y C_z U x^{2-n}} \cdot \exp\left[-x^{n-2}\left[\frac{y^2}{C_y^2} + \frac{z^2}{C_z^2}\right]\right], \tag{2}$$

where Q is the release rate, U is the mean wind speed, C_y and C_z are the respective horizontal and vertical diffusion coefficients, and n is a parameter ($0 < n < 1$) dependent on the vertical profile of wind velocity. The origin of the coordinate system is the location of the source with the x axis aligned in the direction of the mean wind, the y axis in the crosswind direction, and the z axis in the vertical. The dispersion coefficients are functions of the atmospheric turbulence, terrain roughness, and vertical windspeed profile. Sutton (1953, p. 292) suggests that with light winds, neutral atmospheric conditions, and level ground, the following approximate value can be used: $C_y = 0.4\ \text{cm}^{1/8}$, $C_z = 0.2\ \text{cm}^{1/8}$, and $n = 0.25$. We have utilized these "typical values" in our test of Sutton's model, as did Wright (1958), Bossert and Wilson (1963), and nearly

all subsequent applications of Sutton's model to pheromone dispersion. We tested the model performance against a 3-min sampling interval of wing-fanning behavior because that is the interval for which the equation was derived. This version of the Sutton model applies to a source located on the surface of a reflecting plane. For a perfectly adsorbing surface the concentration at all points in space is exactly half that given in Eq. (2). For an elevated source, as in this experiment (1.6-m height), Sutton suggests the following equation:

$$C_{xyzh} = \frac{Q \exp(-y^2/C_y^2 x^{2-n})}{\pi C_y C_z U x^{2-n}} \left\{ \exp\left[-\frac{(z-h)^2}{C_z^2 x^{2-n}}\right] + \exp\left[-\frac{(z+h)^2}{C_z^2 x^{2-n}}\right] \right\}, \quad (3)$$

where h is the height of the source above ground. For all sample locations at the same height as the source ($z = h$), as in this experiment, the estimated concentrations for an elevated source are the same as for a ground-level source if the ground is perfectly adsorbing. If the ground below an elevated source is perfectly or partially reflecting, the concentration in the plane (x, y, h) is elevated by a factor of <2, which represents the limiting case. In our test of Sutton's model we have used Eq. (2) since that is the version used by most pheromone biologists regardless of source height. Within the horizontal plane (x, y, h) the difference in concentration for an elevated versus a ground-level source and for a reflecting versus an adsorbing ground surface is miniscule compared to the large range in concentrations (covering many orders of magnitude) estimated for different locations within the plane.

Gaussian Plume Model. In the Gaussian plume model (Pasquill, 1961; Fares, et al. 1980),

$$C_{xyzh} = \frac{Q}{2\pi\sigma_y\sigma_z U} \cdot \left\{ \exp\left[-\frac{y^2}{2\sigma_y^2}\right] \right\} \cdot \left\{ \exp\left[-\frac{(z-h)^2}{2\sigma_z^2}\right] + \alpha \cdot \exp\left[-\frac{(z+h)^2}{2\sigma_z^2}\right] \right\}, \quad (4)$$

all variables are defined as in the Sutton model, except that the horizontal and vertical dispersion coefficients (σ_y, σ_z) are functions of the downwind distance (x) instead of the constants C_y and C_z of the Sutton model, and α is a constant ($0 < \alpha < 1$) that depends upon the degree of adsorption of material to the ground.

The differences among various applications of the Gaussian plume model are the values or functions chosen for the dispersion coefficients, which are determined experimentally for a specific sample interval and a given set of terrain and atmospheric conditions. Here we use the Pasquill "Prairie Grass" coefficients calculated from the graphs given by Gifford (1968, p. 102), which are appropriate to a 10- to 60-min sample interval. We also use the coefficients of Fares et al. (1980) from the TRC-343 data, which were acquired in a forest

with a 15-min sampling time. The appropriate stability class for each version of the Gaussian plume model (A–F in the Pasquill treatment; buoyant, neutral, or inversion in the model of Fares et al.) was determined from the standard deviation of the wind azimuth measured by the bivane during each run according to Pasquill's formula given by Gifford (1968, p. 102). This method of selecting the appropriate stability class is thus based upon a direct measure of wind turbulence rather than upon the vertical temperature profile. The latter measure pertains to atmospheric turbulence, whereas our method also incorporates the effects of mechanical turbulence, which is substantial beneath a forest canopy. Furthermore, the vertical temperature profile in a woodlot on a typical sunny day is likely to be an inversion below the canopy, with a large lapse rate above and with complex effects on the wind turbulence measured on the ground.

RESULTS

Validity of the Assay. The moth response to the presence of pheromone was unmistakable at all distances tested (Figure 2, Table 1). Despite some spontaneous wing-fanning activity, the simultaneous initiation of wing fanning (within a 30-sec period) by most of the 30 moths at a given rack almost never occurred during the prepheromone period but occurred with regularity during the pheromone release period (Table 1). The response to pheromone was highly correlated with the wind direction, at nearly all distances (Table 1). During the pheromone release period, whenever the wind direction shifted directly upwind of the source, as indicated by the meandering track of the bivane, wing fanning usually ensued within a few seconds (Figures 3a and b).

There was no evidence of any response interaction between the moths in a grid. A sample of 200 wing-fanning events during the prepheromone period revealed that initiation of wing fanning by a particular moth did not significantly increase the probability that adjacent moths would fan (χ^2 test, $P > 0.05$).

Following removal of a pheromone source the wing-fanning activity of the moths persisted for several minutes until returning to the background spontaneous rate (Figure 2). The same phenomenon is obvious in Figures 3a and b, in which the response continued at a particular rack even though it was no longer directly downwind of the pheromone source. Consequently, wing-fanning behavior is a sensitive indicator of the onset but not the cessation of a pheromone stimulus.

Moth Response vs Model Predictions. The occurrence of wing-fanning activity or percentage time spent wing fanning at each of the 15 racks of moths during the 3-, 10-, and 15-min sample intervals corresponds in a rough

TABLE 1. NUMBER OF MALE GYPSY MOTH WING-FANNING RESPONSES AND MEAN OF MAXIMUM PERCENTAGE RESPONDING WITHIN ANY 30-SEC INTERVAL AT VARIOUS DISTANCES FROM THE SOURCE DURING THE FIRST 10 MIN PRIOR TO PHEROMONE RELEASE AND DURING PHEROMONE RELEASE[a]

Pheromone dispenser dose (μg)	Distance from source (m)	No. of observations[b]	Before pheromone release		During pheromone release		Proportion of > 50% responses coinciding with wind blowing in rack direction ($\pm 10°$)[d]
			No. of racks with > 50% response[c]	Mean of maximum % response per rack per 30 sec	No. of racks with > 50% response[c]	Mean of maximum % response per rack per 30 sec	
100	20	50	2	9.5	39	61.7	0.43
	40	58	1	6.7	32	48.0	0.54
	80	54	0	6.3	19	31.3	0.70
10	20	70	0	5.9	51	55.7	0.57
	40	75	0	3.4	32	38.1	0.56
	60	47	0	5.5	13	33.2	0.81
	80	30	0	5.4	4	17.4	0

[a] The proportion of responses which coincide with a wind direction measured previously at the source blowing in the direction of the rack ($\pm 20°$) and correlation of wind direction and subsequent percentage response at different distances from the source.

[b] An observation consists of 30 moths observed for 10 min before pheromone release followed by 10 min during release.

[c] A response consists of 50% or more of the available moths per 30-moth observation rack initiating wing fanning within a 30-sec interval. Available moths are those moths present which were not already fanning. No response was counted if fewer than 15 moths were available.

[d] The wind direction samples chosen were those which were taken at the time of wing-fanning response minus the estimated transit time between source and rack based upon the wind speed. Of five consecutive wind samples ($t \pm 24$ sec), we selected one that was closest to the rack direction.

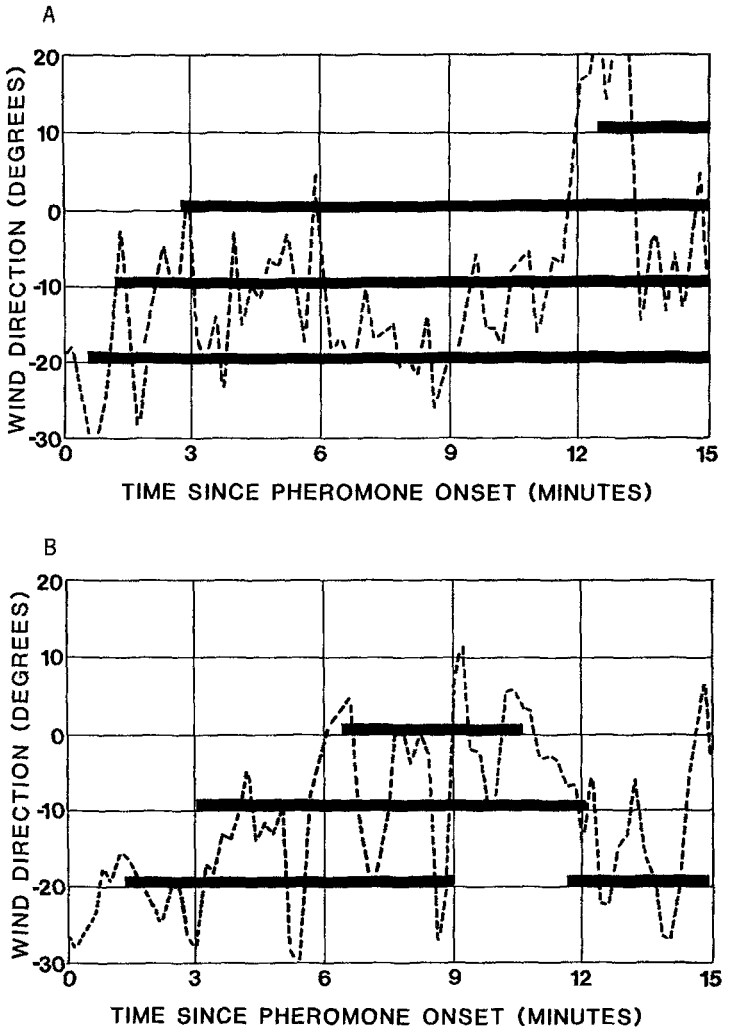
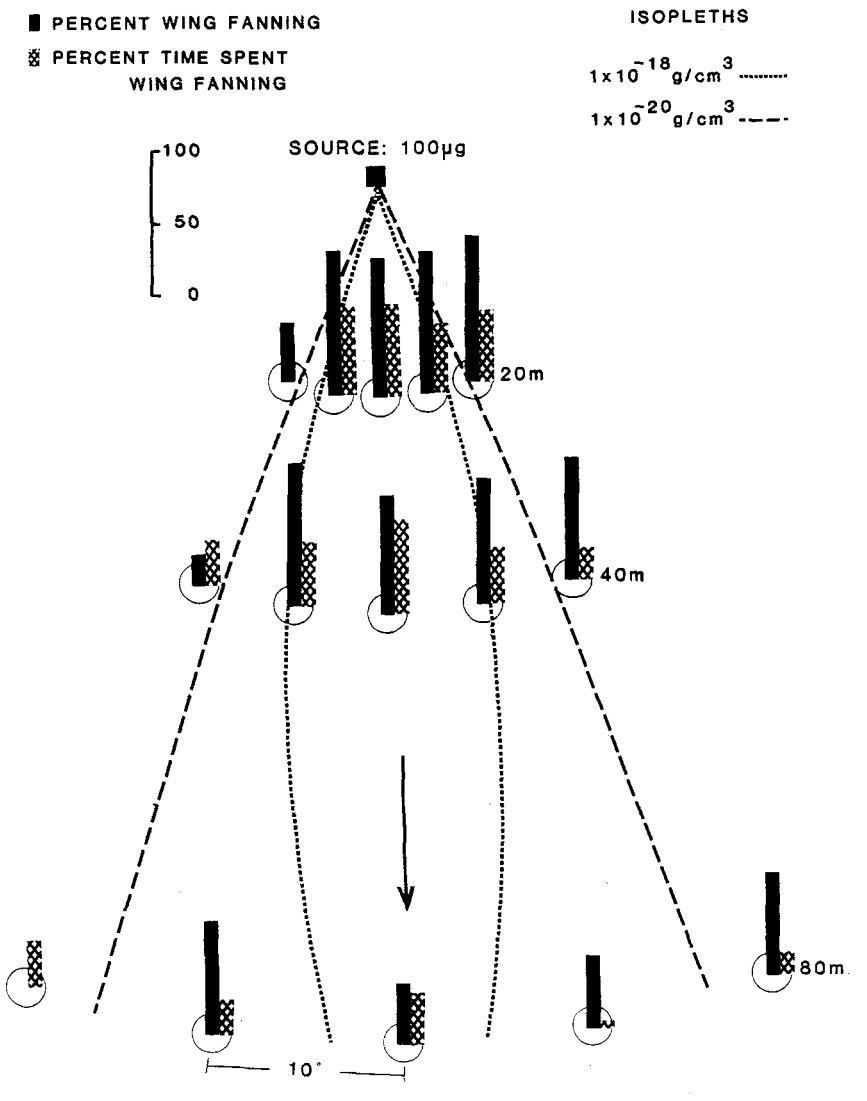


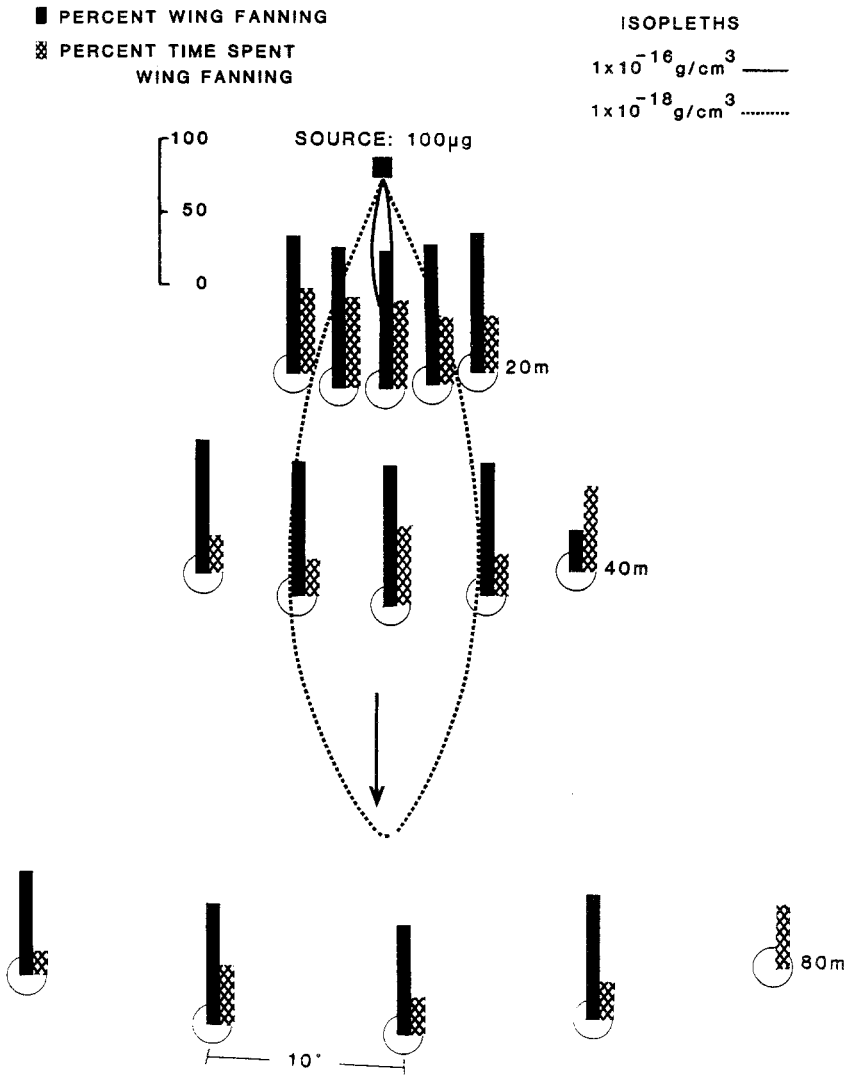
FIG. 3. Coincidence of wind direction (dashed line) measured at the source and the onset of wing-fanning activity (solid bars) among at least 50% of the males within any 30-sec interval after pheromone release at five different locations (−20, −10, 0, +10, and +20°) at a distance of 20 m from the source.

qualitative way to the direction and shape of the three Gaussian models considered (Figures 4 and 5). In each case, however, wing fanning occurred at locations well outside of the 1×10^{-18} g/cm^3 isopleths predicted by the three models. This isopleth approximates a concentration which produces a 50% wing-fanning response in the wind tunnel (Cardé and Hagaman, 1983; Figure



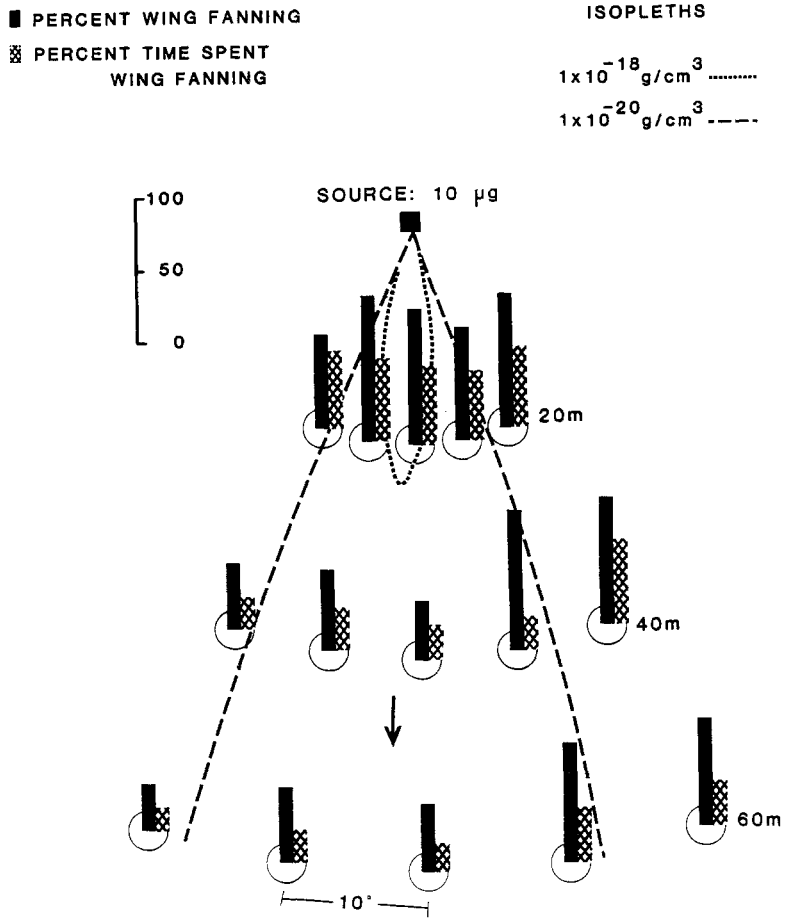
(a)

FIG. 4.(a–d) Concentration isopleths predicted by the Gaussian plume model with Pasquill dispersion coefficients and the percentage wing-fanning response at 15 locations over a 10-min interval on four different days with a 100- or 10-μg pheromone source. The solid bars represent the percentage of males that wing fanned (corrected for background) during the interval. Hatched bars represent the percentage of time spent wing fanning corrected for background. The 1×10^{-18} isopleth is approximately the threshold concentration known to produce a 50% wing-fanning response in a wind tunnel.



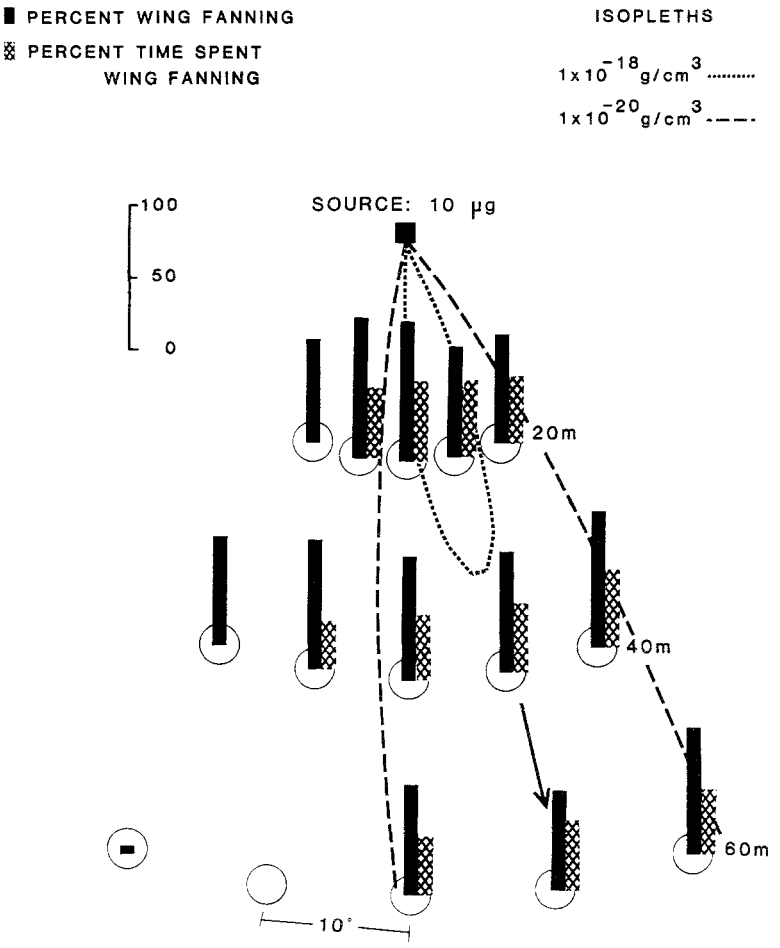
(b)

FIG. 4. (*Continued*)



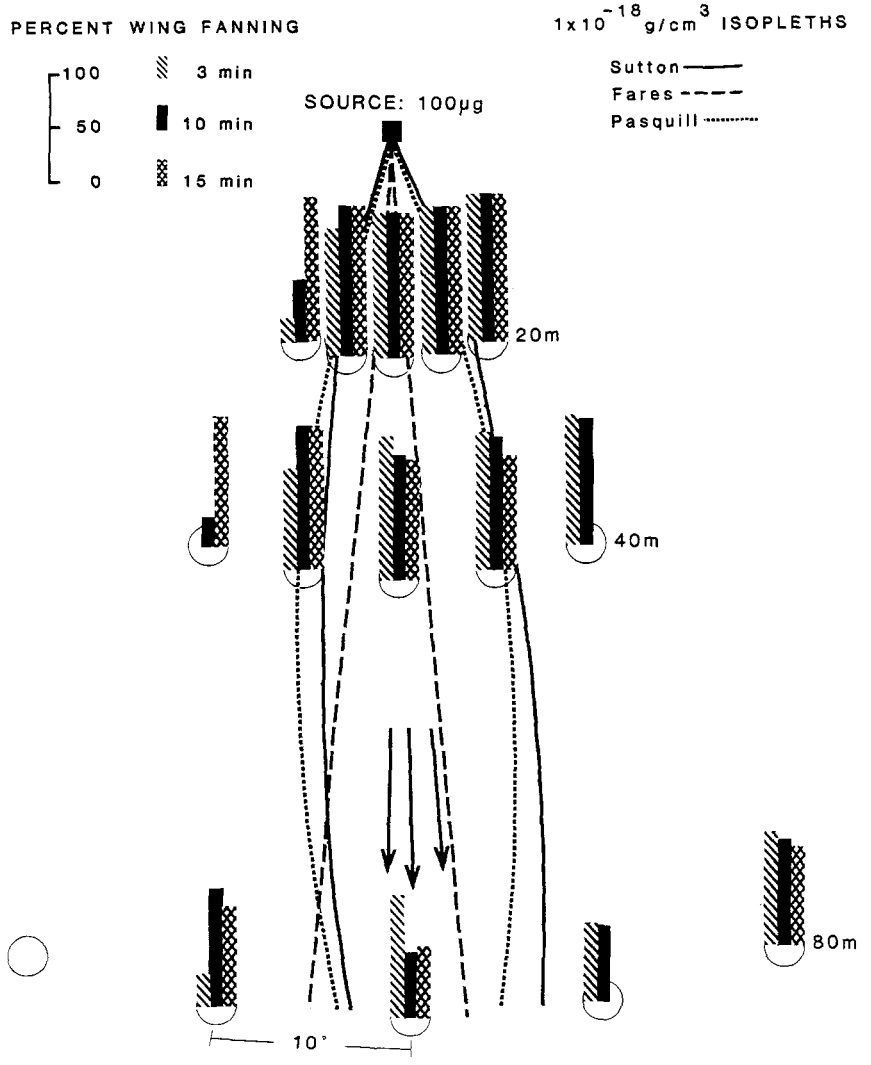
(c)

FIG. 4. (*Continued*)



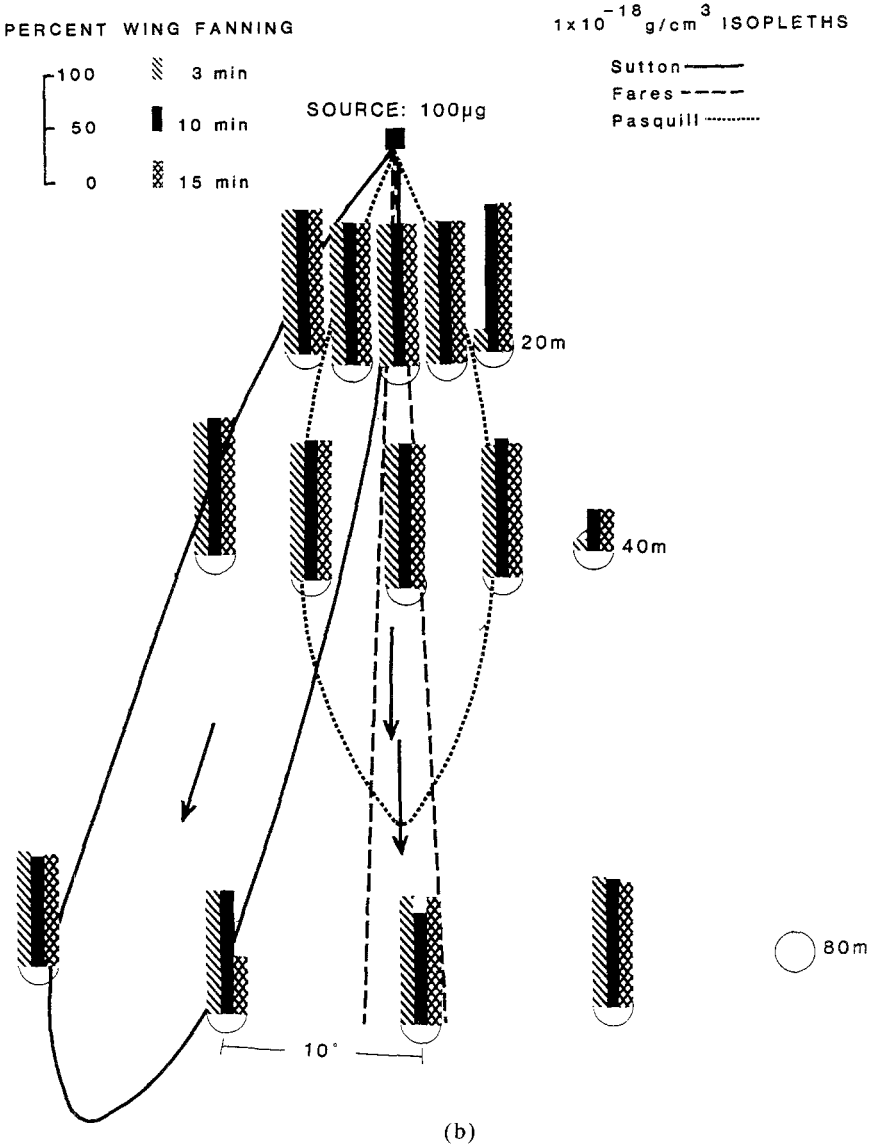
(d)

FIG. 4. (*Continued*)



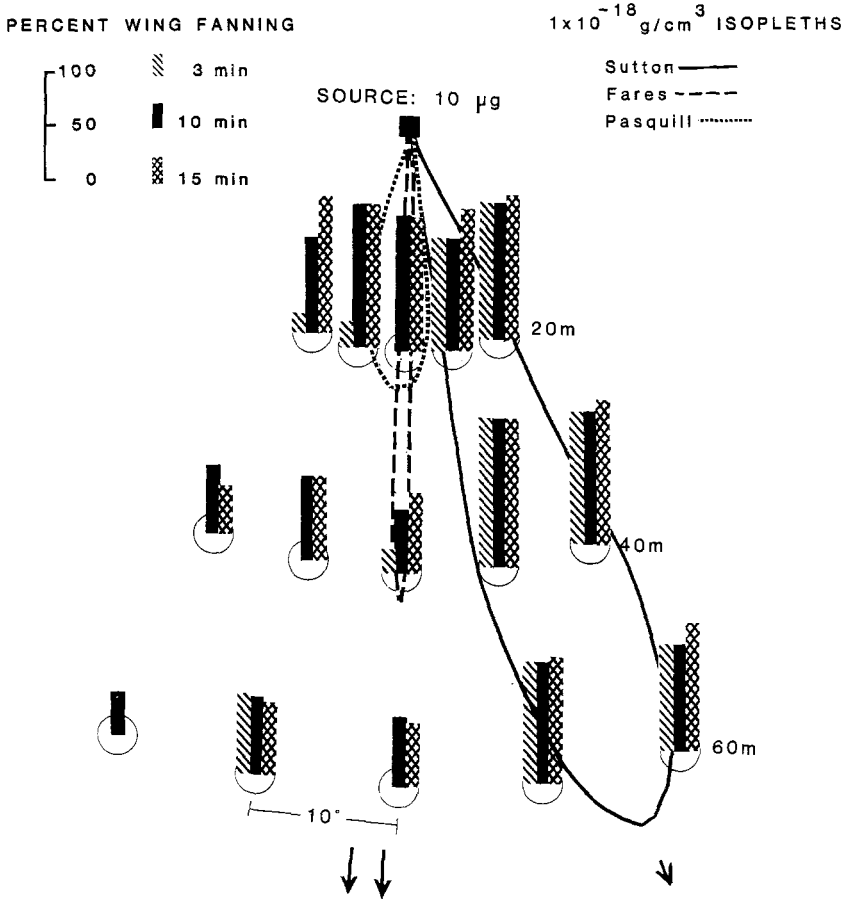
(a)

FIG. 5.(a–d) The 1×10^{-18} concentration isopleths predicted by the Sutton model and the Gaussian plume model with Pasquill and Fares dispersion coefficients for a 3-, 10-, and 15-min sample interval, respectively, versus the percentage of males that wing fanned (corrected for background) during the three intervals at 15 locations downwind of a pheromone source. The plume centerlines for the three models differ due to changes in the mean wind direction as the sample interval increased. The percentage wing fanning at some locations actually decreased between 3 and 10 or 15 min because of the effects of the correction for background wing-fanning activity.



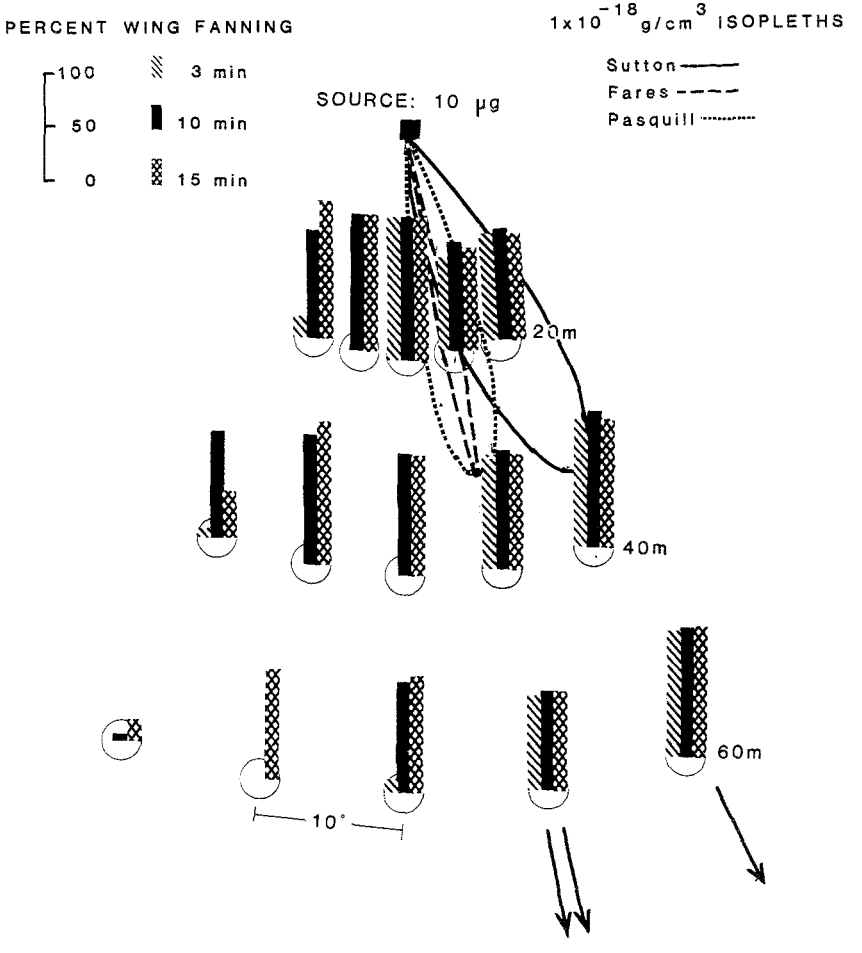
(b)

FIG. 5. (*Continued*)



(c)

FIG. 5. (*Continued*)



(d)

FIG. 5. (*Continued*)

6). In other words, all these models underestimate the width of the active space at each distance.

This conclusion is corroborated by the probit analyses (Figure 7, Table 2), which indicate that there are no significant correlations among observed responses and the concentrations predicted by any of the three models. [If the models were accurate descriptions of concentrations of pheromone, the observed regression line should be similar to that obtained from the wind tunnel (Figure 6).] At all distances from the source (20 and 80 m) and at both release rates (100- and 10-μg dispensers), the slope of the regression line was not significantly different from zero. Conversion of pheromone concentration to pheromone flux had a negligible effect on the calculated regression lines for any model because the conversion entailed multiplying the concentration by the wind speed (values close to 1 m/sec). The difference caused by this conversion was miniscule when plotted on an abscissa covering many orders of magnitude.

At 20 and 80 m the width of the plume predicted by the Gaussian plume model using the dispersion coefficients of Fares et al. (1980) is much smaller than the width of the Sutton and Pasquill models, despite the facts that the sample intervals compared to the Sutton model were longer (15 vs 3 min) and

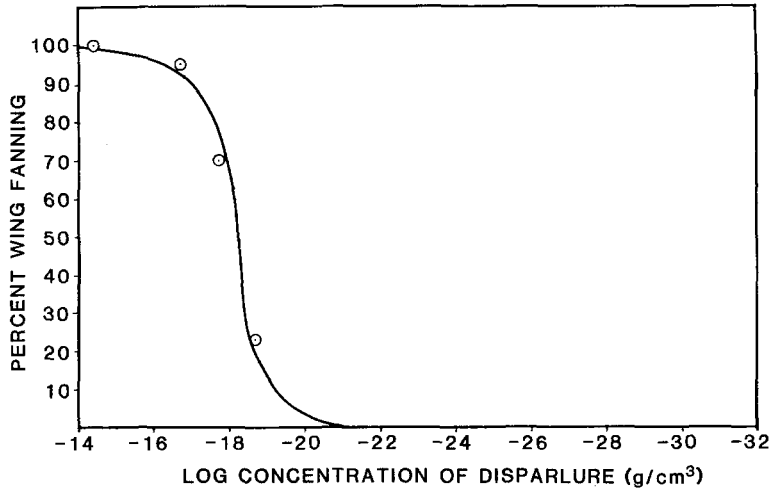


FIG. 6. Percentage wing-fanning responses versus estimated (+)-disparlure concentration in a wind tunnel at 24° C (Hagaman and Cardé, 1983). Concentrations estimates assume that (+)-disparlure is evenly distributed within a 15-cm-wide plume at the site of the males. Release rates for dispenser doses of 1000 and 100 ng were measured by residue analysis (Cardé and Hagaman, 1983) and extrapolated in decade steps to 10 and 1 ng from the measured rate at 100 ng. The probit regression line was calculated by the method of Finney (1971).

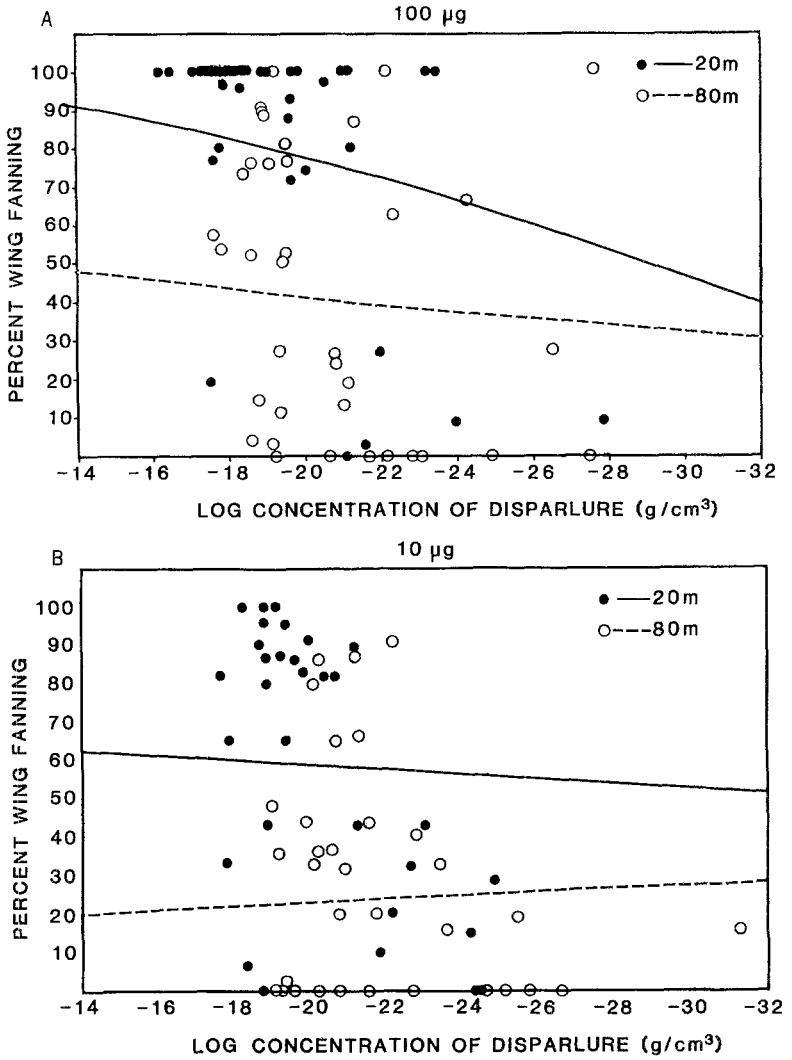


FIG. 7.(a, b) Regression lines generated by probit analysis at each rack location of the pheromone concentration predicted by the Sutton and Gaussian plume models for a 10- and 100-μg source, respectively, at 20 and 80 m versus the observed percentage wing-fanning response (corrected for background) for each rack.

the dispersion coefficients were derived from studies conducted beneath a forest canopy, where mechanical turbulence is enhanced. Furthermore, the model of Fares et al. predicted higher pheromone concentrations at 80 m than at 20 m along all directions displaced by more than ca. 2° from the mean plume centerline in contrast to the predictions of the other two models.

TABLE 2. PROBIT ANALYSIS OF PHEROMONE CONCENTRATION PREDICTED BY THREE GAUSSIAN DISPERSION MODELS VERSUS THE OBSERVED PERCENTAGE WING-FANNING RESPONSE OF 30 MALE GYPSY MOTHS AT 20 AND 80 M FROM THE PHEROMONE SOURCE

Model	Dispenser dose (μg)	Distance from source (m)	No. of observations	Probit regression coefficient		ED_{50} (conc.)[a]	χ^{2b}
				Intercept (probits)	Slope (probits/log conc.)		
Sutton	100	20	45	7.9	0.095	0.14×10^{-25}	802
		80	42	17.0	0.170	0.17×10^{0}	670
	10	20	30	6.1	0.044	0.27×10^{-26}	547
		80	25	6.5	0.102	0.79×10^{-13}	380
Pasquill	100	20	42	7.8	0.008	0.23×10^{-31}	1053
		80	43	5.1	0.016	0.38×10^{-10}	607
	10	20	35	5.6	0.016	0.14×10^{-36}	494
		80	35	3.9	−0.013	0.56×10^{-75}	381
Fares	100	20	29	6.4	0.002	$<1 \times 10^{-100}$	803
		80	33	5.2	0.005	$<1 \times 10^{-100}$	443
	10	20	31	5.7	0.004	$<1 \times 10^{-100}$	416
		80	34	4.4	0.002	$<1 \times 10^{-100}$	362

[a] ED_{50} is the dose estimated by probit analysis to produce a 50% wing-fanning response. The values have little meaning when the slopes are not significantly different from zero.
[b] All χ^2 values are significant at $P < 0.01$, indicating a poor fit between the data and the probit regression lines.

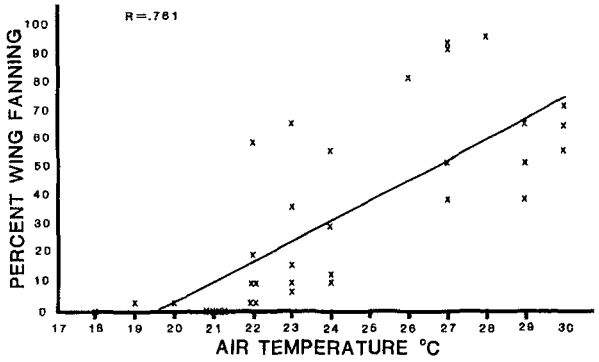


FIG. 8. Correlation of air temperature and percentage of males exhibiting spontaneous wing-fanning activity (i.e., 15 sec or longer) during the first 10 min of the period prior to pheromone release.

The spontaneous wing-fanning activity during the prepheromone period increased with increasing temperature (Figure 8). It is also likely that increasing temperature lowered the threshold of the wing-fanning response to pheromone as shown in wind tunnel experiments (Cardé and Hagaman, 1983). Temperature also has a slight effect upon the rate of pheromone release from the dispenser, but this effect is small compared to the effect of changes in concentration on the moth's responsiveness.

DISCUSSION

Characteristics of Time-Average Gaussian Models. The three models are "Gaussian" in that they assume that the average concentration of airborne material released from a point source follows a normal (Gaussian) distribution along any axis perpendicular to the mean downwind direction. These models were derived from experiments in which tracer substances were released from a source and recovered in samplers arrayed downwind for the duration of a specific sample interval. The width of the plume, which is governed by the rate of turbulent dispersion, is expressed in terms of the vertical and horizontal dispersion coefficients. In general, longer sample intervals give rise to wider plumes (Slade, 1968, p. 57; Mason, 1973). The material is distributed at a lower concentration over a larger area. Few pheromone biologists have appreciated that these models apply only to the same specific sample interval as the experiments from which the dispersion coefficients were derived. For this reason we have correlated the model predictions to the wing-fanning responses that occurred *during the appropriate interval* (3 min for Sutton's model, 10 min for Pasquill's dispersion

coefficients, 15 min for coefficients used by Fares et al.). The rate of dispersion and the values of the dispersion coefficients also depend upon the terrain and atmospheric conditions.

Comparison of Sutton and Gaussian Plume Models. In Sutton's model (1947, 1953) the vertical and horizontal dispersion coefficients C_z and C_y are constant under a given set of terrain and atmospheric conditions. Most applications of Sutton's model to pheromone dispersion have utilized the "typical" values," $C_z = 0.2\,\text{cm}^{1/8}$, $C_y = 0.4\,\text{cm}^{1/8}$, and $n = 0.25$, which Sutton (1953, p. 292) suggested were appropriate for neutral atmospheric conditions over level ground under light winds. In theory, Sutton's dispersion coefficients could be calculated for any number of terrain and atmospheric conditions. However, atmospheric science has largely abandoned the Sutton model in favor of the more general Gaussian plume model in which the dispersion coefficients are measured experimentally for a given set of conditions instead of the theoretical derivations from other variables including the vertical wind speed profile attempted by Sutton (Mason, 1973). In contrast to Sutton's model, the dispersion coefficients in the Gaussian plume model are functions of the downwind distance (x), which does not appear explicitly in Eq. (4). These coefficients are equivalent to the standard deviation of the vertical and horizontal distribution of concentration along any axis perpendicular to the mean wind direction. Thus, a great advantage of the Gaussian plume model is that it can be easily applied to a variety of atmospheric stabilities.

Neutral atmospheric conditions as required by the typical values of Sutton's dispersion coefficients only sometimes prevail. Unstable conditions occur most often during the day, when the ground absorbs heat from the sun and transmits it to the air immediately above, giving rise to convective updrafts and vertical mixing. Stable or inversion conditions occur most often at night, when radiant heat loss from the ground leads to cool air at ground level with warmer air above. However, daytime inversions may prevail beneath a closed forest canopy because the air at canopy level is heated by the sunlit foliage, with cooler air below. Therefore, the argument of Fares et al. (1980), that higher turbulence associated with midday temperature causes rapid dispersion of the plume, may not apply in forests with a closed canopy.

The differences among various forms of the Gaussian plume model mainly involve different values or functions chosen for the dispersion coefficients. The coefficients used in the model of Fares et al. (1980) were derived from a study conducted beneath a forest canopy. Given the long sampling time (15 min) compared with the Sutton model and the mechanical turbulence introduced by the trees, it is surprising that the dispersion coefficients in Fares' model increase so slowly with downwind distance compared with the other Gaussian models.

Effect of Wind Speed: Flux vs Concentration. All the Gaussian dispersion models are inverse functions of wind speed. The size of the active

spaces shrinks as the wind speed increases. Several previous studies have documented this effect with pheromone communication (Nakamura, 1976; Shapas and Burkholder, 1978). This phenomenon is caused principally by the dilution effect of eluting a given amount of pheromone into a larger "initial" volume. Wind speed also affects the rate of expansion of the pheromone plume. Wind speed affects the power spectrum of turbulent eddies that determine the rate of dispersion. For this reason the dispersion coefficients (C_y, C_z) in the Sutton equation are themselves functions of the mean wind speed. The latter effect is ignored when typical values are used for C_y and C_z, and in any case, it is small relative to the dilution effect. We did not attempt to document the effect of wind speed on the maximum distance of communication because we made observations at only three distances simultaneously and therefore we did not have a good measure of the *length* of the active space.

The moth response, moreover, is mediated by the number of molecules per second adsorbed by the antennae, a phenomenon more closely related to the flux (g/cm^2 sec) of the pheromone through the antennae than the concentration (g/cm^3). Since flux increases in direct proportion to the wind speed, the dilution effect is canceled. In our experiments conversion from concentration to flux did little to improve the performance of the models in predicting percentage wing fanning.

Instantaneous vs Time-Average Models. The Sutton model and the Gaussian plume model considered here estimate the average concentration (or flux) of pheromone for sample intervals of several minutes. Wright (1958) and Aylor et al. (1976) have noted that the actual plume experienced by a moth at any instant in time is by comparison very narrow and high in concentration. The plume meanders with the large-scale turbulent eddies such that a sampler fixed in space downwind of a source will experience no pheromone most of the time, interspersed by short intervals of high concentration. Moths presumably respond not to average concentrations of pheromone but to the "instantaneous" concentrations above a certain threshold. A brief exposure to a sufficiently high concentration will produce a wing-fanning response, even though the average concentration (over several minutes) at a particular site is below threshold. Therefore the "active space" of a pheromone (defined as the volume in space within which the concentrations are above the moth response threshold) will be larger for a given sample interval than that predicted by the Sutton or the Gaussian plume models. This explains why we observed wing-fanning responses at locations where the average concentration predicted by any of the three Gaussian models was several orders of magnitude below that which produced a similar response in the wind tunnel. This occurred even though we applied the Gaussian model to wing-fanning behavior that occurred over the appropriate time intervals for which they were designed.

Unfortunately there exists to date no comprehensive treatment of

dispersion that can supplant the Gaussian models. The instantaneous plume models derived from the work of Batchelor (1952) and developed for pheromone dispersion by Aylor et al. (1976) and Miksad and Kittredge (1979) predict concentrations at locations fixed in reference to the meandering plume centerline. To apply such models to locations fixed in space we would need a description of plume meander as well.

Correlation of Wind Direction with Moth Response. The association between the wind direction measured at the source and the subsequent onset of wing-fanning activity at locations downwind along that direction (Figure 3, Table 1) provides circumstantial support for the simple dispersion model of David et al. (1982). The model conceives of the pheromone plume as a continuous series of pheromone "parcels," each of which travels in a straight line after leaving the source, although the trajectory of consecutive parcels varies continually, as measured by the changing direction of a windvane. However, the fact that the association is not very strong suggests that, at least for the woodlot situation, at distances of 20 m or more the model is at best an approximation. Presumably the trajectory of individual parcels is influenced by collision with trees. However, a substantial proportion of the lack of correlation between the measured wind direction and the subsequent occurrence of wing fanning is undoubtedly due to the facts that wind direction samples occurred only at 12-sec intervals and that the wind direction sensor was positioned 10 m from the source.

Characteristics of the Behavioral Assay. Neither of the two methods for estimating percentage wing-fanning response was a completely satisfactory measure of *average* pheromone concentration during the sample interval. The first method, which focused on the proportion of moths that responded at any time during the interval (corrected for background), is clearly a measure of peak, not average, concentration. The second method, based upon the mean number of seconds spent wing fanning, is related to average concentration, but it incorporates a bias caused by the continuation of wing-fanning behavior following removal of the pheromone stimulus (Figure 2). Rack locations where the response occured late in the 15-min interval would show less time spent wing fanning than racks where the response occured early, even though both experienced the same average concentration. This would occur because the wing-fanning response would continue at the early location throughout much of the interval.

The threshold of wing-fanning response was also undoubtedly influenced by the air temperature as demonstrated in the wind tunnel (Cardé and Hagaman, 1983). Baker and Roelofs (1981) have shown that the size of the active space of the oriental fruit moth, *Grapholitha molesta,* pheromone varies with air temperature. Some of the variability associated with the probit regression lines (Figure 7, Table 2) was undoubtedly due to daily variation in temperature.

SUMMARY AND CONCLUSION

Time-average Gaussian dispersion models have been used to describe the size and shape of the pheromone active space or to estimate behavioral thresholds ever since they were first applied to pheromones by Wright (1958) and Bossert and Wilson (1963). This has occurred despite the arguments of Wright (1958) and Aylor et al. (1976) that instantaneous rather than average concentrations of pheromone determine responses and thus determine the size and shape of the active space. Furthermore, these models have generally been used by pheromone biologists without regard to the sample intervals or the terrain and atmospheric conditions to which they apply. Our results underscore the inadequacies of Gaussian dispersion models for describing pheromone active spaces. Even though we applied these models to the appropriate sample intervals and even though we attempted to use dispersion coefficients appropriate to the measured wind turbulence (standard deviation of the wind azimuth) and (for Fares' coefficients) appropriate to forest conditions, the pheromone concentrations predicted by these models at locations where we observed male gypsy moth wing fanning were several orders of magnitude lower than the minimum concentrations known to elicit wing fanning in a wind tunnel. This undoubtedly occurred because the moths were responding to the peak instantaneous concentrations rather than the average concentrations predicted by these time-average Gaussian models.

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A SUBSTANCE WHICH ACTS AS A pH INDICATOR FROM THE MOTH *Euchloron megaera* L.

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Abstract—Wing scales of the moth *Euchloron megaera L.* (Sphingidae) contain a water-soluble green pigment showing pH indicator properties. This pigment has been isolated. The UV-visible spectra, the color changes, the reduction by dithionite, the polarity on SiO_2 TLC, and the high molecular weight relate this pigment to an anthocyanidin derivative derived from the plant food. This result is discussed in connection with the presence of similar substances in plants of the family Apocynacea and in other moth species of the Sphingidae group, in which they play aposematic roles. As a hypothesis, it is suggested that the green pigment results from a reaction between *Nerium* sp. anthocyanidins (plant food) and NH_2 groups of basic constituents present in the animal.

Key Words—Pigment, anthocyanidin derivative, *Euchloron megaera*, Sphingidae, moth, plant-insect relationship, pH indicator.

INTRODUCTION

During the course of the last 20 years, we have been investigating blue-green pigments of insects. Three original substances belonging to the IXγ series of the bile pigments have been isolated in a systematic survey of butterfly and moth species, namely, pterobilin, phorcabilin, and sarpedobilin, and the corresponding structures have been established (Barbier, 1981). Different hypotheses have been advanced concerning the biological functions of pterobilin. This pigment could have a photoreceptive role related to the diapause determination or a photoprotective role in butterfly larvae as reviewed in preceding publications (Barbier, 1981; Bois-Choussy and Barbier, 1983a, b). Such green substances may also play a role in the capture of energy-rich photons, thus ensuring heat transfers necessary to flight in adults. The biomimetic significance is highly probable, chlorophyll being the most fre-

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quent background in nature. Because it may at least enter into one of these biochemical or biophysical considerations, we have been investigating the green pigment of *Euchloron megaera.* The isolation of this substance does not seem to have been reported as yet, and its properties are completely different from those of other butterfly blue–green pigments.

Euchloron megaera is a big Sphingidae of about 10-cm wing span, widely distributed in Africa, showing on its forewings a brilliant emerald-green pigmentation. This substance is water soluble and behaves as a pH indicator. The unstability of the substance in neutral media, the difficulties encountered in isolation and the relative scarcity of the animals have greatly impaired further analysis. The results so far obtained are discussed in relation to the biochemical and biophysical hypothesis.

METHODS AND MATERIALS

The animals used in this work were collected in Central Africa, the Bangui area. Larvae of this moth are believed to feed on oleander (*Nerium* species).

The green pigment present in the scales of the forewings can be extracted with boiling water. The extraction is easier in slightly alkaline media, and in a typical procedure, 10 wings were treated with 20 ml of boiling water containing 2 drops of concentrated ammonia. The filtered (cotton plug), vacuum-concentrated solution (deep emerald-green color) was submitted to a series of preparative thin-layer chromatographies (TLC). By using SiO_2 plates, 1 mm thick, a first development with the mixture ethyl acetate–methanol (3:2) allowed the elimination of many impurities, as the pigment had an R_f of 0 under such conditions and remained well separated. A second run of the same TLC was performed over a short distance, as most impurities migrated to the upper part of the plate. Developing with a mixture of acetic acid–water–HCl (30:10:2) yielded the pigment as a red band, R_f 0.70. Elution from SiO_2 TLC was obtained by treatment of the scraped powder with the same solvent mixture. The solution was concentrated under high vacuum at room temperature, using KOH traps, resulting in a dark-green glassy material. The final product was washed with a little ether and cold water, leading to an amorphous substance which was recovered through centrifugation.

The same product can be obtained by first extracting the wings with 2 *N* HCl at 80°C (red solution), then performing the same series of chromatographic separations and other washings.

By this method, ca. 0.4 mg of substance was obtained from 10 forewings of *Euchloron megaera;* a check of homogeneity through control TCL showed only one spot.

RESULTS AND DISCUSSION

The green pigment which accumulates within the scales of the forewings in the moth *Euchloron megaera* (Sphingidae) decomposes above 300°C (amorphous on a Kofler apparatus). This pigment acts as a pH indicator, giving a green or blue-green solution between pH 14 and pH 8, a yellow solution between pH 5 and pH 2, and a red solution below pH 1.5. UV-visible absorptions in water–ammonia (blue-green solution) were found at 280 and 630 nm, while the 2 *N* HCl solution gave absorptions at 290 and 500–510 nm. These solutions were discolored by sodium dithionite, with the formation of a yellow substance, R_f 0.60 [acetic acid–water–HCl (30:10:2), cellulose film], which could not be investigated further due to the minute amounts. The isolated pigment was unsoluble in the usual organic solvents. Weak acidic, basic, or neutral aqueous solutions furnished decomposition products. We did not succeed in obtaining a mass spectrum by using either electron impact, chemical ionization, or fast atom bombardment methods. The substance was precipitated from its solutions by lead tetraacetate, forming a blue-green complex from which the original pigment could not be recovered due to decomposition into a series of derivatives. No pyrrolic fragments were obtained by chromic acid oxidations, confirming that the substance does not belong (Bois-Choussy et al., 1974) to the bile pigment series.

The observed color changes and UV-visible absorptions are characteristic of anthocyanidins, and the formation of pale yellow pseudobases in weak acids (Harborne, 1967; Ribéreau-Gayon, 1968) agrees with the corresponding chromophore in the substance. Strong acidic solutions are required to get the stable forms in the anthocyan family as previously reported. The pseudobases are unstable and easily decompose at rather low pH ranges. The reduction by dithionite is an argument in favor of the presence of this chromophore in the structure. However, the high polarity as exemplified by the R_f of 0 in the classical butanol–acetic acid–water (4:1:5) system (cellulose film) and the green color at higher pH levels definitely differentiate the product from an ordinary anthocyanidin. The green color above pH 5 is in agreement with two chromophoric groups, one yellow, related to the pseudobase of an anthocyanidin structure, and the second blue, corresponding to the anionic form.

The properties observed for this substance are similar to those reported for products extracted from the flowers of *Nerium odorum* (Apocynacae) acting as pH indicators (Sanyal and Das, 1956), giving red solutions in acidic media (under pH 5) and green solutions above pH 8. Many Sphingidae moths are known to feed on *Nerium* spp., such as the oleander hawk-moth *Daphnis nerii*, feeding on *Nerium oleander*, which also presents a characteristic green wing pattern.

Dried red flowers of the common oleander collected in southern France, standing at room temperature in a dilute solution of ammonia, effectively give a green solution, but the pigment is quite unstable and cannot be extracted by the method used for the moths. This green form changes to red with HCl, but repetition leads to brown decomposition products. Extraction of the flowers with dilute HCl also gives the red solution of the pigment, which turns brown with the addition of ammonia, and in all cases, defined products could not be observed in chromatographical attempts. It appears, then, that the green pigment could occur from a reaction between plant anthocyanidins and basic NH_2 groups present in the animal, leading in the case of *Euchloron megaera* to a relatively more stable derivative. The flavonoids of *Nerium oleander* have been investigated by Paris and Duret (1972a,b) and contain flavonol glycosides derived from quercetol and koempferol, in which rutoside and nicotifloroside predominate.

The phenomenon of accumulation of a plant anthocyanidin derivative in Sphingidae moths could then be more general than thought at first. Having studied one specimen of the giant (12-cm wing span) South American *Eumorpha labruscae*, we have been able to establish the presence of a water-soluble green pigment in the wings, but not leading to defined products due to decompositions during the extractions.

Euchloron megaera, like many moths, sleeps during the day with the forewings covering the hind wings horizontally, and obviously the green pigment, also present within the body scales, has a determinant mimetic role. For such animals under these conditions, the chlorophyll green of plant leaves is the most common background, and the pigment which is accumulated from the plant food during the larval stage leads to a good camouflage. This adaptation is, of course, the result of the ecological niche moth–plant, ensuring the preservation of the species by an incidental use of a plant metabolite. However, other biochemical uses of this substance during larval development are possible, with the same significance as proposed before for butterfly blue-green bile pigments, as they are all sensitive photoreceptors of the red wavelengths. Thus, the heat accumulation necessary for flight recuperation in these animals, which fly long distances, is rendered possible by the green pigment, a specific receptor of the calorific, energy-rich photons.

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LIGHT-DEPENDENT TOXICITY OF α-TERTHIENYL AND ANTHRACENE TOWARD LATE EMBRYONIC STAGES OF *Rana pipiens*

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Abstract—Alpha-terthienyl is toxic to late embryonic stages of *Rana pipiens* in the presence of sunlight. Neither α-terthienyl alone in the dark nor a previously photolyzed solution of α-terthienyl has comparable activity. The LC_{50} was 0.11 ppm with 30 min of exposure and 0.018 ppm with 2 hr of exposure to sunlight. Anthracene, a representative example of polycyclic aromatic hydrocarbons widely distributed in the environment, also showed similar phototoxicity, with an LC_{50} of 0.065 ppm after 30 min of exposure and 0.025 ppm after 5 hr.

Key Words—*Rana pipiens*, ultraviolet light, sunlight, photosensitization, α-terthienyl, anthracene, water.

INTRODUCTION

Alpha-terthienyl (Scheme 1) occurs naturally in *Tagetes erecta* and in many other planting species in the family Compositae. It has been found to display light-enhanced toxicity toward a number of biological organisms, such as bacteria and yeasts (Chan et al., 1975; Towers et al., 1977; Arnason et al., 1981a; Kagan et al., 1980; Kagan and Gabriel, 1980; Downum et al., 1982), nematodes (Gommers, 1972), and the larvae (Arnason et al., 1981b; Wat et al., 1981; Kagan et al., 1983) and eggs (Kagan and Chan, 1983) of insects. It is also capable of preventing the germination of seeds and killing plant seedlings (Campbell et al., 1982), as well as acting as an herbicide (Harvey, Jr., 1963).

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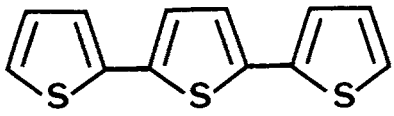

SCHEME 1. Alpha-terthienyl.

The activity level of α-terthienyl against larvae of mosquitoes compares favorably with that of DDT (Arnason et al., 1981b), and this high activity level, the absence of halogens in the molecule, its natural origin, and its ease of photodegradation to inactive products suggested that this compound might become useful as a photoactive insecticide, particularly since larvae of *Aedes aegypti* mosquitoes from strains which had acquired resistance to DDT and to Dieldrin were affected by α-terthienyl to the same extent as the nonresistant ones in the laboratory (Kagan et al., unpublished results). Even though α-terthienyl does occur naturally, its potential for practical applications as an insecticide and as an herbicide will critically depend upon its environmental acceptability. Little is known in this area.

Although the phototoxicity of α-terthienyl against certain algae has been studied (Arnason et al., 1980), nothing was known about the susceptibility of other important aquatic organisms to this powerful sensitizer. We now describe that late embryonic stages of *Rana pipiens*, an organism widely distributed throughout the world, are very severely affected by the presence of α-terthienyl in water.

METHODS AND MATERIALS

Alpha-terthienyl was synthesized in the laboratory (Beny et al., 1982). Anthracene (sensitizer grade) was purchased from Baker and used without further purification. The organisms were collected at the inlet of the "Fifty-Fifth Street Pond" in Downers Grove, Illinois, on June 5, 1983, and were kept in aerated pond water. They were identified as *Rana pipiens*, embryonic stage Schumway 25. All the experiments were carried out within 2 days in beakers or petri dishes, each containing 20 ml of pond water, 20 organisms, and the amount of α-terthienyl necessary for achieving the concentrations shown in Figure 1. The sensitizer was dissolved in 95% ethanol, and 10–80 μl of a suitably diluted solution was used in these experiments. A control experiment in which the organisms were kept in the dark was performed with the highest nominal concentration of α-terthienyl used (8 ppm). For the control experiments, testing in the dark the toxicity of the photoproducts from α-terthienyl, an 8-ppm solution was irradiated with sunlight for 3 hr. The organisms were placed in this solution and kept in the dark for 24 hr. With anthracene, the same experiment used a solution containing 2 ppm. One

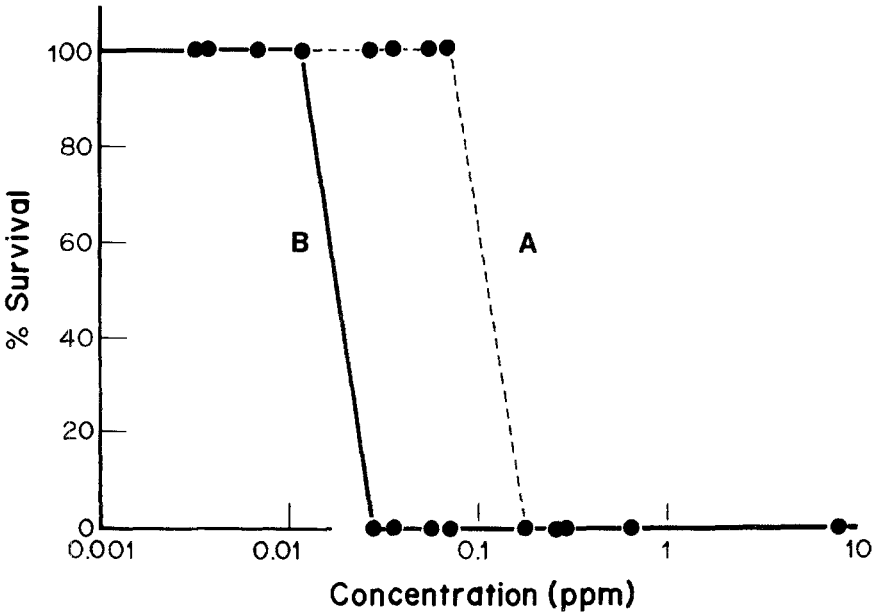

FIG. 1. The phototoxicity of α-terthienyl to embryonic stages of *R. pipiens:* (A) 30-min irradiation; (B) 2-hr irradiation.

control experiment was performed with each series of photolyses, where the organisms were irradiated in water containing 80 μl of ethanol without any added sensitizers. No mortality was induced by 2.5 hr of exposure.

RESULTS AND DISCUSSION

The initial experiment in which the phototoxicity of α-terthienyl was tested utilized 20 ml of solution containing 8 ppm of sensitizer. The organisms were added and they were immediately exposed to sunlight. Within 20 min, all had died. In control experiments conducted in the dark, the same concentration of sensitizer had no effect on the organisms over the same time period. The sensitizer had some intrinsic toxicity, however, and while all the organisms were still alive after 8 hr of contact in the dark, they were dead 12 hr later. The observed light-enhanced toxicity, which was independent of the presence of ethanol, could not be due to the photochemical conversion of α-terthienyl into toxic products, because an identical solution exposed to sunlight for the same length of time did not kill the organisms which were later introduced into it. In these experiments in which the activity of the α-terthienyl photoproducts was tested, the organisms were still alive after 36 hr of exposure to the solution.

The control experiments prove that the phototoxicity of α-terthienyl depends on the simultaneous presence of the organisms with the sensitizer and with the light and was not due to the toxicity in the dark of products which might have been formed by the photolysis of the sensitizer.

In order to obtain a more quantitative assessment of the phototoxicity, a series of experiments was performed in which the concentration of α-terthienyl was varied (Figure 1).

The population of embryonic stages of *R. pipiens* which was tested was very homogeneous and, in every experiment where mortality was observed, all the organisms had died within minutes of each other. The higher concentrations of sensitizer led to a faster death of the organisms. All the experiments with concentrations of sensitizer between 8 and 0.028 ppm required between 20 min and 2 hr of exposure to sunlight in order to achieve 100% mortality. At lower concentrations (down to 3.4 ppb), the organisms survived a total of 20 hr of irradiation over 2 days, but they had died by the morning of the third day.

The mortality curves corresponding to 30 min and 2 hr of exposure to sunlight are presented in Figure 1. The LC_{50} values are 0.11 and 0.018 ppm, respectively. Further studies will attempt to measure phototoxicity effects on a larger pool of organisms at different developmental stages over longer periods of time. Perhaps such studies will produce survival curves with intermediate points between 0 and 100% mortality which will define the LC_{50} values more closely. While this would certainly be desirable, the sharpness of the breaks observed in Figure 1 provides sufficient confidence that the errors of the values herein reported cannot be very large. In any case, these values cannot be more than an imprecise indicator of phototoxicity, because they depend on a large set of mostly irreproducible parameters defining the biological organisms in their previous natural environment, as well as the conditions of the irradiation such as fluence, wavelength distribution, and temperature.

The mechanism by which the toxicity is produced is unknown, as is the relationship (if any) between the process in the dark and that in the presence of light. Alpha-terthienyl has been shown to sensitize the denaturation of several important enzymes *in vitro* (Bakker et al., 1979; Gommers et al., 1980), and a correlation between phototoxicity and inactivation of acetylcholinesterase activity has now been demonstrated *in vivo* with insect larvae (Kagan and Grynspan, to be published). The requirement for the simultaneous presence of sunlight, the organisms, and the sensitizer in the light-enhanced toxicity is in agreement with an oxygen-dependent process, as was proved to be the case in the phototoxicity toward microorganisms and nematodes. While the powerful singlet-oxygen sensitizing ability of α-terthienyl has been demonstrated *in vitro*, it is impossible to perform the neces-

sary control experiments in the absence of oxygen with organisms which are oxygen dependent, as in the present instance. Glass filters which cut off radiation below 330 nm did not protect the organisms at all when tested at 0.15 ppm. On the other hand, filters which cut off radiation below 410 nm completely protected the organisms at the same concentration level of sensitizer which led to the death of all the unprotected organisms within 20 min. Consequently, the active wavelengths must be between 330 and 410 nm, the region of maximum absorption of the sensitizer itself (λ_{max} = 350 nm in ethanol).

The rate of degradation of α-terthienyl in the environment is probably dependent upon many variables, which remain to be studied. A crude experiment supported views based upon laboratory data in organic solutions which showed that the continued irradiation of α-terthienyl led to extensive degradation into products which were inactive in the dark as well as in the presence of light. The exposure to sunlight of a solution of α-terthienyl (4 ppm) for about 7 hr markedly reduced the phototoxicity. For example, a group of tadpoles was killed in 45 min under the sunlight in a fresh solution containing

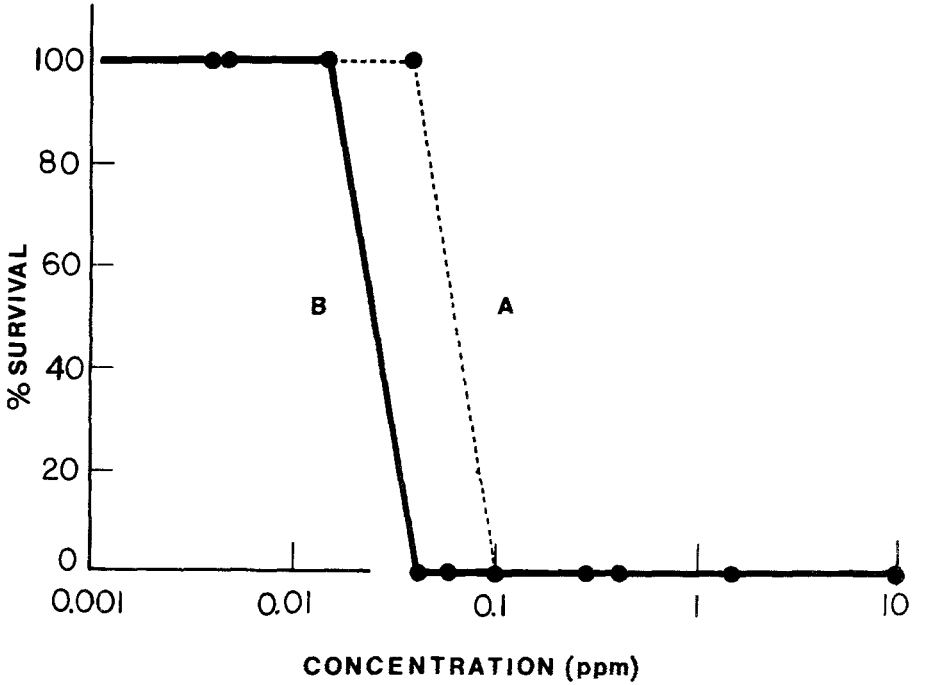

FIG. 2. The phototoxicity of anthracene to embryonic stages of *R. pipiens:* (A) 30-min irradiation; (B) 5-hr irradiation.

0.1 ppm of sensitizer, while a similar group survived longer than 150 min in the irradiated solution which had been diluted to the same concentration level.

Although a review article by Towers (1980) disclosed that phenylheptatriyne was phototoxic to juvenile rainbow trout (*salmon gairdneri*), a recent study provided the first thorough discussion of phototoxicity of an organic molecule to fish (Bowling et al., 1983). In that work, anthracene, a widely distributed polycyclic aromatic hydrocarbon, was found to be toxic to juvenile bluegill sunfish (*Lepomis macrochirus*) at a concentration of 0.0127 ppm. We had previously observed the phototoxicity of several polycyclic aromatic hydrocarbons toward the larvae of the mosquito *Aedes aegypti* and of the fruit fly *Drosophila melanogaster* (to be published), and we also found that anthracene was toxic to the immature *R. pipiens* in the presence of sunlight but not in its absence. The nominal LC_{50} value for 30 min of exposure, without lengthy prior contact with the sensitizer, was 0.065 ppm. After 5 hr, it was 0.025 ppm (Figure 2). This value is not very different from that reported for the bluegill sunfish.

CONCLUSION

The results presented in this report indicate that *Rana pipiens* is very sensitive to the photosensitizing effects of very low concentrations of chemicals in water. The toxicity of α-terthienyl toward late embryonic stages of *R. pipiens* has been shown to be greatly enhanced in the presence of long-wavelength ultraviolet light (330–410 nm). Since α-terthienyl is produced by many plants in the family Compositae, its release into the environment through any mechanism may be responsible for subsequent photosensitized killing of many organisms, including some in aquatic environments. Also, while this sensitizer is susceptible to light-induced degradation, one must still be concerned with the possibility that any applications of α-terthienyl for insect control might lead to the presence of small amounts in natural waters and be responsible for the death of desirable aquatic organisms. Further work is required to determine the rate of degradation of α-terthienyl in natural environments and to ascertain the importance of this plant constituent in affecting the survival of distant organisms in their surroundings. Similarly, the widespread dispersion of anthracene and other polycyclic hydrocarbons in the environment may have dramatic effects on the survival of many organisms. Our results, which expand the scope of the observations on fish by Bowling et al. (1983), suggest the need for more detailed studies in this area. These authors pointed out that while photo-induced mortality may not occur in deeper, more turbid, waters or in shaded areas, juveniles of most fish are found in shallow areas of the littoral zone or on the surface as pelagic larvae,

where they would be extremely vulnerable. The same considerations probably apply to many other aquatic organisms, such as *R. pipiens*, which is discussed in this report.

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RESPONSE OF *Diabrotica virgifera virgifera*,[1] *D. v. zeae*,[1] AND *D. porracea*[1] TO STEREOISOMERS OF 8-METHYL-2-DECYL PROPANOATE[2]

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Abstract—The four stereoisomers of 8-methyl-2-decyl propanoate were tested in the United States and Mexico for attractiveness to *Diabrotica virgifera virgifera* LeConte, the western corn rootworm, *D. v. zeae* Krysan and Smith, the Mexican corn rootworm, and *D. porracea* Harold. Males of *D. v. virgifera* and *D. v. zeae* responded strongly to the (2R,8R)-isomer and secondarily to (2S,8R), while *D. porracea* responded exclusively to the (2S,8R)-isomer. The (2S,8S)- and (2R,8S)-isomers were inactive in all tests. Synergism or inhibition was not detected when various mixtures of the isomers were tested with *D. v. virgifera*. These phenomena were not tested with *D. v. zeae* and *D. porracea*.

Key Words—Coleoptera, Chrysomelidae, *Diabrotica*, western corn rootworm, Mexican corn rootworm, sex pheromone, stereospecificity.

INTRODUCTION

The sex pheromone of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) (WCR), was recently shown to be the propionate ester of 8-methyl-2-decanol (Guss et al., 1982). This compound was the first pheromone to be identified from the family Chrysomelidae, and, like most known coleopteran pheromones, it is chiral (two

[1]Coleoptera: Chrysomelidae.
[2]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

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asymmetric centers). Lack of sufficient quantities of pure pheromone precluded elucidation of the specific configuration of the natural pheromone, but at low doses (about 350 ng), racemic synthetic 8-methyl-2-decyl propanoate dispensed from cotton wicks was equal to a similar amount of pure natural pheromone in its ability to attract feral WCR males to baited traps (Guss et al., 1982).

Racemic 8-methyl-2-decyl propanoate has been shown to be attractive to a number of *Diabrotica* spp. (Guss et al., 1982). The Mexican corn rootworm, *D. virgifera zeae* Krysan and Smith (MCR), is attracted to racemic 8-methyl-2-decyl propanoate in a manner identical to that of the WCR with respect to time-of-day arrival at baited traps. These two taxa are subspecies and have been shown to intergrade in those areas where their ranges contact (Krysan et al., 1980). Another congener, *D. porracea* Harold, which cohabits the same areas as the MCR in this study, also responds to the racemic synthetic pheromone (Guss et al., 1982), but no information is available concerning reproductive interaction between the MCR and *D. porracea*.

Synthesis of the four stereoisomers of 8-methyl-2-decyl propanoate at high isomeric purity has been accomplished (Sonnet and Heath, 1982; Carney et al., unpublished), and here we report the response of the WCR, MCR, and *D. porracea* to these individual isomers. The data yield an insight into the stereochemical composition of the natural pheromone produced by WCR females.

METHODS AND MATERIALS

Syntheses of the two stereoisomers used in this study involved a convergent approach in which two fragments, each containing one asymmetric center, were joined to complete the required sequence. The configurational purity of each fragment, which assured the configurational purity of the final product, was determined absolutely by GLC and/or HPLC using diastereomeric derivatives (Sonnet and Heath, 1982; Carney et al., unpublished). The isomeric purity of the target isomers in these preparations ranged from 97.4 to 98.8% (Table 1).

Usually, the individual isomers or specific mixtures were diluted to appropriate concentrations in hexane and dispensed into the "cup" portion of rubber septa (A.H. Thomas No. 8753-D22) in 50-μl quantities to produce the pheromone sources. In Mexico cotton wicks (30 $\times$ 10-mm diameter) were used as dispensers to compare the activities of unfractionated WCR volatiles with synthetic pheromone because the volume of the unfractionated preparation (1 ml) was too large to be accommodated by rubber septa. When cotton wicks were used, synthetic pheromone was diluted with hexane containing 10% trioctanoin to regulate coarsely volatilization of the phero-

TABLE 1. ISOMERIC COMPOSITION OF 8-METHYL-2-DECYL PROPANOATE PREPARATIONS

Excess isomer	% isomeric composition			
	2*S*,8*S*	2*R*,8*S*	2*S*,8*R*	2*R*,8*R*
2*S*,8*S*	98.8	0.4	0.8	—
2*R*,8*S*	0.9	98.3	—	0.8
2*S*,8*R*	1.9	—	97.4	0.7
2*R*,8*R*	—	1.9	0.3	97.8

mone (Guss, 1976). Collection of unfractionated volatiles from WCR females is described elsewhere (Guss et al., 1982).

Field traps for studies with the WCR consisted of plastic (PVC) cylinders (45 × 5-cm diameter) wrapped in waxed paper and coated with Stickem Special. The traps were placed between corn rows on wooden stakes (2.5 cm^2 × 1.3 m) equipped with pegs to control trap height. The trap height at the top was normally first ear height or about 1 m, and the distance between traps was about 20 m. For studies with the MCR and *D. porracea* in Mexico, traps consisted of wax-coated cylindrical drink cups (11.2 × 9.0-cm diameter) coated inside and out with Stickem Special. Pheromone sources were anchored to the tops of the traps, and the traps were monitored daily.

Experiments involving the WCR were done near Brookings, S.D., during August 1981, and those involving the MCR and *D. porracea* were done near Ameca in the state of Jalisco, Mexico, during September and October 1981.

RESULTS AND DISCUSSION

Western Corn Rootworm. Under field trapping conditions, the response threshold of racemic 8-methyl-2-decyl propanoate for the WCR is about 10 ng when dispensed from rubber septa (Guss et al., 1982). When assessing the activity of the individual isomers, we at first chose a loading of 250 ng in order to obviate as much as possible influences that might be attributable to the small amounts of nontarget isomers in these preparations (see Table 1). Thus, in most cases with the 250-ng sources, the amount of any given nontarget isomer was less than that found in 10 ng of racemic pheromone. In the case of (2S,8S) in (2S,8R) and 2R,8S) in (2R,8R), the amount of nontarget isomers was slightly more than that found in 10 ng of the racemate.

With the 250-ng sources, data in Table 2 show that (2R,8R) was the preferred configuration, being about four times as active as (2S,8R). Both (2S,8S) and (2R,8S) were inactive. With 1-μg sources, (2R,8R) was about twice as active as (2S,8R), while (2S,8S) and (2R,8S) were again inactive.

TABLE 2. RESPONSE BY *D. v. virgifera* MALES TO STEREOISOMERS OF 8-METHYL-2-DECYL PROPANOATE

Isomer	Mean No. of males/trap ± SD[a]	
	250 ng	1 μg
Racemate	104.3 ± 19.7 b	—
2*R*,8*R*	148.3 ± 44.7 a	607.3 ± 66.1 a
2*S*,8*R*	39.0 ± 5.3 c	306.7 ± 93.3 b
2*R*,8*S*	2.0 ± 1.0 d	8.0 ± 2.0 c
2*S*,8*S*	4.0 ± 3.5 d	10.7 ± 3.2 c
Solvent blank	6.3 ± 1.2 d	5.3 ± 2.9 c

[a]Means followed by different letters are significantly different at the 0.05 level of confidence (Duncan's multiple range).

The difference in the ratio of trapped insects between (2R,8R) and (2S,8R) at 250-ng and 1-μg loadings may not be meaningful because the experiments were carried out at different locations with different population densities. Also, 250 ng of (2S,8R) may be approaching the response threshold for this isomer under these conditions. Thus, at 250 ng the mean for (2S,8R) was significantly different at the 0.05 level but was not significantly different from (2R,8S), (2S,8S), and the solvent blank at the 0.01 level.

The R configuration at carbon 8 appears to be critical, while either configuration at carbon 2 (site of the ester bond) at least partially satisfies the criteria for positive response for the WCR. Most chiral pheromones thus far identified contain asymmetric centers at or near functional groups (Silverstein, 1979); in several recent cases, however, specific configurations at centers relatively remote from functional groups of pheromones have been shown to be essential (Kraemer et al., 1981) or extremely important (Silverstein et al., 1980); Guss et al., 1983a) for attraction of target insects to baited traps.

The most active isomer, (2R,8R), was combined with the other three isomers, individually, at 1:1 ratios to determine if synergistic or inhibitory phenomena existed. The results in Table 3 indicate that the trap catches obtained with these mixtures were no different from those from traps baited with (2R,8R) only and that the other three isomers, including (2S,8R), neither synergize nor inhibit the response. In further studies involving the two independently active isomers, mixtures containing a constant amount of (2R,8R) plus up to 67% (2S,8R) [1 μg (2R,8R) + 2 μg (2S,8R)] produced captures (35.44 ± 11.22) insignificantly different from those of traps baited with (2R,8R) alone (27.75 ± 5.85).

A dose–response effect was demonstrated with (2R,8R) from 0.25 through 1000 μg, with the data in Table 4 being best characterized by the

TABLE 3. RESPONSE BY *D. v. virgifera* MALES TO MIXTURES OF 8-METHYL-2-DECYL PROPANOATE

Isomer mixture	Mean No. of males/trap ± SD[a]
1 μg 2*R*,8*R*	347.0 ± 123.5 a
1 μg 2*R*,8*R* + 1 μg 2*R*,8*S*	312.5 ± 70.4 a
1 μg 2*R*,8*R* + 1 μg 2*S*,8*R*	277.3 ± 76.2 a
1 μg 2*R*,8*R* + 1 μg 2*S*,8*S*	321.0 ± 92.0 a
Solvent blank	8.3 ± 1.5 b

[a]Means followed by different letters are significantly different at the 0.01 level of confidence (Duncan's multiple range).

equation $y = 136.49 + 90.32 \ln x$ ($R^2 = 0.97$). Notwithstanding the high R^2, the number of WCR captured in traps baited with 1 mg of (2R,8R) may not accurately reflect the number of beetles actually influenced by the source. Several meters downwind from those traps baited with 1 mg of (2R,8R), dramatic increases in beetle density and activity were observed, and it appeared that many of the beetles attracted to the area had ceased searching and were attempting to copulate with other males.

Mexican Corn Rootworm and D. porracea. We showed in an earlier report that both the MCR and *D. porracea* are attracted to traps baited with racemic 8-methyl-2-decyl propanoate (Guss et al., 1982). The response of these two species to the resolved isomers is shown in Table 5, Expt. I. The response of the MCR to (2R,8R) and (2S,8R) is virtually identical to that of the WCR. This was not surprising considering the close relationship of these two taxa (Krysan et al., 1980). The response of *D. porracea*, which cohabited this trap area in very low numbers, was limited exclusively to (2S,8R).

TABLE 4. DOSE-RESPONSE EFFECTS OF 8*R*-METHYL-2*R*-DECYL PROPANOATE ON TRAP CATCHES OF *D. v. virgifera*

Dose (μg)	Mean No. of males/trap ± SD
0.25	66.0 ± 18.5
1	123.3 ± 53.3
10	279.7 ± 41.7
100	524.3 ± 176.6
1000	811.7 ± 405.2
Solvent blank	1.7 ± 2.9

TABLE 5. RESPONSE BY *D. v. zeae* AND *D. porracea* TO STEREOISOMERS OF 8-METHYL-2-DECYL PROPANOATE

Isomer	Experiment I[a] (Mean No. of males/trap ± SD)		Experiment II[b] (Mean No. of males/trap ± SD)	
	D. v. zeae	*D. porracea*	*D. v. zeae*	*D. porracea*
2*R*,8*R*	33.3 ± 3.8	0	285 ± 102	1 ± 1.4
2*S*,8*R*	17.3 ± 4.6	17.0 ± 1.7	119 ± 5.7	35 ± 2.8
2*R*,8*S*	2 ± 1	0	—	—
2*S*,8*S*	0	0	—	—
D. v. virgifera volatiles	—	—	370 ± 98	0
Solvent blank	0	0	0	0

[a]Sept. 15–18, 1981, 10 μg/source dispensed from rubber septa.
[b]Oct. 20–23, 1981, 1 μg of (2R,8R) or (2R,8S) and an estimated 1 μg of active material in *D. v. virgifera* volatiles dispensed from cotton wicks.

At a later date, unfractionated volatiles from WCR females were compared with (2R,8R) and (2S,8R) in the same area (Table 5, Expt. II). As expected, the MCR responded to all three sources, but *D. porracea* again responded overwhelmingly to the synthetic (2S,8R)-isomer and not at all to the unfractionated WCR volatiles. Circumstances associated with this experiment suggest that the two *D. porracea* males captured on one of the (2R,8R) traps may not be accidental. The (2R,8R) preparation is known to contain 0.3% (2S,8R) (Table 1), and the inherently rapid release rate of pheromone from cotton wicks may have resulted in the release of (2S,8R) in quantities sufficient to attract *D. porracea* in the immediate trap area. The very low numbers of *D. porracea* in the trap area would tend to support this hypothesis; intense visual searches failed to produce a single, free-moving *D. porracea* adult except in areas immediately adjacent to traps baited with the (2S,8R) preparation.

The result that *D. porracea* responds specifically to (2S,8R) but also to racemic 8-methyl-2-decyl propanoate indicates that none of the other three isomers inhibits to extinction the response of *D. porracea* to (2S,8R)-8-methyl-2-decyl propanoate. Thus, the failure of *D. porracea* to respond to unfractionated volatiles (known to contain pheromone) from WCR females would indicate that (2S,8R) is not a component of the natural WCR pheromone, despite the fact that WCR males respond to the (2S,8R)-isomer. It would seem remote that either (2S,8S) or (2R,8S) would be a component of the natural WCR pheromone since neither displayed any discernable biological activity, either alone or in conjunction with (2R,8R). Therefore, the

data collected in this study would suggest that the sex pheromone produced by WCR females is exclusively (2R,8R)-8-methyl-2-decyl propanoate.

The untested possibility remains that the unfractionated WCR volatiles could contain a compound(s), other than any of the four pheromone isomers, that inhibits the response of *D. porracea* to the (2S,8R)-isomer, in which case (2S,8R) could be present in the WCR volatiles and be undetectable by the behavior of *D. porracea*. A partial negation of this possibility is available in the response to various pheromone preparations by another congener, *D. barberi* Smith and Lawrence. Thus, *D. barberi* responds strongly to WCR virgin females and unfractionated volatiles therefrom (Guss, 1976; Bartelt and Chiang, 1977), responds only to (2R,8R) among the individual isomers, and is strongly inhibited in its response to (2R,8R) by the presence of very small amounts of the (2S,8R)-isomer (Guss et al., unpublished).

In his review, Silverstein (1979) has listed the response of insects to chiral pheromones in nine possible categories. Assuming from our data that (2S,8R) is not a component of the natural pheromone of the WCR, the response of male WCR to (2S,8R) would place it in category 1, i.e., "The insect produces only a single enantiomer (stereoisomer) and it is more active than the other enantiomer (stereoisomer), which is an artifact." This category of response appears to be the most common among those insects using chiral pheromones.

In the case of the WCR, the relatively high response to (2S,8R) is of no conceivable consequence since there are no other known cohabiting *Diabrotica* that respond secondarily or exclusively to (2S,8R), and therefore, (2S,8R) is probably not encountered by WCR males in their natural environment.

A somewhat different situation exists with the MCR. Since *D. porracea* responds exclusively to (2S,8R), it seems likely that the sex pheromone of this insect is largely, if not exclusively, composed of the (2S,8R)-isomer.

Cohabiting some areas with *D. porracea*, the MCR male, which responds strongly but secondarily to (2S,8R), would probably be exposed to (2S,8R) as an environmental constituent. The consequences, if any, are difficult to predict since relatively little is known about the reproductive behavior of the MCR, and virtually no such information is available for *D. porracea*. It is possible, of course, that the response of *D. porracea* to the (2S,8R)-isomer is fortuitous or that *D. porracea* females, in addition to (2S,8R), produce another compound which inhibits the response of the MCR to (2S,8R), in which cases the cohabiting situation might well be inconsequential.

In addition to those mentioned in this report, three other *Diabrotica* are now known to be attracted to 8-methyl-2-decyl propanoate. They include *D. barberi* (the northern corn rootworm), *D. longicornis* (Say), and an undescribed species found in Peru (Guss et al., 1982; J.L. Krysan, personal communication). These three *Diabrotica* and the three mentioned earlier are

all in the *virgifera* species group (Smith and Lawrence, 1967). This group was erected on morphological grounds, and subsequently, members of this group were found to share similar life histories with respect to host relationships and egg diapause (Branson and Krysan, 1981; Krysan, 1982).

With one exception, all *virgifera* group *Diabrotica*, that we have knowingly exposed to 8-methyl-2-decyl propanoate (racemic and/or certain of the resolved stereoisomers), have responded to baited traps. The exception of which we are aware is *D. cristata* (Harris), a nonpest species found primarily in relict prairies throughout the eastern United States (Wiesenborn and Krysan, 1980). In the summer of 1982, however, we found that males of *D. cristata* strongly respond to both racemic and (2S,8R)-8-methyl-2-decyl acetate (Guss et al., 1983b). It would thus appear that 8-methyl-2-decanol may be a common biosynthetic precursor among *Diabrotica* in the *virgifera* species group.

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RESPONSE OF THE EUROPEAN ELM BARK BEETLE, *Scolytus multistriatus*,[1] TO HOST BACTERIAL ISOLATES

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Abstract—The response of the European elm bark beetle, *Scolytus multistriatus*, to host bacterial isolates was studied qualitatively under field conditions. Initial experiments indicated that such isolates were attractive to in-flight beetles. These isolates, identified as *Bacillus subtilis* (five strains), *B. pumilus*, and *Enterobacter cloacae*, were grown on nutrient agar in glass vials and attached to sticky traps in elm woods. Although beetles were caught on the bacterial isolate-baited traps, the catches were variable, inconsistent, and often contradictory from one experiment to another. High numbers of *S. multistriatus* were caught on traps baited with three strains of *B. subtilis*, but in addition to the *subtilis* strains, there were also aerial contaminants in the treatments, namely *E. aerogenes*, *Corynebacterium* sp., and *Flavobacterium* sp. Also, relatively high catches were recorded on nutrient agar controls. When elm wood-bark plugs, sterilized (by gamma irradiation) and unsterilized, were placed in vials with the host bacterial isolates, the presence or absence of fresh elm, gamma irradiated or not, had no noticeable effect on beetle attractancy.

Key Words—Smaller European elm bark beetle, *Scolytus multistriatus*, Coleoptera, Scolytidae, elm bacterial isolates, gamma irradiation, trapping.

INTRODUCTION

The European elm bark beetle, *Scolytus multistriatus* (Marsham) (Coleoptera: Scolytidae), is attracted to weakened or dying elms by host-produced odors (Meyer and Norris, 1967), which are considered weak attractants (Peacock et al., 1971). However, virgin females, initially attracted by the host

[1]Coleoptera: Scolytidae.

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or primary attractant, produce a pheromone that is responsible for the aggregation of large numbers of male and female beetles (Peacock, 1975). The components of this aggregation attractant have been synthesized, and the mixture, multilure, has been shown to be attractive to endemic *S. multistriatus* populations (Pearce et al., 1975). Other scolytids produce powerful attractant pheromones (Brand et al., 1975), which complicates any investigation of primary attractants (Moeck et al., 1981).

Graham (1968) reported that the major component of the host attractant for ambrosia beetles was some unidentified by-product of anaerobic metabolism in logs. However, Moeck et al. (1981) found no significant numbers of scolytids attracted to pine bolts previously held under vacuum for several hours. In a laboratory study using Douglas-fir, the scolytid *Gnathotrichus retusus* Le C., when given the choice between fresh logs and logs fully submerged under water for 8 hr, initiated galleries in the latter material only. On visually examining the bacterial colonies cultured from the three areas comprising the bark–sapwood interface, sapwood, and heartwood, and following the technique of Knutson (1970), there was a significantly greater number of colonies found in the submerged logs compared to the untreated logs, particularly in the bark–sapwood interface (French, unpublished data). Also, when collecting *Xyleborus dispar* L. from a cherry orchard in Oregon (French, 1972), the beetles were found initiating galleries in lenticular tissues and mainly on the underside of branches. On examining the attacked cherry branches, all were infected with the bacterial canker, *Pseudomonas syringae* van Hall. This suggested to one of us (JRJF) at the time that bacterial volatiles may be implicated in some way with host attractants. Person (1931) also proposed the concept of host selections by bark beetles via microbial volatiles. Brand et al. (1975) isolated various microorganisms from the gut of adult male and female *Ips paraconfusus* Lanier and determined their ability to transform α-pinene into *cis*- and *trans*-verbenol. They considered that such microorganisms may play a significant role in the synthesis of certain pheromones occurring in the frass of these bark beetles. Chararas (1977) concluded from feeding experiments involving antibiotics that bacterial conversion of ingested monoterpenes is possible but not essential for pheromone production in certain *Ips* species. White et al. (1980) considered that, in some cases, microorganisms contribute, possibly via mixed-function oxidase involvement, to terpene pheromone production. *Pseudomonas putida* (Trevisan) Migula, when treated with α-pinene, produced a variety of acidic fragments and an olide (Tudroszen et al., 1977). But as White et al. (1980) mentioned, apparently the breakdown pattern and metabolic pathway of α-pinene are as different among various bacterial strains as they are among different mammalian or insect species.

Moeck et al. (1981) have reviewed the literature on primary attraction and outlined alternative hypotheses such as initial random landing of beetles

on various hosts and nonhosts. Hynum and Berryman (1980) reject the theory of primary attraction proposed by Renwick and Vité (1970) and others and the random attack (gallery initiation) theory proposed by Callaham (1952) and Vité and Pitman (1968). They considered that landings of *Dendroctonus ponderosae* Hopkins appear to occur at random over the available vertically distributed surface area, and gallery initiation stimulants determine when and where trees are attacked. Thus, Hynum and Berryman (1980) maintain that a gallery initiation stimulant appears to be the controlling factor in the host selection process.

All the available evidence on primary attraction indicates the complicated and often seemingly contradictory nature of its role in bark beetle attraction (see Wood, 1972; Heikkenen, 1977). With this in mind, and with recent indications that multilure baits are not as attractive to *S. multistriatus* beetles as odors emitted from multilure-elm log combinations (J.W. Peacock, personal communication), it seemed appropriate to reexamine the role of host bacteria in elm bark beetle attraction.

In a preliminary experiment in Melbourne, billets (300 mm long, 100- to 150-mm diameter) of fresh elm (*Ulmus procera* Salisb.) were end-coated with paraffin wax and placed separately in polyethylene bags for 1 week at 26° C. After this treatment, the billets were removed from the bags and hung on elms together with untreated billets. Both sexes of *S. multistriatus* were attracted to the treated billets within a week. In comparison, very few beetles were found on the untreated billets. Cultures of bacteria isolated from these "sealed" elm billets were attached to sticky traps on elms and found attractive to in-flight *S. multistriatus* (French, unpublished data).

This paper presents the results of field experiments conducted over several years showing the response of endemic *S. multistriatus* populations to elm bacterial isolates. Isolates found attractive in Experiment 1 (see below) were used in subsequent experiments. Also, with the knowledge that gamma-irradiated elm is less attractive to *S. multistriatus* than untreated elm (French et al., 1982), we examined the response of these beetles to elm bacterial isolates grown in the presence of sterilized and unsterilized elm.

METHODS AND MATERIALS

Preparation of Elm Billets and Plugs

Billets (300 mm long, 100- to 150-mm diameter) of fresh elm (*U. procera*) were used in Experiments 1 and 2; all the bacterial isolates subsequently used in Experiments 3 and 4 were isolated from the initial billets. Circular plugs of elm (approximately 6-mm diameter) used in Experiment 4 were cut from fresh elm with a hollow steel chisel. Each plug contained bark and wood.

Bacterial Isolation and Culture

Fresh elm billets were scraped at the inner bark/sapwood interface with a sterilized hacksaw blade and the resultant sawdust was sprinkled on the surface of nutrient agar (Difco) in petri dishes (90-mm diameter) (Greaves and Savory, 1965). All cultures were grown at 26° C; various bacteria were separated visually and single colonies cultured. Isolates not immediately required during the spring and summer, and those kept over winter, were stored at 4° C. Duplicate cultures of all isolates obtained were sent to the Commonwealth Mycological Institute (CMI) in Kew, England, for identification.

Experiment 1

In Australia over the last few years, *S. multistriatus* has emerged in early October and continued to fly until late March, with flight peaks in late October–November and late January–early February. This experiment was conducted during November–December 1976 in two Melbourne parks (A and B) 2 km apart. Replicates of each elm bacterial isolate were transferred separately onto nutrient agar (Oxoid) slopes within small glass vials (80 × 25 mm) sealed with metal screw-on caps. Prior to attaching the vial to the center of a white circular plastic lid (230-mm diameter) coated with a nonhardening sticky adhesive (Osticon), a 1-mm-diameter hole was made in the lid of the vial. Fifteen of these traps, representing three replicates for each of the five treatments and nutrient agar and untreated controls, were installed at random about 3 m above ground level on live elm trees in both park A and park B. After 4 weeks, the total number of beetles caught on the individual traps was recorded.

Experiment 2

On 1 December 1977, bacterial isolates from Experiment 1 that had attracted beetles were subcultured onto nutrient agar sent to the CMI for positive identification, and either pure cultures or combinations of the bacterial strains isolated were prepared, within glass vials, as described previously (see Experiment 1). A total of 35 traps containing the various treatments, plus five blank and five nutrient agar controls, was prepared. Each vial was attached at random to individual elms scattered throughout the Royal Botanical Gardens in Melbourne. The number of beetles caught on the traps was recorded after 14 days.

Experiment 3

On 17 February 1978, 39 traps (see Experiment 1) containing various combinations of pure and mixed bacterial colonies as used in Experiment 2

and three nutrient agar controls were attached at random to elms within an elm stand at Johnson's (approximately 6 ha) near Myrtleford in Victoria (300 km northeast of Melbourne). All traps were a minimum of 10 m apart. Over a period of 49 days, seven inspections of the traps were made, and the number of *S. multistriatus* caught was recorded. Beetles were counted and removed from the traps after each inspection, and the accumulative totals added after the final inspection. One bacterial isolate (No. 69), found to be relatively strongly attractive to S. *multistriatus*, was dispatched to the CMI for confirmatory identification.

Experiment 4

This experiment was conducted between 1 March and 20 March 1980 in and around Myrtleford at four locations containing large numbers of elms. Ten milliliters of nutrient agar (Oxoid) was syringed into glass vials, which were capped with metal lids and then sterilized by gamma irradiation from a ^{60}Co source (25 k Gy) (see French et al., 1982). A foam-rubber plug (approximately 50 × 19 mm), similarly sterilized, was used to replace the metal lid, and the vial was covered with aluminum foil held in position with a sticky tape (Cellotape). As gamma irradiation from this particular source does not produce high temperatures, the foam rubber was considered unaffected by heat in this sterilization procedure.

The combined bacterial isolates from fresh elm were cultured in Oxoid nutrient broth No. 2. (25 g/liter) and Oxoid yeast extract (3 g/liter) at pH 7.5. This inoculated broth was designated BB, and the uninoculated broth was designated NB. Onto the nutrient agar in the glass vials were added various combinations of BB, NB, and fresh elm wood-bark plugs (approx. 6-mm diameter) and gamma irradiated fresh elm wood-bark plugs (see Table 5).

A randomized block design was chosen for the field layout. Three replicate groups were hung at Murmungee, Lower River Road, and Johnson's, and five groups at Carley's Crossing on 29 February 1980. Each group contained seven traps, with each trap having attached a single glass vial (see Experiment 1).

To eliminate the effects of probable, undetected sources of natural pheromones (see von Keyserlingk, 1982), the traps were hung 300 mm apart between elms on a string line, 2 m above the ground, at each of four sites several kilometers apart in and around Myrtleford. These sites were at Murmungee, Lower River Road, Carley's Crossing, and Johnson's. Just prior to leaving each site, a small (1-mm diameter) hole was made in the aluminum foil covering the foam-rubber plug of each vial. The number of beetles caught on the traps was counted after 11 and 20 days. The data were analyzed using logarithmic transformations and a double split plot analysis of variance. In the event that there was an unusual pattern of response to various treatments, we examined whether or not there was a significant serial correlation

between beetle counts on neighboring traps over the sites within each location. The serial correlation was calculated for beetle counts on neighboring traps, one apart, two apart, and three apart. Calculations were made with log-transformed data and adjusted for site differences. There appeared to be little evidence of serial correlation among the counts of beetles on the traps, and it was concluded tentatively that 300 mm was an adequate distance between traps.

RESULTS

Experiment 1

After 4 weeks, the total number of beetles caught on traps baited with the various bacteria was recorded as shown in Table 1. The bacteria were identified as *Bacillus subtilis* (Ehrenberg) Cohn, comprising two and three strains, respectively, and one strain of *Enterobacter cloacae* (Jordan) Hormache and Edwards (Table 2). In both parks, baits containing the three-strain mixture of *B. subtilis* were more attractive to in-flight *S. multistriatus* beetles than was any other bacterial bait (Table 2). In park A, traps baited with *E. cloacae* proved more attractive than the traps baited with the two-strain *B. subtilis* and the nutrient agar controls. No beetles were caught on untreated traps.

Experiment 2

The number of *S. multistriatus* caught after 14 days on the various baited traps on elms in the Royal Botanical Gardens are shown in Table 3. Overall, few beetles were recorded on the majority of the baited traps (e.g., 20 of 35

TABLE 1. TOTAL NUMBER OF *S. multistriatus* CAUGHT ON TRAPS BAITED FOR 4 WEEKS WITH BACTERIA ISOLATED FROM SOUND LIVING ELM WOOD (THREE REPLICATES PER TREATMENT): EXPERIMENT 1, NOVEMBER–DECEMBER 1976

Treatment	Number of bacterial strains	CSIRO specimen numbers	Total number of beetles caught	
			Park A	Park B
Bacillus subtilis	2	65	30	2
B. subtilis	3	66	521	210
Enterobacter cloacae	1	63	134	4
Nutrient agar controls	—	—	44	5
Untreated controls	—	—	0	0

TABLE 2. ELM WOOD BACTERIAL CULTURES IDENTIFIED AT THE COMMONWEALTH MYCOLOGICAL INSTITUTE, KEW (U.K.)

CSIRO specimen No.	Identification	Bacterium*
62	*Bacillus subtilis* (Ehrenberg) Cohn (agreeing with	*B. subtilis*[1]
65	the description of the type of culture in all tests)	"
66		"
72		"
73		"
74		"
75		"
76		"
77		"
78		"
79		"
80		"
81		"
62	Agreeing with *B. subtilis*, except Voges-Proskauer	*B. subtilis*[2]
65	(VP) test, negative	"
66	Agreeing with *B. subtilis*, except nitrate reductase, negative	*B. subtilis*[3]
66	Agreeing with *B. subtilis*, except NO_3 reductase	*B. subtilis*[4]
68	and VP, both negative	"
69		"
70		"
71	Agreeing with *B. subtilis*, except no growth in 7%	*B. subtilis*[5]
77	NaCl	
69	*Bacillus pumilus* Meyer and Gottheil; NO_3 and starch both positive	*B. pumilus*[6]
63	*Enterobacter cloacae* (Jordan) Hormache and	*E. cloacae*
64	Edwards	"

*Superscripts on *B. subtilis* 1-5 refer to strain number.

traps had no beetles), and when beetles were caught in high numbers, there was no pattern or consistency of attraction. For instance, the numbers of *S. multistriatus* caught on isolate No. 66 traps were 0, 1, and 524, respectively. Similar results were shown throughout with other isolates; for example, catches were highly variable for Nos. 62, 63, 64, 65, and 66. Of interest is that isolate No. 66, composed of the three-strain mixture of *B. subtilis* (*B. subtilis* 1, 3, and 4; Table 2), attracted the highest number of beetles (524). This isolate was subcultured from our original *B. subtilis* culture, which also recorded the highest catch of beetles when used in the previous season in two Melbourne parks.

Relatively high numbers of beetles were attracted to isolates No. 63 (*E. cloacae*) and No. 65 (*B. subtilis* 1 and 2) but, again, the distribution was

TABLE 3. TOTAL NUMBER OF *S. multistriatus* CAUGHT ON TRAPS BAITED FOR 14 DAYS WITH BACTERIAL ISOLATES IN THE ROYAL BOTANICAL GARDENS: EXPERIMENT 2, DECEMBER 1977

Specimen No.	Bacteria	Number of strains in original culture	Trap No.	Number of beetles caught per trap
62	*B. subtilis*	1	1	3
			2	3
			3	0
			4	0
			5	0
62	*B. subtilis*	2	1	0
			2	0
			3	0
			4	2
			5	0
62	*B. subtilis*	2	1	60
			2	0
			3	0
63	*E. cloacae*	1	1	3
			2	0
			3	196
64	*E. cloacae*	1	1	10
			2	79
			3	0
64	*E. cloacae*	1	1	0
			2	16
			3	0
			4	0
			5	30
65	*B. subtilis*	2	1	0
			2	151
			3	0
			4	8
			5	0
65	*B. subtilis*	2	1	45
			2	0
			3	0
66	*B. subtilis*	3	1	0
			2	1
			3	524
Nutrient agar		—	1	0
			2	0
			3	0
			4	40
			5	16
Blank controls		—	1	0
			2	4
			3	0
			4	0
			5	0

uneven. Unexpectedly, high numbers of beetles were trapped on two of the five nutrient agar controls. This may suggest some atttraction to the nutrient agar medium, though contaminants may have played a part in this attractancy.

Experiment 3

The total number of beetles caught on the traps on elms at Johnson's after 49 days is presented in Table 4. Although all the beetles caught on the traps were removed at each inspection in order to reduce any insect-emitted pheromones, there was an increase in total numbers caught after 27 days. More beetles were trapped using the No. 69 isolates than any other isolate, with the highest catches on one of the three No. 69 replicates. When cultures of traps 1 and 2 of the No. 69 isolate were reexamined at the CMI, the following was reported.

Cultures from No. 69 trap 1: *Enterobacter aerogenes* (Kruse) Hormache and Edwards.
B. subtilis reductase and Voges-Proskauer, both negative.
Flavobacterium sp.
Corynebacterium sp.

Cultures from No. 69 trap 2: *E. aerogenes*, *Corynebacterium* sp., and *Flavobacterium* sp.

In both cultures *E aerogenes* was strongly predominant (and may have been the cause of the extra attractiveness). The *Corynebacterium* and *Flavobacterium* spp. were both present in only very small numbers (J.F. Bradbury, personal communication). Two attempts to isolate a spore former from trap 2 failed. Other airborne bacterial "contaminants" were able to grow within the vials, which may have proved attractive to in-flight *S. multistriatus*.

Experiment 4

The numbers of beetles caught after 11 and 20 days on the traps baited with sterilized and unsterilized elm wood-bark plugs at the locations near Myrtleford are presented in Table 5. The highest number of beetles (650) was caught at Carley's Crossing, which had five replicates, two more than at the other locations. The lowest number of beetles (19) was caught at Johnson's, even though this site had the largest elm population. This may be an indication that the traps competed poorly with host- and beetle-produced attractants in the test area, or being close to the end of the flight period, fewer beetles were flying.

The ANOVA using the log transformation is shown in Table 6. There are a mild location effect ($P < 0.05$) and substantial time ($P < 0.001$) and location–time ($P < 0.001$) effects. There is a significant treatment–time

TABLE 4. TOTAL NUMBER OF *S. multistriatus* CAUGHT ON TRAPS ON ELMS BAITED WITH BACTERIAL ISOLATES OVER 49 DAYS AT JOHNSON'S, NEAR MYRTLEFORD: EXPERIMENT 3, FEBRUARY–MARCH 1978

CSIRO specimen No.	Trap No.	Bacterial strain in mixture	Number of beetles caught per trap, days after installation of bait traps							Accumulative total of beetles caught	Grand total of 3 traps per mixture
			3	4	9	14	17	27	49		
50	1	*B. subtilis* 1, 2[a]	0	1	0	1	2	2	2	8	62
	2		1	1	1	2	2	1	14	22	
	3		0	1	1	0	4	11	15	32	
51	1	*B. subtilis* 1	0	1	0	0	0	0	2	3	
	2		0	1	0	1	1	6	0	9	48
	3		2	0	3	2	6	4	19	36	
52	1	*B. subtilis* 1, 4	1	0	0	2	0	3	19	25	
	2		1	0	0	1	2	0	5	9	45
	3		1	1	0	1	3	2	3	11	
53	1	*B. subtilis* 1	0	2	2	2	2	2	4	14	
	2		1	0	0	0	0	0	6	7	66
	3		4	1	5	1	8	3	23	45	
54	1	*B. subtilis* 1, 2	0	0	0	0	0	1	2	3	
	2		1	2	0	0	4	10	8	25	34
	3		0	0	0	0	3	0	3	6	
55	1	*B. subtilis* 1, 2	1	0	0	0	2	2	4	9	
	2		0	0	1	1	1	2	4	9	19
	3		0	1	0	0	0	0	0	1	

56	1	*B. subtilis* 1, 2	1	0	0	0	0	1	2	4	
	2	*E. cloacae*	1	1	3	3	0	8	21	37	42
	3		0	0	0	0	0	0	1	1	
62	1	*B. subtilis* 1, 2	0	1	1	0	1	9	10	22	
	2		0	0	0	0	0	1	3	4	26
	3		Damaged in transit								
63	1	*E. cloacae*	0	1	0	2	0	1	5	9	
	2		0	1	0	0	0	1	3	5	14
	3		Damaged in transit								
66	1	*B. subtilis* 1, 3, 4	1	0	1	0	2	4	5	13	
	2		0	0	0	0	1	0	5	6	30
	3		1	0	0	0	2	3	5	11	
69	1	*B. subtilis* 4	2	4	1	15	24	78	262	386	
	2		4	1	2	3	0	10	26	46	453
	3		1	0	2	1	2	4	11	21	
71	1	*B. subtilis* 5	0	1	1	0	2	0	2	6	
	2		0	0	0	0	0	0	0	0	24
	3		2	1	2	2	3	3	5	18	
74	1	*B. subtilis* 1	0	1	0	0	1	3	4	9	
	2		1	0	0	0	0	0	7	8	58
	3		0	3	4	1	2	8	23	41	
SC	1	Nutrient agar	0	0	0	0	0	0	4	4	
	2		0	0	1	1	1	0	12	15	34
	3		2	0	2	0	5	4	2	15	
Grand totals			29	27	33	42	86	187	551	955	955
Totals less No. 69			22	22	28	23	60	95	252	502	502

[a]Bacterium as listed in Table 2.

TABLE 5. TOTAL NUMBER OF *S. multistriatus* CAUGHT ON TRAPS WITH STERILIZED AND UNSTERILIZED ELM WOOD-BARK PLUGS AFTER 11 AND 20 DAYS AT FOUR LOCATIONS NEAR MYRTLEFORD: EXPERIMENT 4, MARCH 1980

		Treatment[a]														All treatments		
		A		B		C		D		E		F		G				
Site	Replicates	11	20	11	20	11	20	11	20	11	20	11	20	11	20	11	20	
Murmungee	3	12	32	6	21	12	23	14	11	2	21	9	11	17	9	72	128	200
Lower River Road	3	32	34	25	49	48	64	36	44	23	71	33	31	42	15	239	308	547
Johnson's	3	1	2	2	2	1	3	3	3	1	6	5	1	1	2	14	19	33
Carley's Crossing	5	63	89	74	127	56	109	77	92	74	112	49	64	85	29	478	622	1100
Totals		108	157	107	199	117	199	130	150	100	210	96	107	145	55	803	1077	1880
		265		306		316		280		310		203		200		1880		

[a]A, nutrient agar (NA) + nutrient broth (NB); B, NA + bacterial broth (BB); C, NA + NB + fresh elm wood-bark plug (FEW); D, NA + BB + FEW; E, NA + NB + FEW (gamma irradiated); F, NA + BB + FEW (gamma irradiated); G, blank.

TABLE 6. ANOVA LOG-TRANSFORMED DATA FOR EXPERIMENT 4 AT MYRTLEFORD, MARCH–APRIL 1980

Source of variation	df	SS	MS	VR
Site stratum	13	124.9243	9.6096	
Locations	3	81.6806	27.2269	6.30*
Error (a)	10	43.2437	4.3244	
Between-traps stratum	84	39.0451	0.4648	
Treatments	6	5.7232	0.9539	2.10(ns)[a]
Locations · treatments	18	6.0897	0.3383	0.74(ns)[a]
Error (b)	60	27.2321	0.4539	
Within-traps stratum	95	50.2380	0.5288	
Time	1	19.7170	19.7170	103.09***
Locations · time	3	8.3221	2.7740	14.50**
Treatments · time	6	4.2542	0.7090	3.71**
Locations · treatments · time	18	5.1299	0.2850	1.49(ns)
Error (c)	67	12.8148	0.1913	

[a] Not significant.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

interaction ($P < 0.01$), but all other effects involving treatment, *viz.*, treatment, location–treatment, and location–treatment–time, were not significant. For the purpose of this experiment the main effect was the treatment–time interaction. The treatment means (transformed data) after 11 and 20 days are given in Table 7, together with the least significant differences at the 5 and 1% levels of significance for comparing various mean differences. On day 11, treatments C (nutrient agar + nutrient broth + fresh elm wood, and E (nutrient agar + nutrient broth + fresh elm wood, gamma irradiated) were significantly greater than the other treatments, except A (nutrient agar + nutrient broth); none of the other treatments differed from each other. By day 20, treatments A to F (see Table 5) did not differ significantly from each other, and all except F (nutrient agar + bacterial broth + fresh elm wood, gamma irradiated) were significantly greater than G (control blank). There was a decrease in mean beetle catches between day 11 and day 20 for treatments A, C (nutrient agar + control broth + fresh elm wood), E, and G, a mild decrease for B (nutrient agar + bacterial broth) and F, and no significant decrease for D (nutrient agar + bacterial broth + fresh elm wood).

The presence or absence of plugs of fresh elm wood bark, whether gamma irradiated or not, did not have a noticable effect on attractancy. The important factor seems to concern whether a treatment contained nutrient broth (NB) or bacterial broth (BB) (B, D, and F, Table 7). The treatments

TABLE 7. TREATMENT MEANS (TRANSFORMED DATA) AFTER 11 AND 20 DAYS OF TRAPPING AT MYRTLEFORD: EXPERIMENT 4, MARCH 1980

Treatment[a]	Day 11	Day 20
A	1.793	1.097
B	1.541	1.144
C	2.015	1.166
D	1.405	1.242
E	2.105	1.088
F	1.437	1.034
G	1.548	0.632
Between:	LSD, 5%	LSD, 1%
Times	0.331	0.440
Treatments	0.429	0.571

[a]A, nutrient agar (NA) + nutrient broth (NB); B, NA + bacterial broth (BB); C, NA + NB + fresh elm wood-bark plug (FEW); D, NA + BB + Few; E, NA + NB + FEW (gamma irradiated); F, NA + BB + FEW (gamma irradiated); G, blank.

containing NB (A, C, and E, Table 7) performed better than their counterparts with BB on day 11, and the presence of fresh elm wood (FEW) plugs (gamma irradiated or not) did enhance this difference; there was no significant difference between treatment A and treatment B (the two treatments without FEW) on day 11. By day 20, these differences were negligible. The performance of the treatments compared with the control blank differed on both occasions. On day 11, only those treatments involving NB in the presence of FEW (C) and gamma-irradiated FEW (E) were significantly greater than the control blank, while on day 20, only treatment F was not significantly greater than the control blank, although it was almost so.

DISCUSSION

In the earlier experiments, there was some indication that bacterial isolates from elms were attractive to *S. multistriatus* beetles. Even though these and subsequent experiments indicated qualitatively the response of *S. multistriatus* to these isolates, the results in sequential experiments were variable, inconsistent, and often seemingly contradictory. The variation in beetle catches on the different replicates throughout the study indicates our lack of information on the endemic populations and dispersal habits of *S. multistriatus* in any particular area. Peacock et al. (1971), Lanier et al. (1976), and Wollerman (1979) concluded that, even after intensive trapping, with and without multilure, *S. multistriatus* counts did not provide an

estimate of actual beetle populations; rather they provided an index of relative endemic populations during the flight season. A similar experience was reported by Gardiner (1981) for the native elm bark beetle, *Hylurgopinus rufipes* (Eichh.), in central Ontario. Although the bacterial isolates from elm (*B. subtilis*, mixture of strains 1, 3, and 4) appeared attractive to *S. multistriatus* in 1976, 1977, and 1978, there was considerable unevenness in the number of beetles caught on the traps. Also, the relatively high number of beetles caught on the nutrient agar-baited (control) traps (see Table 1) may be partly due to volatiles from aerial contaminants that entered the glass vials and grew on the agar slopes, together with the original bacterial isolates. This was indicated on examination of the two attractive bacterial isolates (No. 69, *B. subtilis* 4) after the 49-day trapping period. This contained, in addition to *B. subtilis*, other bacteria such as *E. aerogenes*, *Corynebacterium* sp., and *Flavobacterium* sp. Even after redesigning the glass vial "caps" and employing a random block design to compare treatments (see Experiment 4), beetle numbers varied considerably, with again, relatively high catches on the nutrient agar controls. In another study, irradiating elm billets with gamma rays had a significant effect in reducing the attractancy of such billets to in-flight *S. multistriatus* beetles (French et al., 1982). But it was not clear whether or not this was due to killing the microorganisms, or the plant cells in the bark–sapwood interface (leading to the chemical rearrangement of plant volatiles), or a combination of these and other factors. On pursuing this point (see Experiment 4) we found that the presence of absence of plugs of fresh elm wood bark, whether gamma irradiated or not, had no noticeable effect on beetle attractancy.

Further work is underway to examine the volatiles from dead, dying, and live elm material and the accompanying microorganisms with regard to their role in attracting *S. multistriatus*.

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LETTER TO THE EDITOR

REDEFINING "PHARMACOPHAGY"

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In his classification of *Papilio* butterflies, Haase (1892) named a subgenus *Pharmacophagus*. The larvae of some of the species were known to feed on *Aristolochia* plants, and Haase assumed that they utilized noxious chemicals obtained from their hosts for their own defense. Subsequently, a few authors informally referred to other insects as "pharmacophagous" because they shared this habit of feeding on "toxic" plants. Considering today's much more detailed knowledge of insect–plant relationships, I plead here for a redefinition of this term so that it can be used with a precise meaning to characterize a particular type of insect–plant association.

Chemical techniques applied during the last 20 years or so have demonstrated that noxious secondary plant substances are indeed sequestered by a huge array of insects (for review and references, see Blum, 1981; Brower, 1984). It appears that the only feature which insects feeding on so-called toxic plants all have in common is that they take advantage of food sources avoided by most herbivores. However, this does not imply that the insects recognize the respective secondary plant chemicals or that they utilize them. On the one hand, insects can sequester toxic plant products without being able to detect them, i.e., they merely take them up automatically with their food.[1] On the other hand, ingested toxic plant substances are not necessarily retained or stored by insects, there being many examples where they are degraded

[1]In the case of *Danaus plexippus* (Lepidoptera: Danainae), for example, a classic aposematic insect frequently protected by cardiac glycosides obtained from milkweed plants on which the larvae feed (for review and references, see Brower, 1984), neither the egg-laying females nor the caterpillars show an apparent interest in cardenolides (Dixon et al., 1978; Cohen and Brower, 1982). As a consequence, cardenolide-free asclepiadaceous plants are also chosen as food, and so storage of cardenolides is not a constant feature of the species. Only the potential to do so is.

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and/or eliminated (see, e.g., Rothschild, 1972). Thus, pharmacophagy in the broad sense, as previously used (see above), embraces fundamentally different relationships between insects and plants.

Generally, plants are sources of nutrients for insects, with their particular secondary plant substances often mediating herbivory by serving either as attractive or as repellent stimuli. Sequestration of secondary plant products in most cases is only a side effect of the consumption of food (see above). In recent years, however, we have learned of insect–plant associations which are independent of the need to obtain energy and which concern the plants' allelochemicals only. It has been recognized that several insects require—and even can depend on—certain secondary plant substances and that they can gather these by specific behavior which can be different from and additional to ordinary feeding. Examples include the following: adult danaine butterflies are attracted to withered plants containing pyrrolizidine alkaloids (PAs); with their proboscises, they apply a fluid capable of dissolving PAs and then reimbibe it. Under certain conditions, they can obtain PAs by scratching fresh leaves and ingesting the sap oozing out. Gathering PAs is thus a special activity and is separate from feeding behavior (although from some plants, insects can get PAs with nectar, i.e., with food). This is indicative of a peculiar importance of PAs, which has been shown: both sexes store PAs for defense, and males depend on PAs as precursors for the biosynthesis of a pheromone component essential for courtship success. (For details and references, see Boppré, 1978, 1984.)

This example demonstrates that plants are not only "grocery stores" but can also be "pharmacies" which—sometimes in addition to food but even exclusively—supply insects with chemicals not needed for their primary metabolism but significantly affecting their fitness. In the insects, basically different adaptations are involved if interactions with plants concern food, on the one hand, or "drugs," on the other, and so separate terminology should be applied. This leads me to suggest restricting the term "pharmacophagous" to the following usage: *insects are pharmacophagous if they search for certain secondary plant substances directly, take them up, and utilize them for specific purpose other than primary metabolism or (merely) foodplant recognition.*[2] Thus, calling an insect pharmacophagous requires demonstration that it takes up plant allelochemicals in pure form—even if these are normally ingested together with food—and evidence that this is of advantage for its fitness. Of course, the plant chemical(s) must be known; it may be, but need not be, noxious to other organisms.

Redefining pharmacophagy is not just a semantic issue. To apply the

[2]It is stressed that for convenience, i.e., to avoid the need for further terms, pharmacophagy is not to be understood literally; in this context, "pharmaco-" means secondary plant substances in general and not only those having curative effects, and "-phagy" means gathering, which can, but need not, be ingestion.

term, experiments are needed and these should give some insight into the function(s) of the plant metabolite(s) for the insect. That tests for pharmacophagy can be worth pursuing is shown by example of *Creatonotos* (Lepidoptera: Arctiidae): the males emit an odor from their androconial organs which was assumed to be derived from PAs. By breeding insects with or without access to PAs and checking whether PAs are detected and ingested by the larvae in pure form, we established not only that PAs serve as precursors for the odor, but also that PAs specifically regulate the growth of the androconial organs (Schneider and Boppré, 1981; Schneider et al., 1982; Boppré and Schneider, in preparation).

Apart from Danainae and *Creatonotos*, which were used as examples only, other Lepidoptera have been proved to be pharmacophagous or seem likely to be. Many Ithomiinae, Arctiidae, and Ctenuchiidae obtain PAs from dry plants (in experimental situations they are attracted to and ingest pure PAs; cf. Figure 1) and store them, and some use them additionally as pheromone precursors (references given by Pliske, 1975; Boppré, 1978, 1984). PAs also bait various species of flea beetles (Chrysomelidae) and grasshoppers (*Zonocerus*), which also ingest crystals of PAs (Boppré and Scherer, 1981; Boppré et al., 1984; Boppré, unpublished). A rather different example which also meets the definition of pharmacophagy appears to occur in golden bees (Hymenoptera: Euglossinae): the males visit flowers of orchids and some other plants which do not contain nutrients but certain fragrances. They collect the fragrances in their hind legs and utilize them to attract other male bees of their species and form leks where mating takes place (such a pheromonal role of these plant substances in the behavior of the bees needs further substantiation). Fragrance components displayed in the field are attractive and are collected. [See, e.g., Vogel (1966), Dodson (1975), and Dressler (1982) for details and references.]

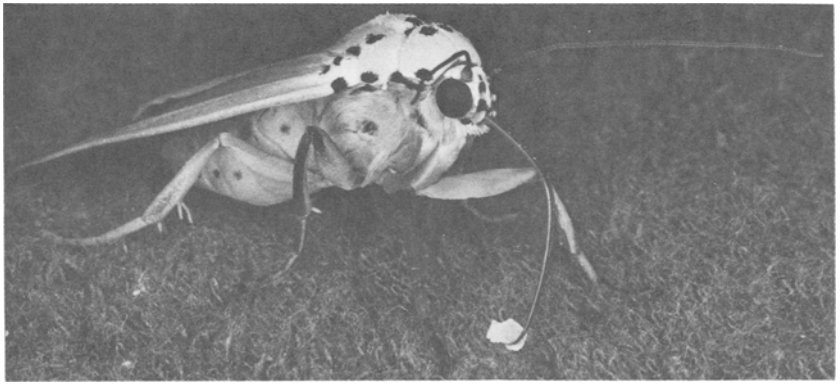

FIG. 1. *Rhodogastria phaedra* (Arctiidae) ingesting monocrotaline by dissolving a crystal of this PA with a fluid applied via its proboscis and reimbibing it.

It seems very probable that many other insects are pharmacophagous, but few have been investigated in this light.

In analogy to the definition of pharmacophagy given above, one might create a term for those insects which utilize certain secondary plant substances for a specific purpose other than primary metabolism but do obtain them together with food exclusively. However, to be complete, other types of insect–plant relationships would also need to be named, and it is thought that this should await a thorough classification of insect–plant interactions.

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BOOK REVIEW

Les Phéromones. By Michel Barbier. Masson, Paris, 1983, 140 pp., 145FF (in French).

The growing interest in chemical ecology of students and scientists in France is in strong contrast with corresponding lack of French literature in this area.

In 100 pages, Barbier reviews the pheromones produced by more than 200 species of animals and plants and presents much information otherwise available only through the English literature. The first chapter is a general introduction (classification and definitions of semiochemicals); perception of pheromones is briefly summarized. Four chapters are devoted to the study of insect pheromones, the management of insect pests with pheromones, and the synthesis of insect pheromones. Chapter 6 deals with the chemical communication among fungi and algae, while chapter 7 deals with chemical signals in vertebrates including humans.

To attempt to cover this vast range, large concessions must be made to superficiality, but Barbier, at least, has been successful in introducing these topics to the French readers; he cites 270 references and provides an adequate index. The chemical aspects are treated factually and competently. The biological aspects are sometimes obscured by philosophical discussions that seem to lack focus. The presentation is also marred by some uncritical reporting of the literature and by a number of errors and misinterpretations; several are listed here as examples.

Page 35. The "contradictory" claims for the pheromone of *Trogoderna inclusum* are noted, but Barbier misses the point that the aldehyde is in fact the pheromone and was detected by aeration of the "calling" females; the alcohol and ester are probably precursors. On page 45, the erroneous conclusions of Rossi are accepted despite the subsequent clarification by Levinson and Mori of the chirality of the pheromone of *T. granarium.*

Page 40. Frontalin is omitted as one of the three components of the aggregation pheromone of *Dendroctonus brevicomis.*

Page 41. The account of experiments in Germany on *Scolytus multistriatus* is garbled and the subsequent clarification by Blight is ignored.

Page 42. The important point that *Gnathotrichus sulcatus* produces and uses both enantiomers of sulcatol is ignored.

Page 38. A reference is given for the synthesis of the pheromone of *Periplaneta americana* but not for its isolation and identification.

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Pages 60 and 48–52. The discussions of the termite trail pheromones and of the mode of action on bees of *trans*-9-oxo-decenoic acid and other compounds are oversimplified.

Page 40. The European elm bark beetle, *Scolytus multistriatus*, is referred to as "le coleoptère americain *Ulmus americana*."

Page 93. Slime mold is translated "amibe."

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COMPARATIVE STUDY BY ELECTROPHYSIOLOGY OF OLFACTORY RESPONSES IN BUMBLEBEES (*Bombus hypnorum* and *Bombus terrestris*)

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Abstract—Electrophysiological data (EAG) were recorded on adult bumblebees stimulated with floral and/or pheromonal pure odorants at different concentrations. The responses of queen, worker, and male bees are compared and the sensitivities of these insects to the pure odorants tested are discussed.

Key words—Apoidea, *Bombus hypnorum, Bombus terrestris,* Hymenoptera, sex, caste, floral odorants, pheromones, electroantennography, olfactory equipment.

INTRODUCTION

Bumblebees (Apoidea, Hymenoptera) that live in groups grow up in a complex chemical environment, whether inside or outside their nests. Intraspecific relations seem to be determined, to a great extent, by chemical cues: brood pheromone (Heinrich, 1974), queen pheromone (Röseler and Röseler, 1974; Röseler, 1975, 1977; Plowright and Pendrel, 1977; Van Honk et al., 1980; Röseler et al., 1981), sexual pheromones (Kullenberg et al., 1970, 1973; Svensson and Bergström, 1977; Van Honk et al., 1978; Ågren et al., 1979; Svensson, 1979), trail pheromone (Cederberg, 1977b), and alarm pheromone (Cederberg, 1977a). Chemical volatiles cooperate with other mechanisms (spatial, temporal, mechanical) as premating isolating mechanisms and so prevent inbreeding (Svensson, 1980; Bergström et al., 1981).

On the other hand, these insects, which are active foragers, learn to

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recognize the visual, chemical, and morphological signals of nectariferous plants. For the honeybee (*Apis mellifica*), another pollinator, chemical signals are the most effective signals (von Frisch, 1923; Kriston, 1973; Koltermann, 1973). Comparative behavioral experiments, applied to whole colonies of honeybees and bumblebees, have shown that conditioning to pure odorants (geraniol, limonene) is similar for both Hymenoptera (Pham-Delegue et al., 1983). Therefore it appears that volatile pheromones and plant aromas are essential sensory criteria for bumblebees to ensure the survival of the species.

It was then interesting to analyze, at different levels (perception, identification, and message integration), the nervous mechanisms which represent the key to the behavioral responses induced by chemical stimuli. We first studied the antennal olfactory equipment of *Bombus hypnorum* (Fonta and Masson, 1982) and quantified, by means of scanning electronic microscopy, sensilla placodea distribution on adult insect antennae. As has been well demonstrated in honeybees, plate organs are sensory structures whose nervous cells are specially adapted to detect volatile compounds (Lacher and Schneider, 1963; Kaissling and Renner, 1968; Esslen and Kaissling, 1976). For each antennal segment, the average density of the sensilla placodea is similar for male, worker, and queen bumblebees, yet the male antenna flagellum is one segment longer and the s. placodea population is denser on the distal segment (Fonta and Masson, 1982). However, differences in the number of sensory neurons, as has been found between male and worker of *Apis* (Esslen and Kaissling, 1976), or in the function of the placodea neuroreceptors (discrimination ranges and sensitivities) might exist between sexes and/or castes at this peripheral level.

The present work aims to conduct further studies on the electrophysiological recordings from single cells to analyze the insect olfactory detection level and perception characteristics. The antennal electric activity was measured by electroantennography recordings (EAG). Neurophysiological studies on bumblebees which have been published previously deal only with the visual system (Meyer-Rochow, 1980).

METHODS AND MATERIALS

Biological Materials. Two species (*Bombus hypnorum* L. and *Bombus terrestris* L.) of adult bees of diverse ages were studied. The experimental animals belonged to different colonies that were reared in the laboratory. The individual size variation inside each caste was kept narrow. The worker bees are foragers; the males have reached their sexual maturity whereas the queens are potential foundresses which have not yet hibernated.

Odorants. Eight pure substances (purity = 99.9%) were tested: butanol (BUT), the usual reference compound for functional studies in olfaction, limonene (LIM), geraniol (GER), nerol (NER), citral (CIT), eugenol (EUG), vanillin (VAN), and isoamylacetate (ISO), floral aroma constituents (Loper, 1972; Lawrence, 1978; Etievant et al., 1984) and/or pheromonal elements of various Hymenoptera (e.g.: *Apis:* Boch and Shearer, 1962; Pickett et al., 1980, 1981; *Bombus:* Kullenberg et al., 1970; Svensson and Bergström, 1977; Bergström et al., 1981; *Andrena:* Bergström and Tengö, 1974; Tengö and Bergström, 1976, 1977; Francke et al., 1981; Bergström et al., 1982; *Anthophoridea:* Vinson et al., 1982).

Methods. Experiments were carried out on live insects, immobilized in a special restraining device.

The recording electrode (a drawn out glass microcapillary, 1 mm inner diameter) was set up on the left antenna tip when the distal part of the last segment had been removed. The reference electrode (inner diameter: 0.5 mm) covered the right antenna. The two electrodes were filled with 2 M NaCl solution (116.88 g/liter). They were connected to an impedance adapter, which was itself connected to a storage cathodic oscilloscope.

The stimulation device was an olfactometer with dynamic and controlled gas dilution (Masson and Friggi, 1974; Masson et al., in preparation). The head of the insect was swept by pure nitrogen between the stimulations or by odoriferous nitrogen during the stimulations. The carrier air was scented when passed through the enclosed spaces containing the odorants and delivered at about 4 mm from the longitudinal axis of the left antenna. This flow is associated with a constant nitrogen flow (7.3 liter/hr) which is necessary for the antennal mechanoreceptors to be habituated and which cleans the circuits, followed by the scented air flows. The carrier air pressure and thus the concentration of the stimulating odorous molecules were controlled by a water manometer.

The following equation gives the number of molecules in each milliliter of carrier air (Masson, 1973; Masson and Friggi, 1974):

$$ni = \frac{(6 \times 10^{23}) \times 273\ Pi}{22400 \quad T \times 760} \sim 0.96 \times 10^{19} \frac{Pi}{T}$$

where ni = number of molecules/ml of carrier gas; Pi = vapor pressure in mm Hg at 20°C; and T = temperature in degrees Kelvin.

Protocol. Stimulation with pure odorants used alone lasted 2 sec, which is the time required for the stimulus to reach the neighborhood of the antenna. Between two successive stimulations, a 2-min interval ensured desorption of the membrane neuroreceptors.

Each of the eight odorants was tested at three concentrations (Table 1) which were linked to the three nitrogen flows: D_1 = 1.2 liter/hr, D_2 = 3.6

TABLE 1. NUMBER OF MOLECULES (*Ni*) OF A 2-SEC STIMULATION[a]

	Stimulus Quantity					
	D_1 = 1.2 liter/hr		D_2 = 3.6 liter/hr		D_3 = 6.05 liter/hr	
Odorants	*Ni*	log *Ni*	*Ni*	log *Ni*	*Ni*	log *Ni*
Butanol (BUT)	1.40×10^{17}	17.146	4.22×10^{17}	17.625	7.10×10^{17}	17.851
Geraniol (GER)	4.45×10^{14}	14.648	1.34×10^{15}	15.127	2.26×10^{15}	15.354
Nerol (NER)	1.53×10^{15}	15.185	4.62×10^{15}	15.664	7.77×10^{15}	15.890
Citral (CIT)	1.26×10^{15}	15.100	3.80×10^{15}	15.580	6.39×10^{15}	15.806
Isoamylacetate (ISO)	1.21×10^{17}	17.083	3.66×10^{17}	17.563	6.16×10^{17}	17.790
Limonene (LIM)	3.04×10^{16}	16.483	9.17×10^{16}	16.962	1.54×10^{17}	17.188
Eugenol (EUG)	3.00×10^{14}	14.477	9.05×10^{14}	14.957	1.52×10^{15}	15.182
Vanillin (VAN)	3.69×10^{12}	12.567	1.11×10^{13}	13.045	1.87×10^{13}	13.272

[a] D_1, D_2, D_3: the three flow rates of the carrier air.

liter/hr, D^3 = 6.05 liter/hr. For each flow rate, stimulations were randomized. The maximal amplitude of depolarization induced by the stimulation was recorded.

The results reported and discussed here represent the mean of the responses of 10 insects; the substances were tested on each animal and 1–3 trials were conducted with each experimental insect.

Average amplitude of responses is figured by a function of decimal logarithm of the odorant molecules concentration.

RESULTS

The odorous chemicals used in this work stimulate the neuronal activity of the insect peripheral olfactory system.

The EAG responses, which are functions of the stimulating molecule concentrations, are represented by linear functions in the range of chosen concentrations.

In *Bombus hypnorum* (Figure 1), the EAG responses of workers and males were similar for each stimulus, response amplitudes increasing in the same way for both. The future foundresses of this species gave, in general, slightly higher responses. The largest difference was shown to be between queens and workers and males for the highest concentrations of butanol used in our experimental situation (2:1 quotient). The responses of queens and workers increased in parallel, except for butanol and eugenol. In comparing males and workers, a more irregular evolution was found in the response amplitude ratios.

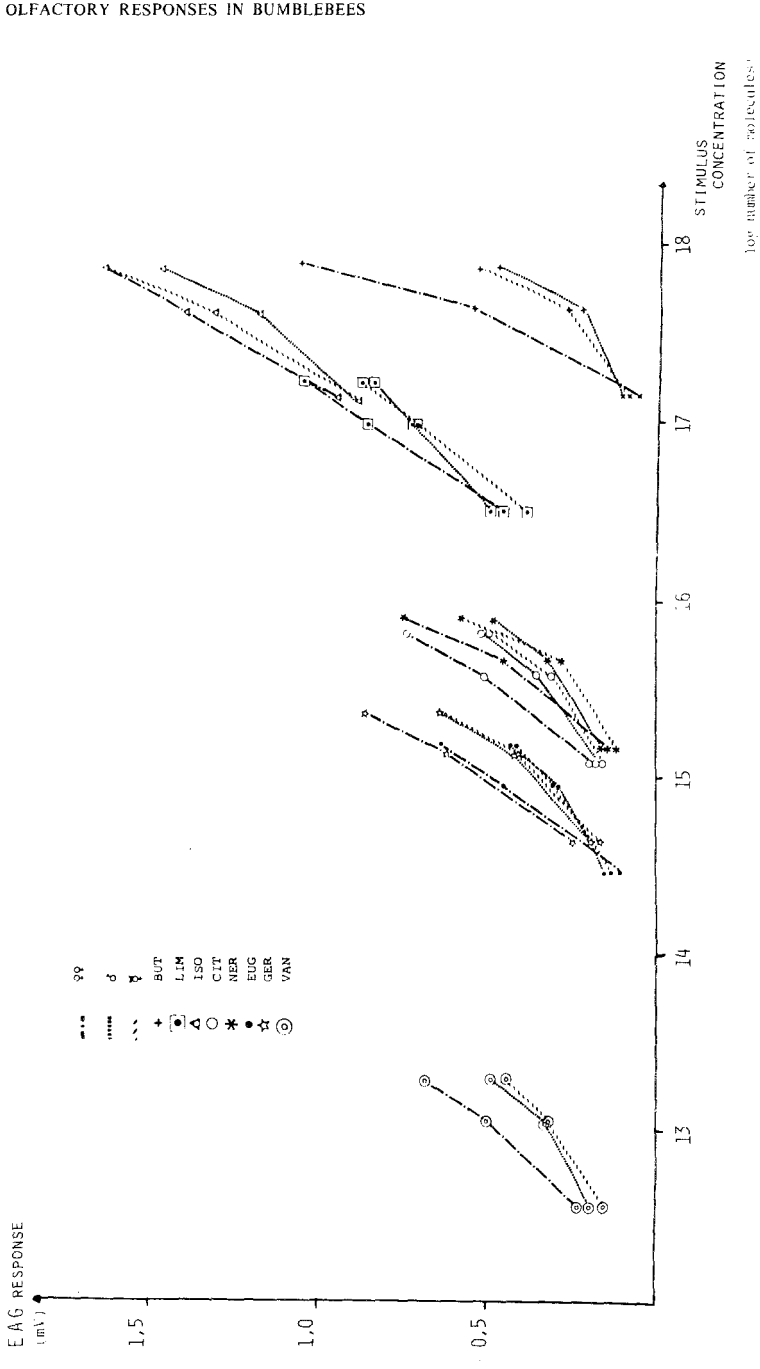

FIG. 1. EAG responses of *Bombus hypnorum* (in millivolts) to odoriferous stimulations at different concentrations (in decimal logarithm of the number of molecules). BUT: butanol; CIT: citral; EUG: eugenol; GER: geraniol; ISO: isoamylacetate; LIM: limonene; NER: nerol; VAN: vanillin.

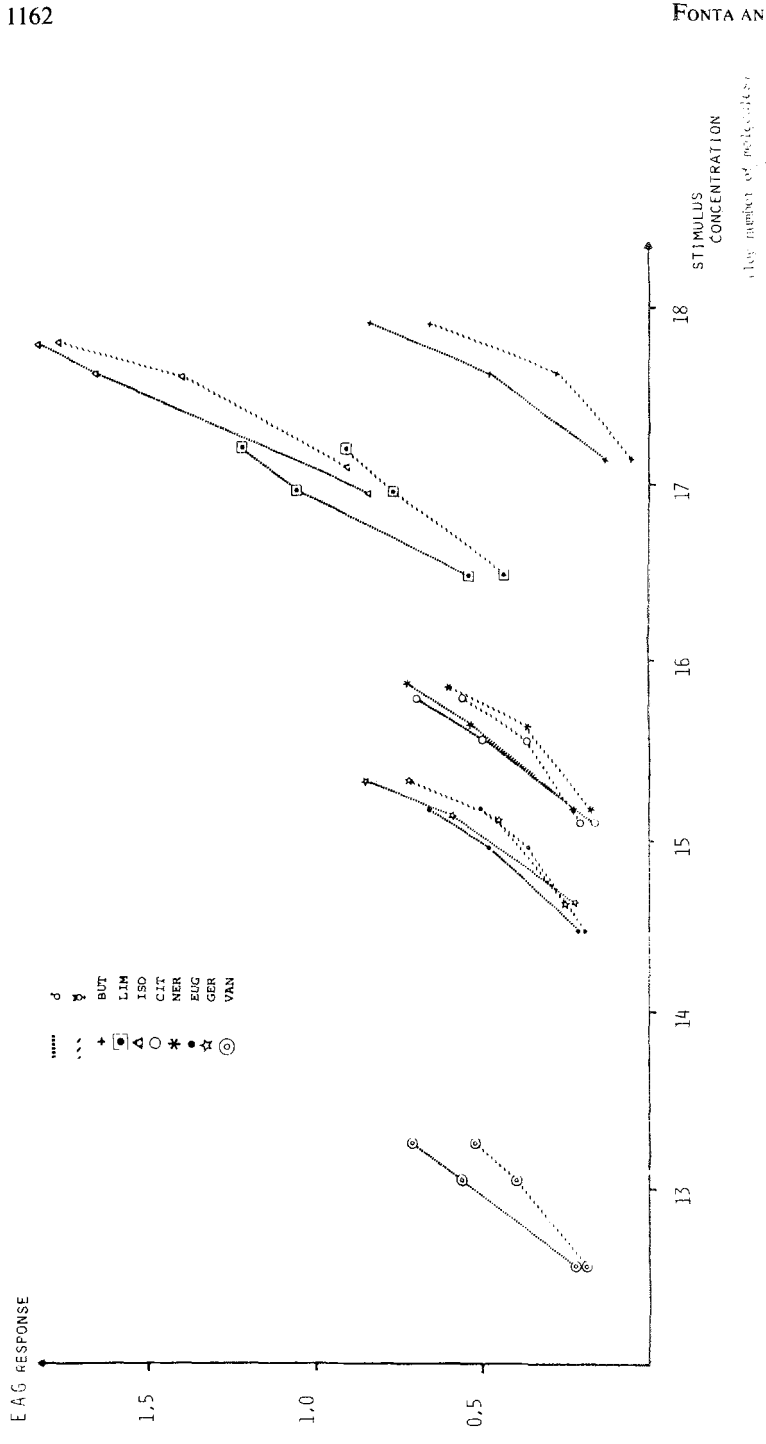

FIG. 2. EAG responses of *Bombus terrestris* (in millivolts) to odoriferous stimulations at different concentrations (in decimal logarithm of the number of molecules). BUT: butanol; CIT: citral; EUG: eugenol; GER: geraniol; ISO: isoamylacetate; LIM: limonene; NER: nerol; VAN: vanillin.

In *Bombus terrestris* (Figure 2), the responses of workers and males varied in an analogous way with the stimulus quantity. The depolarization amplitudes were higher for males than for females; this difference was emphasized with the upper concentration of limonene. Generally, the curves of stimulus concentration–response are not parallel for the two sexes for each odorant; amplitude ratio variation is different for substances tested in comparable concentration ranges (for example, limonene and butanol).

A linear regression ($y = mx + b$) has been calculated with three coordinates for each function derived experimentally. Correlation coefficient values range from 0.92 to 0.99. Consequently, the stimulus concentration values (x) obtained from the linear regressions can be relied on for a response amplitude (y) of 0.5 mV.

The results can be analyzed for the two species together. The eight pure odorants tested can be classified in five groups if the stimulus intensity required to obtain a 0.5-mV amplitude detection response (Table 2) is used as the classification criterion: (1) VAN ($\sim 10^{13}$ molecules), (2) GER-EUG ($\sim 10^{15}$ molecules), (3) CIT-NER ($\sim 3 \times 10^{15}$ to 10^{16} molecules), (4) LIM-ISO (~ 3–6×10^{16} molecules), and (5) BUT ($\sim 3 \times 10^{17}$ to 10^{18} molecules).

There is a 10^4 factor between groups 1 and 5, the first compound (VAN) being clearly distinct from the second class of compounds (GER-EUG). This hierarchy in sensitivity and detection method for the eight substances is similar within each species, each caste and each sex.

DISCUSSION

All the EAG responses obtained in these experiments are expressed as depolarizations. The results, by quantitative analysis, do not show strong differences in the responses of the three kinds of individuals except for butanol; this odorant molecule induces an antennal electrical activity higher for the young queens than for workers and males of *Bombus hypnorum.*

As demonstrated here all the responses of the queens are more pronounced, and the response amplitudes of males are superior to those of the workers. Two alternative explanations could account for these observations: (1) A first-order sensory fibers equipment difference for the three types: various numbers of neuroreceptors in the sensilla placodea and/or a different s. placodea population, in absolute value (on the one hand the body size and therefore the antennal area of queens is bigger than workers and males; on the other hand, the antennal flagellum of the male has one additional segment). (2) Sensitivity and discrimination power of the peripheral sensory neurons may show characteristics for each caste and/or each sex. Until now, no study has been undertaken at the unitary level with bumblebees.

TABLE 2. NUMBER OF ODOR MOLECULES (N) INDUCING A 0.5-mV EAG AMPLITUDE (y)[a]

Insects	Stimulation	Pure Odorants							
		BUT	GER	NER	CIT	ISO	LIM	EUG	VAN
hypnorum									
♀♀	x	17.501	14.963	15.645	15.522	16.618	16.496	15.012	13.010
	N	3.170×10^{17}	9.183×10^{14}	4.416×10^{15}	3.327×10^{15}	4.150×10^{16}	3.133×10^{16}	1.028×10^{15}	1.023×10^{13}
♂	x	18.031	15.198	15.993	15.816	16.608	16.530	15.424	13.389
	N	1.047×10^{18}	1.578×10^{15}	9.840×10^{15}	6.546×10^{15}	4.055×10^{16}	3.388×10^{16}	2.655×10^{15}	2.449×10^{13}
☿	x	17.886	15.210	15.878	15.906	16.733	16.644	15.418	13.474
	N	7.691×10^{17}	1.622×10^{15}	7.551×10^{15}	8.054×10^{15}	5.408×10^{16}	4.406×10^{16}	2.618×10^{15}	2.979×10^{13}
terrestris									
♂	x	17.560	14.974	15.592	15.567	16.672	16.434	14.958	12.964
	N	3.631×10^{17}	9.419×10^{14}	3.908×10^{15}	3.690×10^{15}	4.698×10^{16}	2.716×10^{16}	9.078×10^{14}	9.207×10^{12}
☿	x	17.764	15.094	15.786	15.764	16.788	16.581	15.224	13.228
	N	5.808×10^{17}	1.242×10^{15}	6.109×10^{15}	5.808×10^{15}	6.138×10^{16}	3.811×10^{16}	1.675×10^{15}	1.690×10^{13}

[a]The term x determined by linear regression $y = mx + b$; $x = \log N$.

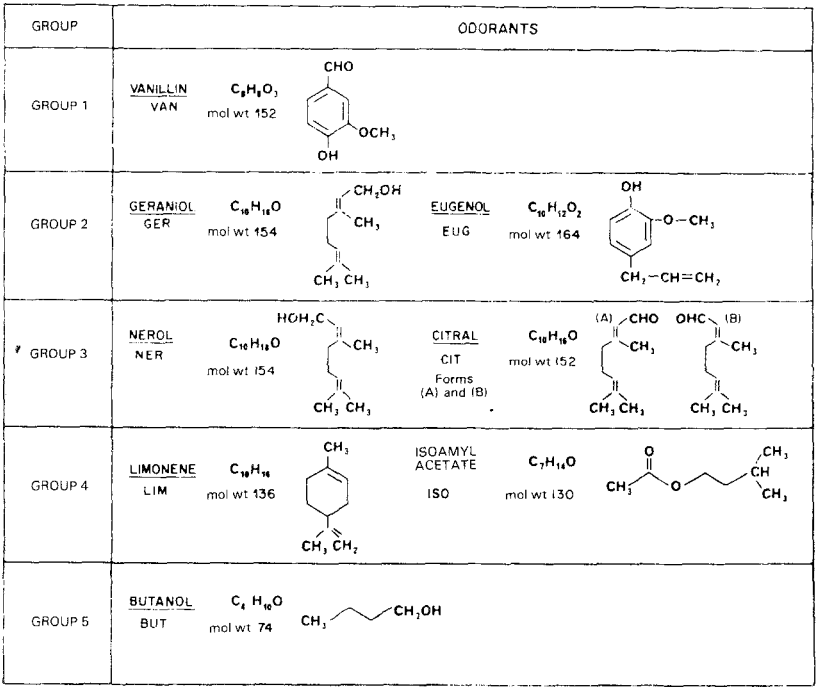

FIG. 3. An attempt to classify the stimuli tested according to the biological responses of bumblebees. MOL WT: molecular weight.

These explanations are necessary to account for the range of olfactory sensitivities expressed by the bumblebees for the tested odorants. The classification in five groups that was hypothesized is now considered to be temporary and arbitrary; the substances studied in this work may be representative of molecular groups whose diverse structural parameters should be decisive in the release (potential receptor) and transmission (generator potential) of the nervous influx along the sensory fiber. We emphasize here that the stereoisomers geraniol and nerol tested at the same concentration induce different EAG responses.

Although fine relationships between the structure and function of odorants cannot be studied with EAG recordings, this work shows that butanol, with the lowest molecular weight (74) and the poorest "response-inducing structure" in terms of chemical functions (one alcohol function only), is not as well perceived as the other stimuli tested (Figure 3). The sensitivity of the receptor cells is more or less linked to the molecular weight (Figure 3); as the number of carbon atoms does not seem to be directly concerned, our results agree with the general hypothesis that molecular

structure and chemical functions are determining factors in the interactions between odorant molecules and neuronal membrane receptors of generalist cells.

Moreover, studies of discriminatory abilities of vertebrate olfactory neuroreceptors (Sicard, 1980) have shown that *d*-limonene is representative of a "terpene group" which does not include isoamylacetate, and butanol appears to be independent of the other odor molecules tested in this work.

The data described suggest that the method used in this work is suitable for the study of the EAG recordings of bumblebees stimulated with odorants belonging to their floral and pheromonal language. These studies have two principal aims. The first is to help, by biological assay, with the chemical identification (by gas chromatography and mass spectrometry) of gland and cuticle extractions, generators of odor substances which are thought to have intra- or interspecific actions (chemical analysis of such odors is now in progress and the results will soon be published). The second is to study the peripheral olfactory nervous system function of a "generalist insect" (Masson and Brossut, 1981) and its part in the identification processing of a pheromonal blend which produces (after the integration of messages in the central nervous system) behavioral or physiological responses from the insect stimulated. Further development of this work requires the measurement of recording at the cellular level.

In *Apis*, wide-spectrum neuroreceptors (Lacher, 1964) and cells responsible for the detection of pheromones (Kaissling and Renner, 1968) have been demonstrated. Are the same features present in bumblebees? In addition, is the sensory olfactory equipment of bumblebees organized in specific cell groups as has been demonstrated in *Apis* (Vareschi, 1971)?

If the inherent potentialities of the olfactory sense of bumblebees were known, then one can determine the complexity and elaboration level of chemical communication and elucidate the actual importance of olfaction to the sensory spectrum of these insects.

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EFFECTS OF FERULIC ACID AND SOME OF ITS MICROBIAL METABOLIC PRODUCTS ON RADICLE GROWTH OF CUCUMBER[1]

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Abstract—An initial survey of the effects of aqueous solutions of ferulic acid and three of its microbial metabolic products at pH 4.5, 6.0, and 7.5 was determined on radicle growth of 11 crop species in Petri dishes. These bioassays indicated that cucumber, ladino clover, lettuce, mung bean, and wheat were inhibited by ferulic, caffeic, protocatechuic, and/or vanillic acids and that the magnitude of inhibition varied with concentration (0–2 mM), phenolic acid, and pH of the initial solution. The pH values of the initial solutions changed considerably when added to the Petri dishes containing filter paper and seeds. The final pH values after 48 hr were 6.6, 6.8, and 7.1, respectively, for the initial 4.5, 6.0, and 7.5 pH solutions. The amounts of the phenolic acids in the Petri dishes declined rapidly over the 48 hr of the bioassay, and the rate of phenolic acid decline was species specific. Cucumber was subsequently chosen as the bioassay species for further study. MES buffer was used to stabilize the pH of the phenolic acid solutions which ranged between 5.5 and 5.8 for all subsequent studies. Inhibition of radicle growth declined in a curvilinear manner over the 0–2 mM concentration range. At 0.125 and 0.25 mM concentrations of ferulic acid, radicle growth of cucumber was inhibited 7 and 14%, respectively. A variety of microbial metabolic products of ferulic acid was identified in the Petri dishes and tested for toxicity. Only vanillic acid was as inhibitory as ferulic acid. The remaining phenolic acids were less inhibitory to noninhibitory. When mixtures of phenolic acids were tested, individual components were antagonistic to each other in the inhibition of cucumber radicle growth. Depending on the initial total concentration of the mixture, effects ranged from 5 to 35% lower than the

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sum of the inhibition of each phenolic acid tested separately. Implications of these findings to germination bioassays are discussed.

Key Words—Ferulic acid, vanillic acid, caffeic acid, cucumber, radicle growth, antagonism, germination bioassays, allelopathy, phytotoxicity.

INTRODUCTION

Plants produce a variety of secondary metabolic products that have been implicated in allelopathic interactions (Whittaker and Feeny, 1971; Rice, 1974, 1979). One of the more obvious manifestations of allelopathy is the modification of seed germination and plant growth (Rice, 1974, 1979). The observed magnitude of allelopathic interactions depends on the compounds involved, the concentration and stability of such compounds in the environment, and plant resistance to such compounds and their microbial metabolic products.

Among the phytotoxins that have been identified are the simple phenolic acids. One such compound is ferulic acid (Wang et al., 1967; del Moral and Muller, 1970; McPherson, 1971; Rasmussen and Einhellig, 1977). It is commonly found in plants (Bates-Smith, 1956), has been isolated from soil (Whitehead, 1964; Guenzi and McCalla, 1966; Wang et al., 1967; Lodhi, 1975; Whitehead et al., 1981, 1982), and is a product of lignin degradation (Flaig, 1964; Turner and Rice, 1975; Martin and Haider, 1976). Microbial metabolism of ferulic acid results in the production of a variety of compounds, including vanillic acid, caffeic acid, and protocatechuic acid (Evans, 1963; Flaig, 1964; Dagley, 1971; Turner and Rice, 1975; Martin and Haider, 1976). Vanillic acid and caffeic acid have both been isolated from soils (Whitehead 1964; Guenzi and McCalla, 1966; Wang et al., 1967; Lodhi, 1975; Whitehead et al., 1981, 1982), and have been implicated as allelopathic agents (Wang et al., 1967; del Moral and Muller, 1970; McPherson, 1971).

The presence of various combinations of phenolic acids in soil solutions has been demonstrated (Whitehead et al., 1982). Little, however, is known about how these compounds might interact to bring about the observed inhibition of germination and seedling growth. Rasmussen and Einhellig (1977) observed that the total inhibition of grain sorghum germination by equal molar concentrations of ferulic and *p*-coumaric acids was greater than the sum of the inhibition of each phenolic acid tested separately. This effect was most evident 48 hr after seeds were placed into the appropriate 5×10^{-3} M solutions. Williams and Hoagland (1982) observed that the combination of coumarin plus *p*-hydroxybenzaldehyde, each at 10^{-3} M, had an additive effect in the inhibition of hemp sesbania and prickly sida germination. They also observed for hemp sesbania that coumarin in combination with chlorogenic acid (each at 10^{-3} M) was no more inhibitory than coumarin by itself. Chlorogenic acid by itself was not inhibitory to hemp sesbania.

The objectives of this study were: (1) to develop an adequate means by which potential interactions of phenolic compounds could be determined on radicle growth, and (2) to determine how ferulic acid in combination with some of its microbial metabolic products might affect radicle growth.

METHODS AND MATERIALS

Cucumis sativus cv Early Green Cluster, *Triticum aestivum* cv Roy, *Zea mays* P10 3780, *Trifolium repens* cv Tillman, *Phaseolus lunatus* cv Henderson Baby Lima, *Lactuca sativa* cv Grand Rapids, *Brassica juncea* cv Giant Southern Curled, *Glycine max* cv Hood, *Vigna radiata*, *Raphanus sativus* cv Cherry Belle, and *Phaseolus vulgaris* cv Mountain White Half Runner seeds were obtained from Wyatt Quarles Seed Company, Raleigh, North Carolina; Sphar and Company, Mt. Carmel, Illinois; or the Department of Crop Science, North Carolina State University, Raleigh, North Carolina.

Seeds were placed into plastic Petri dishes (100 × 15 mm) containing No. 3 Whatman paper and 5–7 ml of the solution to be tested. Twenty-five seeds per Petri dish were used for all but *Phaseolus* sp., *Glycine*, and *Zea*. For these species 15 seeds per Petri dish were used. All phenolic acids, except for cinnamic acid were obtained from Sigma Chemical Company, St. Louis, Missouri. Cinnamic acid was obtained from Fisher Scientific Co., Raleigh, North Carolina. Predetermined amounts of these acids were dissolved in deionized water or 15 mM MES buffer [2-(*N*-morpholino)ethanesulfonic acid]. The pH of each solution was adjusted with $\leqslant$0.1 N NaOH to pH 4.5, 5.8, 6.0, and/or 7.5 depending on the experiment. Seeds were germinated in the dark in an incubator at 25–27° C. For the initial species survey, radicle lengths were placed into two size classes, i.e., less than or greater than the mean control minus one standard deviation of the control. For all other experiments actual radicle lengths were measured. Unless specified, all radicles were measured 48 hr after seeds had been placed into the Petri dishes.

The pH values of the solutions in Petri dishes were monitored with a surface pH probe inside the dish. Concentrations of the various phenolic acids were determined initially and at various times during the germination period by injecting samples from the Petri dishes into a high-performance liquid chromatograph (HPLC). The solutions were passed through a 0.2-μm membrane filter before injection. A Waters HPLC with a model 440 absorbance detector set at 254 nm and a μ Bondapak C_{18} column was used. Isocratic elution was achieved with a solvent consisting of 35% methanol, 1% ethylacetate, and 2% acetic acid and a flow rate of 2 ml/min. Gradient elutions, to separate ferulic acid from its physiochemical breakdown and microbial metabolic products, was achieved with a model 660 solvent programmer (curve No. 10). The initial concentration was 90% of solvent A (2.5% meth-

anol, 0.25% ethylacetate, and 0.5% acetic acid) and 10% of solvent B (35% methanol, 1% ethyl acetate, and 2% acetic acid). Final concentrations (20% of solvent A and 80% of solvent B) were reached in 30 min. A flow rate of 1.8 ml/min and a total run time of 45 min was used. Physiochemical breakdown and microbial metabolic products of ferulic, vanillic, and caffeic acid were identified by comparison of retention time for known and unknown phenolic acids. Sterile techniques were employed in setting up the germination experiments. All solutions were filter-sterilized, and all other materials (filter paper, pipets, etc.) were autoclaved. Initial attempts to surface-sterilize the seeds with sodium hypochloride were discontinued since concentrations necessary to do so modified radicle growth. To test the effectiveness of the sodium hypochloride treatments, seeds were rinsed with distilled water and samples of this water were placed on Trypticase Soy agar.

Data (number/Petri dish or mean value/Petri dish) were analyzed using the Statistical Analysis System (SAS®) programs for analysis of variance, and linear and multiple regressions (Helwig and Council, 1979). First-, second- and third-order polynomial equations were fitted to the data. Final model selection criteria were the significance level (<0.05) and R^2 value. Experimental designs for the various experiments are summarized in Table 1.

RESULTS

Initial Species Survey. Since this survey was intended only to identify likely candidates for further study, a statistical analysis of the data is not presented. Of the 11 species tested in ferulic acid solutions (initial pH 4.5), lettuce, cucumber, ladino clover, wheat, and mung bean had the greatest number of seeds with radicle lengths less than the mean radicle length of the water control minus one standard deviation of the control (Figure 1). Of the three microbial metabolic products of ferulic acid, vanillic acid appeared to be more inhibitory whereas caffeic and protocatechuic acid appeared less inhibitory than ferulic acid. Although there appeared to be no clear-cut relationship between seed size and the sensitivity of a species, the four species with the largest seeds appeared to be among the least sensitive under the experimental conditions used in this study. This may have been due to the fact that for the larger seeds radicles were not always in direct contact with the solution being tested. The use of a stratified inhibition medium such as Kimpak should eliminate this problem.

Effects of Ferulic, Vannilic, or Caffeic Acid. Based on our initial screening of species we chose lettuce, cucumber, ladino clover, wheat, mung bean, and radish for further study. Since the least apparent inhibition of radicle length was observed for protocatechuic acid, it was excluded from further study.

TABLE 1. EXPERIMENTAL DESIGN OF VARIOUS EXPERIMENTS

Experiment	Species	Phenolic acids	Concentrations (mM)	pH	Solvent	Seeds/ petri dish	Number Petri dishes per treatment
Initial species survey	Lettuce	Ferulic	0–6.4	4.5	Deionized water	25	4
	Ladino clover	Vanillic				25	
	Cucumber	Caffeic				25	
	Mustard	Protocatechuic				25	
	Wheat					25	
	Corn					15	
	Soybean					15	
	Lima bean					15	
	Radish					25	
	Mung bean					25	
	Bush bean					15	
Effects of phenolic acids	Ladino clover	Ferulic	0–2	4.5	Deionized water	25	3
	Cucumber	Vanillic		6.0			
	Lettuce	Caffeic		7.5			
	Mung bean						
	Radish						
	Wheat						
Total μg of phenolic acids in Petri dishes over time	Ladino clover	Ferulic	1	4.5	Deionized water	25	2
	Cucumber	Vanillic		7.5			
	Lettuce						
	Mung bean						
	Radish						
	Wheat						

TABLE 1. Continued

Experiment	Species	Phenolic acids	Concentrations (mM)	pH	Solvent	Seeds/ petri dish	Number Petri dishes per treatment
Phenolic acid combinations	Cucumber	Ferulic Vanillic	0–2 and various combinations	5.8	15 mM MES Buffer	25	5
Phenolic acid combinations	Cucumber	Ferulic Vanillic Caffeic	0–1.5 and combinations	5.8	15 mM MES Buffer	25	4
Effects of phenolic acids over time	Cucumber	Ferulic Vanillic Caffeic	0–1.5	5.8	15 mM MES Buffer	25	4
Effects of phenolic acids	Cucumber	Cinnamic Gallic *p*-Coumaric *p*-Hydroxybenzoic	0–20	5.8	15 mM MES Buffer	25	4

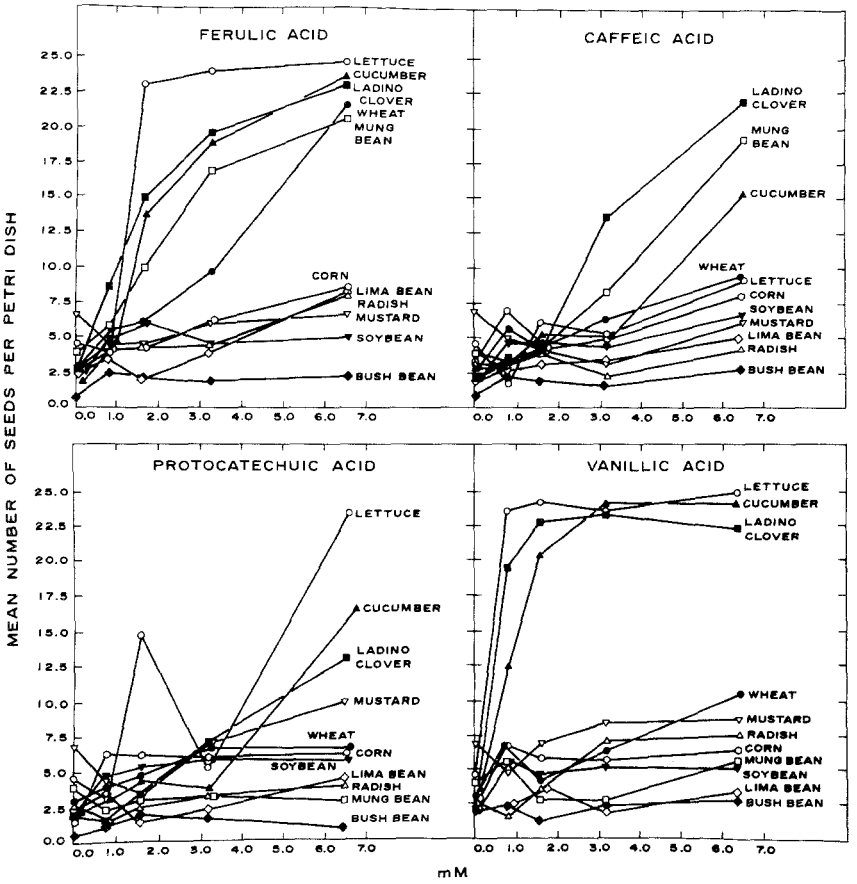


FIG. 1. The mean number of seeds per Petri dish with radicle length less than the control mean minus one standard deviation. Seeds were germinated in the dark at 25–27° C for 48 hr in various concentrations of ferulic, vanillic, caffeic, and protocatechuic acid. $N = 4$. Number of seeds per Petri dish was 25 or 15. See Table 1 for details.

For this series of bioassays, the phenolic acid solutions were adjusted to pH 4.5, 6.0, or 7.5. Since deionized water containing these phenolic acids has little buffering capacity, it was not surprising that the pH values in the Petri dishes were modified even in the absence of seed. After 2 hr, the pH values of the solutions in the Petri dishes containing filter paper alone were 5.5 ± 0.19 ($\bar{X} \pm$ SE), 6.4 ± 0.08, and 7.0 ± 0.07, and the pH values of solutions in Petri dishes containing filter paper and cucumber seeds were 5.5 ± 0.19, 6.1 ± 0.07, and 6.1 ± 0.06 for the initial solution pH values of 4.5, 6.0, and 7.5, respectively. After 48 hr, the pH values of the solution in Petri

dishes containing cucumber seeds were 6.6 ± 0.2, 6.8 ± 0.09, and 7.4 ± 0.2. The pH values for solutions containing other species of seeds were very similar to those given for cucumber. This, of course, made it difficult to clearly interpret the role of pH. The fact that significant differences in radicle length were observed for initial solution pH indicates that pH should be controlled for these types of bioassays. Significant pH and/or pH × concentration effects were observed for cucumber, lettuce, mung bean, and wheat (Table 2).

Radicle emergence was not affected by these phenolic acids. Partial regression coefficients and R^2 values of the models describing the effects of initial solution pH and concentrations of the phenolic acids on radicle length are given in Table 3. Of particular interest (note R^2 values) are the models for (1) ferulic acid and cucumber, (2) ferulic acid and mung bean, (3) vanillic acid and cucumber, and (4) vanillic acid and lettuce. Radicle lengths declined in a linear fashion with increasing concentrations of phenolic acid for: (1) cucumber and ferulic acid, pH 4.5 and 6.0, and (2) cucumber and vanillic acid, pH 6.0. Radicle lengths declined in a curvilinear fashion for the other species and pH combinations.

Total Amounts of Phenolic Acids in Petri Dishes over Time. Petri dishes containing seeds of the various species and phenolic acids were rinsed with

TABLE 2. MEAN SQUARE VALUES FROM ANALYSIS OF VARIANCE FOR RADICLE LENGTH OF SELECTED SPECIES IN PRESENCE OF FERULIC ACID, VANILLIC ACID, AND CAFFEIC ACID

Source[a]	*df*		Clover	Cucumber	Lettuce	Mung bean	Radish	Wheat
Ferulic acid								
pH	2		2.52	5.04	4.10	3.22	1.78	8.37*
Conc	8		31.88*	130.10*[b]	9.48*	138.07*	9.16*	3.58*
pH × Conc	16		5.33	8.85*	1.74	2.63	0.89	2.29
Error	54		3.23	2.73	1.86	3.19	1.76	1.54
Vanillic acid								
pH	2		0.57	55.98*	12.50*	8.05	4.24	18.24*
Conc	8		52.50*	114.65*	95.96*	15.93*	36.18*	14.24*
pH × Conc	16		2.67	8.79*	1.72	4.83	1.24	5.46*
Error	54		3.51	2.11	1.27	4.55	1.97	1.32
Caffeic acid								
pH	2	2	4.38	14.09		40.31*		
Conc	8	16	8.64*	28.19*		36.19*		
pH × Conc	16	32	2.95	11.95*		21.31*		
Error	54	100	3.43	6.85		7.68		

[a]pH = initial solution pH of 4.5, 6.0, and 7.5; Conc =concentrations 0–2 in 0.25 mM steps for all but caffeic acid and cucumber where concentrations ranged from 0 to 4 mM. Second column for df is for cucumber only.
[b]Significance level: * ≤0.05 level of probability.

TABLE 3. PARTIAL REGRESSION COEFFICIENTS AND R^2 VALUES FOR RADICLE LENGTH OF SELECTED SPECIES IN PRESENCE OF FERULIC ACID, VANILLIC ACID[a]

Species	Phenolic acid	pH	Line intercept	Linear	Quadratic	Cubic	R^2
Clover	Ferulic (F)	4.5, 6.0, 7.5	13.325	−2.397			0.37
	Vanillic (V)	4.5, 6.0, 7.5	11.951	−6.146	1.508		0.54
	Caffeic (C)	4.5, 6.0, 7.5	14.402	−1.216			0.16
Cucumber	F	4.5	29.691	−6.614			0.92
		6.0	29.212	−5.461			0.70
		7.5	30.243	−12.230	4.143		0.75
	V	4.5	29.513	−13.751	4.069		0.89
		6.0	28.593	−4.232			0.72
		7.5	29.977	−10.303	2.617		0.89
	C	4.5	27.611	−5.099	0.724		0.42
		6.0	20.794	1.338	−0.520		0.23
		7.5	21.864	−0.659			0.12
Lettuce	F	4.5, 6.0, 7.5	18.154	−1.403			0.31
	V	4.5, 6.0, 7.5	17.226	−16.482	10.799	−2.501	0.83
	V	4.5	15.409	−9.989	3.007		0.88
		6.0	18.555	−22.513	16.696	−4.068	0.87
		7.5	16.811	−9.17	2.388		0.85
Mung bean	F	4.5, 6.0, 7.5	23.286	−10.585	2.556		0.82
	V	4.5, 6.0, 7.5	22.292	−1.606			0.19
	V	4.5	24.604	−10.769	4.023		0.37
		6.0	20.535	−2.191			0.23
		7.5					
Radish	F	4.5, 6.0, 7.5	10.910	−2.904	0.859		0.30
	V	4.5, 6.0, 7.5	10.562	−2.746			0.60
Wheat	F	4.5, 6.0, 7.5	15.646	−4.450	6.678	−2.418	0.13
		4.5	16.441	−9.402	11.042	−3.584	0.32
		6.0					
		7.5					
	V	4.5	16.172	−14.725	14.499	−4.303	0.66
		6.0	14.298	−5.607	−7.987	2.311	0.63
		7.5	15.131	−10.719	14.526	−5.041	0.29

[a]See Table 1 for experimental details.

deionized water at the times indicated in Figure 2. Total micrograms of ferulic and vanillic acid in these solutions were determined by an HPLC. It was apparent that the total micrograms within each Petri dish declined with time and that the rate of decline varied with species. The initial pH of the solution apparently had little effect on the decline of both ferulic and vanillic acid in this experiment. The disappearance of these compounds was due either to adsorption/absorption by the seeds and filter paper and/or to microbial metabolism. Seeds were not surface sterilized. Physiochemical degradation

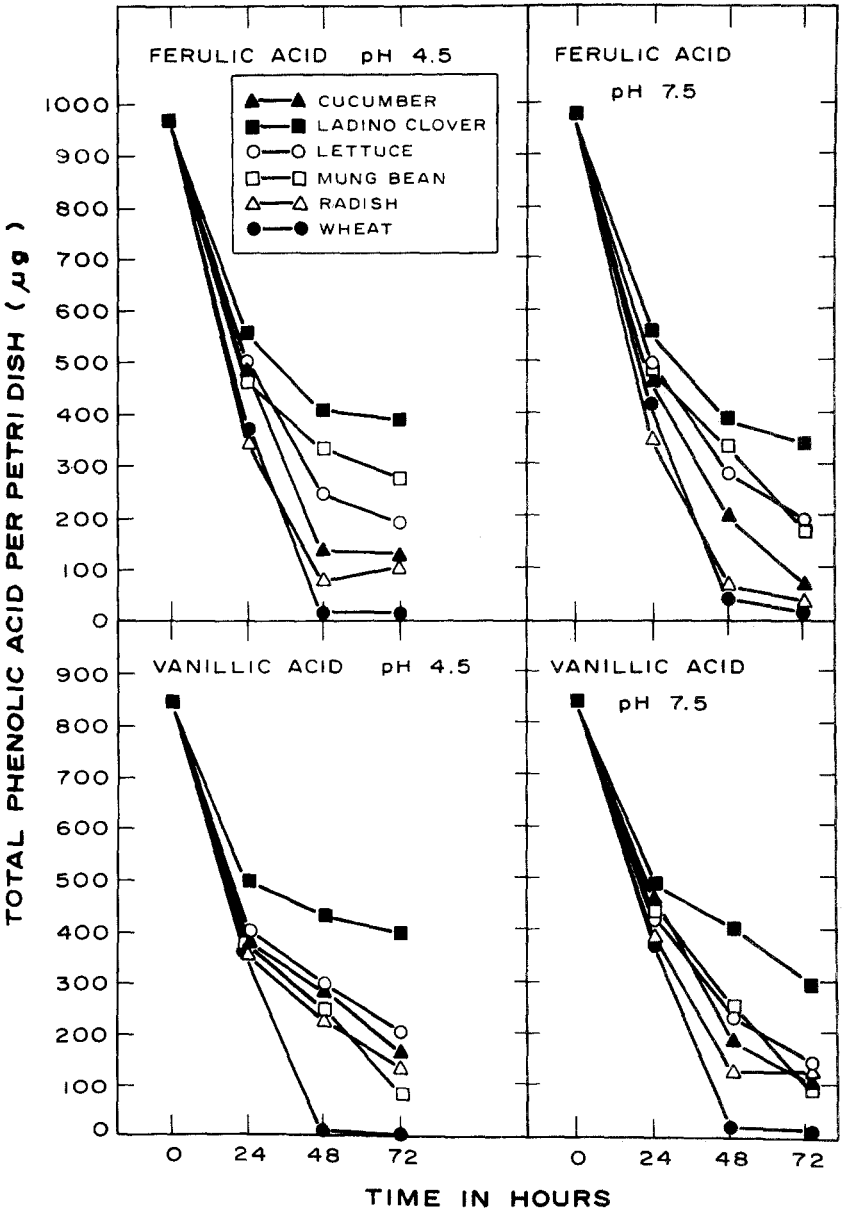


FIG. 2. Total micrograms of ferulic and vanillic acid after 48 hr in Petri dishes containing various species of seeds. Initial pH of the water solutions was 4.5 or 7.5. $N = 2$. Each Petri dish contained 25 seeds. Seeds were germinated at 25–27°C.

was of minor importance since these Petri dishes were kept in the dark. Physiochemical degradation increased substantially in the presence of light. Values given in Figure 2 are total micrograms of phenolic acid per Petri dish.

Combinations of Ferulic and Vanillic Acids. We chose to work with cucumber at this point because it was the only species for which models with reasonable R^2 values could be generated for both ferulic and vanillic acid. MES [2-(*N*-morpholino)ethanesulfonic acid] buffer was used to stabilize the pH of the various phenolic acid solutions. MES buffer has a pK_a of 6.15 and a good buffering capacity between 5.5 and 6.5. Initial pH of the various phenolic acid solutions was 5.8. The final pH at 48 hr was 5.5. We had determined earlier that the inhibitory effects of ferulic or vanillic acid on cucumber radicle growth were not significantly modified by the addition of 15 mM MES buffer to solutions containing these phenolic acids.

Cucumber seeds were placed into various concentrations of ferulic or vanillic acid solutions or several solution mixtures of these phenolic acids. The total concentration of the mixtures was 0.5, 1.0, or 2.0 mM. For each mixture concentration, the proportion of ferulic and vanillic acid was as follows: 0.75:0.25 (f:v), 0.5:0.5, and 0.25:0.75. The resulting models for this experiment are presented in Figure 3, and are described by the following equation:

$$\text{Radicle length} = 39.47 - 21.45(\text{F}) - 23.80(\text{V}) + 5.32(\text{F}^2) + 6.42(\text{V}^2) + 13.95(\text{F*V}) - 5.45(\text{V*F}^2) \quad (1)$$

$$\alpha = 0.0001 \qquad R^2 = 0.88$$

where radical length is in mm and F and V represent the ferulic and vanillic acid concentrations in mM, respectively. However, the same data could be described with a much simpler equation:

$$\text{Radicle length} = 39.25 - 21.54(\text{TC}) + 5.00(\text{TC}^2) \quad (2)$$

$$\alpha = 0.0001 \qquad R^2 = 0.87$$

where radicle length is in mm and TC represents the total concentration of the phenolic acid solution being tested in mM. This was possible because of the similarity of the effects of ferulic and vanillic acid on radicle growth. The interaction terms of equation (1) were statistically significant. This indicated that the impacts on radicle growth by ferulic and vanillic acids in the various mixtures were antagonistic rather than additive. Inhibitions of radicle length determined from predicted values of the model were $13.7 \pm 3.0\%$ ($\bar{X} \pm$ SE) below those expected. More specifically, inhibition was 5.3 ± 1.3, 13.0 ± 3.6, and $22.7 \pm 4.5\%$ below expected values for 0.5, 1.0, and 2.0 mM mixtures, respectively.

To determine the role of osmotic effects associated with the increasing

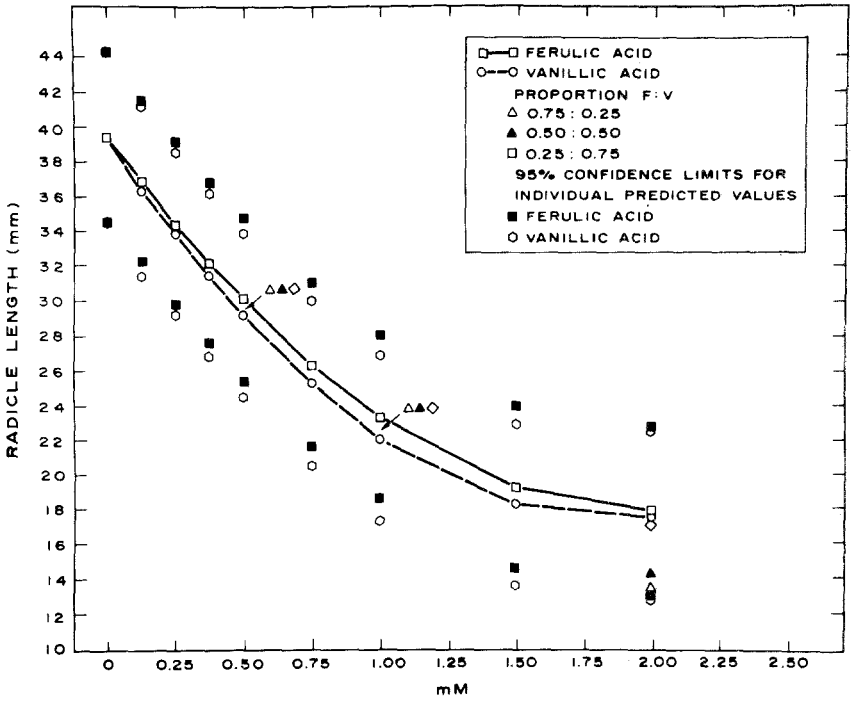


FIG. 3. Cucumber radicle lengths in Petri dishes at 25–27°C in 15 mM MES buffer and ferulic, vanillic, or various mixtures of ferulic and vanillic acids. The pH of the solutions was 5.8 at the start and 5.5 at the end of 48 hr. Lines and confidence limits were determined from the model given in the text. $N = 5$. Each Petri dish contained 25 seeds.

concentrations of the phenolic acids on radicle growth, MES buffer solutions at equivalent osmolalities for the various MES and phenolic acid solutions were tested. Values of the various solutes ranged from 14.5 mosm (milliosmoles) for 15 mM MES (control) to 25 mosm for 15 mM MES plus 3 mM of phenolic acid. No significant effects on cucumber radicle length were observed over this range.

Combinations of Ferulic, Vanillic, and Caffeic Acids. This experiment was similar to the previous experiment with the following differences: (1) seeds were germinated in solutions of ferulic, vanillic, caffeic; ferulic and vanillic; ferulic and caffeic; or vanillic and caffeic acids; (2) only one proportion was used for the mixtures (0.5 mM for each phenolic acid); and (3) there were fewer phenolic acid concentrations (Figure 4). The total concentration of each mixture was 1 mM.

The results for this experiment can be described by the following equations:

FERULIC-VANILLIC ACIDS

$$\text{Radicle length} = 37.27 - 20.88(\text{F}) - 26.57(\text{V}) + 6.07(\text{F}^2) + 10.24(\text{V}^2) + 20.76(\text{F*V}) \quad (3)$$

$$\alpha = 0.0001 \qquad R^2 = 0.89$$

FERULIC-CAFFEIC ACIDS

$$\text{Radicle length} = 37.25 - 20.85(\text{F}) - 13.03(\text{C}) + 6.06(\text{F}^2) + 4.06(\text{C}^2) + 20.39(\text{F*C}) \quad (4)$$

$$\alpha = 0.0001 \qquad R^2 = 0.85$$

VANILLIC-CAFFEIC ACIDS

$$\text{Radicle length} = 35.46 - 21.78(\text{V}) - 6.25(\text{C}) + 7.76(\text{V}^2) + 10.49(\text{V*C}) \quad (5)$$

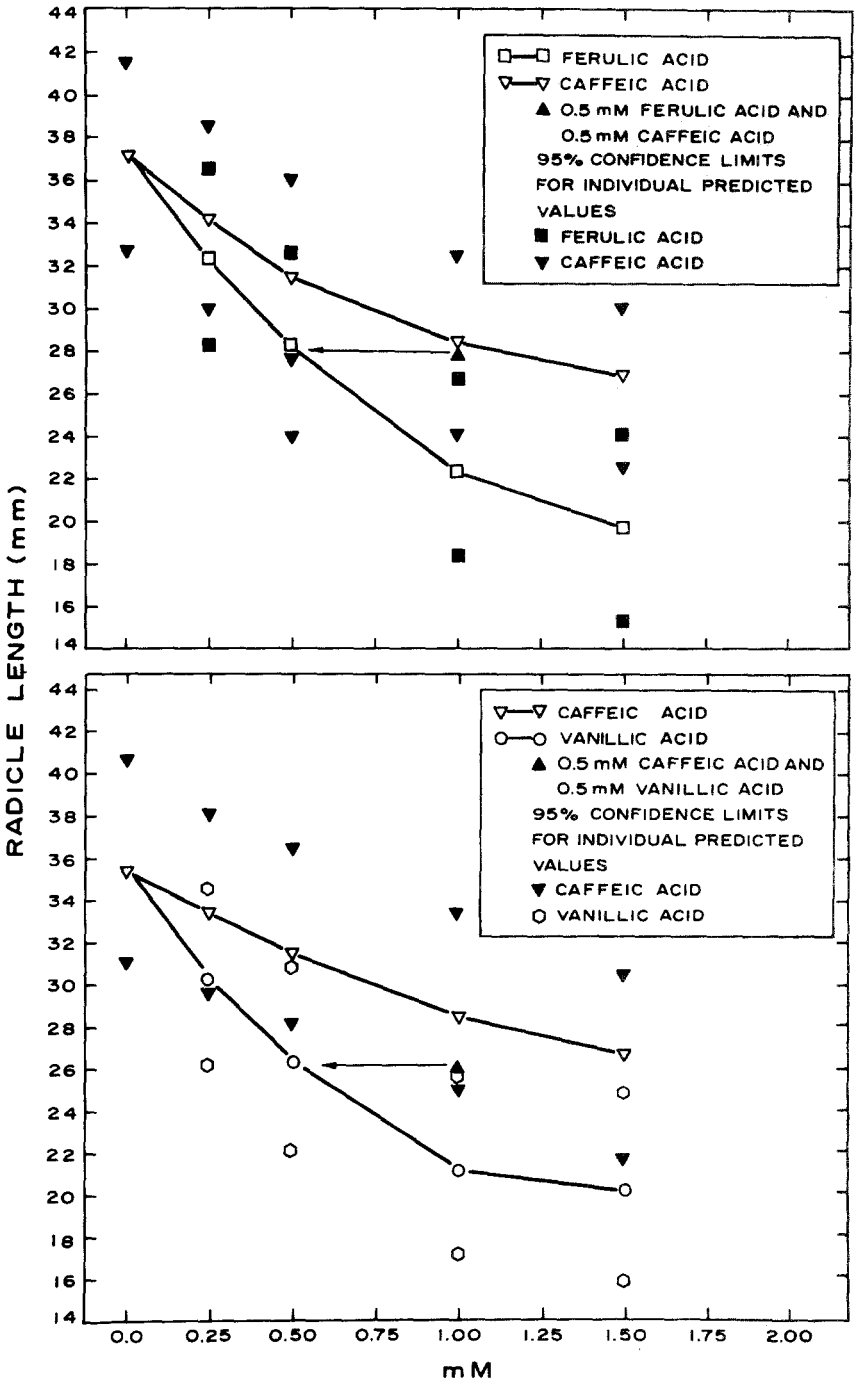
$$\alpha = 0.0001 \qquad R^2 = 0.79$$

where radicle length is in mm and F, V, and C represent the mM concentrations of ferulic, vanillic, and caffeic acids, respectively. Data used to generate equation (3) could also be described by a much simpler equation

$$\text{Radicle length} = 37.11 - 22.82(\text{TC}) + 7.65(\text{TC}^2) \quad (6)$$

$$\alpha = 0.0001 \qquad R^2 = 0.88$$

where radicle length is in mm and TC represents the total concentration of the solution being tested in mM. The interaction term in equation (3) was statistically significant, but was only so when the nonsignificant quadratic terms were included. This supported the conclusions of the previous experiment with mixtures of ferulic and vanillic acids that the effects of ferulic and vanillic acid in mixtures were essentially antagonistic. Inhibition of radicle lengths determined from predicted values of the model were 28% below those expected. The effects of ferulic and caffeic acid singly and in a mixture and vanillic and caffeic acids singly and in a mixture could not be adequately described by a model based on the total concentration of the solutions being tested. This was due to the differential effects of caffeic acid on radicle length when compared with ferulic or vanillic acid. For ferulic, caffeic, and ferulic–caffeic mixture, the R^2 value for total concentration models dropped by 17% and for the vanillic, caffeic, and the vanillic–caffeic acid mixture the R^2


FERULIC ACID
CAFFEIC ACID
0.5 mM FERULIC ACID AND 0.5 mM CAFFEIC ACID
95% CONFIDENCE LIMITS FOR INDIVIDUAL PREDICTED VALUES
FERULIC ACID
CAFFEIC ACID
CAFFEIC ACID
VANILLIC ACID
0.5 mM CAFFEIC ACID AND 0.5 mM VANILLIC ACID
95% CONFIDENCE LIMITS FOR INDIVIDUAL PREDICTED VALUES
CAFFEIC ACID
VANILLIC ACID
RADICLE LENGTH (mm)
mM
0.0
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44

value dropped by 16%. Here also the actions of the mixtures on radicle growth were not additive but antagonistic. Note that the ferulic and vanillic acids in combination with caffeic acid were no more inhibitory than ferulic and vanillic acids by themselves (Figure 4). Inhibition of radicle lengths determined from predicted values of the models were 35% and 22% below those expected for the ferulic acid and caffeic acid mixture and the vanillic and caffeic acid mixture, respectively.

After 48 hr, the phenolic acid concentrations in Petri dishes were reduced substantially, ranging from 100% at lower to 80% at higher concentrations for ferulic and vanillic acid and almost entirely over the whole concentration range for caffeic acid. Concentrations in mixture at 48 hr were similar to those observed for the equivalent concentrations of the individual phenolic acids. Two likely reasons for these observed reductions were: (1) adsorption/absorption of phenolic acids by seeds and radicles, and (2) microbial metabolism of the phenolic acids. Cucumber seeds were not surface-sterilized. We attempted to do this but with little success. Physiochemical degradation was of minor importance. Microbial metabolism appeared to be significant since a variety of metabolic products were detected in Petri dishes containing phenolic acids, but not in the Petri dishes containing the buffer control. It is, of course possible, that some of these products were a result of uptake by cucumber seeds/radicles, metabolism, and subsequent radicle exudation.

Vanillic, cinnamic, and decarboxylated ferulic acids (Liebl and Worsham, 1983) (a styrene derivative) were detected in the Petri dishes supplied with 1 or 1.5 mM ferulic acid. Microbial metabolic products were detected for all treatment concentrations of caffeic acid. The following compounds were isolated and identified: gallic, protocatechuic, vanillic, *p*-coumaric, ferulic, and cinnamic acids. Protocatechuic, ferulic, and *p*-coumaric acid were detected in all the caffeic acid treatments. The products detected for the mixtures were identical to those observed for the pure equivalent concentrations of the individual phenolic acid treatments. As might be expected, concentrations of these metabolic products were small, ranging from <1 ppm to 4.5 ppm. For this experiment, solutions remaining in the Petri dish were injected into the HPLC without dilution. Finally, there were also a number of other compounds with absorbance at 254 nm in the Petri dishes which were not identified.

FIG. 4. Cucumber radicle lengths in Petri dishes at 25–27° C in 15 mM MES buffer and ferulic, vanillic, caffeic, and various mixtures of ferulic and caffeic and vanillic and caffeic acids. The pH of the solutions was 5.8 at the start and 5.5 at the end of 48 hr. Lines and confidence limits were determined from the appropriate models given in the text. Arrows indicate levels of inhibition of radicle growth for seeds germinating in 0.5 mM ferulic or vanillic acid. The mixtures of the phenolic acids contained 0.5 mM of both phenolic acids. $N = 4$. Each Petri dish contained 25 seeds.

Effects of Phenolic Acids over Time. The effects of ferulic acid on cucumber radicles at 24 and 48 hours could be described by the following models (Figure 5):

$$\text{Radicle length at 24 hr.} = 9.17 - 1.75(F)$$

$$\alpha = 0.0001 \qquad R^2 = 0.78$$

$$\text{Radicle length at 48 hr.} = 46.97 - 29.64(F) + 9.42(F^2)$$

$$\alpha = 0.0001 \qquad R^2 = 0.95$$

where radicle length is in mm and F represents the concentration of ferulic acid in mM. For vanillic acid the models were as follows (Figure 5):

$$\text{Radicle length at 24 hr.} = 9.47 - 5.91(V) + 5.25(V^2)$$

$$\alpha = 0.0001 \qquad R^2 = 0.93$$

$$\text{Radicle length at 48 hr.} = 46.38 - 21.82(V) - 5.59(V^2)$$

$$\alpha = 0.0001 \qquad R^2 = 0.87$$

where radicle length is in mm and V represents the concentration of vanillic acid in mM. Beyond 48 hr (i.e., 72 hr), it was not possible to determine the effects of ferulic and vanillic acids on radicle length of cucumber since seedlings were too crowded in the Petri dishes and water was limiting.

The caffeic acid experiment was carried out independently. No significant effect of caffeic acid on radicle growth was observed at 24 hr. The model for 48 hr was as follows (Figure 5):

$$\text{Radicle length} = 34.31 + 1.20(C) - 2.92(C^2)$$

$$\alpha = 0.0004 \qquad R^2 = 0.70$$

where radicle length is in mm and C represents the concentration of caffeic acid in mM.

As noted earlier, concentrations of phenolic acids in the Petri dishes declined with time (Figure 6). Microbial metabolic products were similar to those described in the previous section. At 24 hr the identified compounds were as follows: (1) for ferulic acid—vanillic and *p*-hydroxybenzoic acid, (2) for vanillic acid—*p*-hydroxybenzoic and protocatechuic acid, and (3) for caffeic acid—protocatechuic, *p*-hydroxybenzoic, vanillic, *p*-coumaric, ferulic, and cinnamic acid. At 48 hr: (1) for ferulic acid—vanillic and cinnamic acid, (2) for vanillic acid—protocatechuic acid, and (3) for caffeic acid—gallic, protocatechuic, vanillic, *p*-coumaric, ferulic, and cinnamic acid. Concentrations for the various microbial metabolic products were below 4 ppm, most below 1 ppm, with two exceptions. Protocatechuic and *p*-coumaric acids in the 1 and 1.5 mM caffeic acid treatments reached 10 ppm in several Petri dishes.

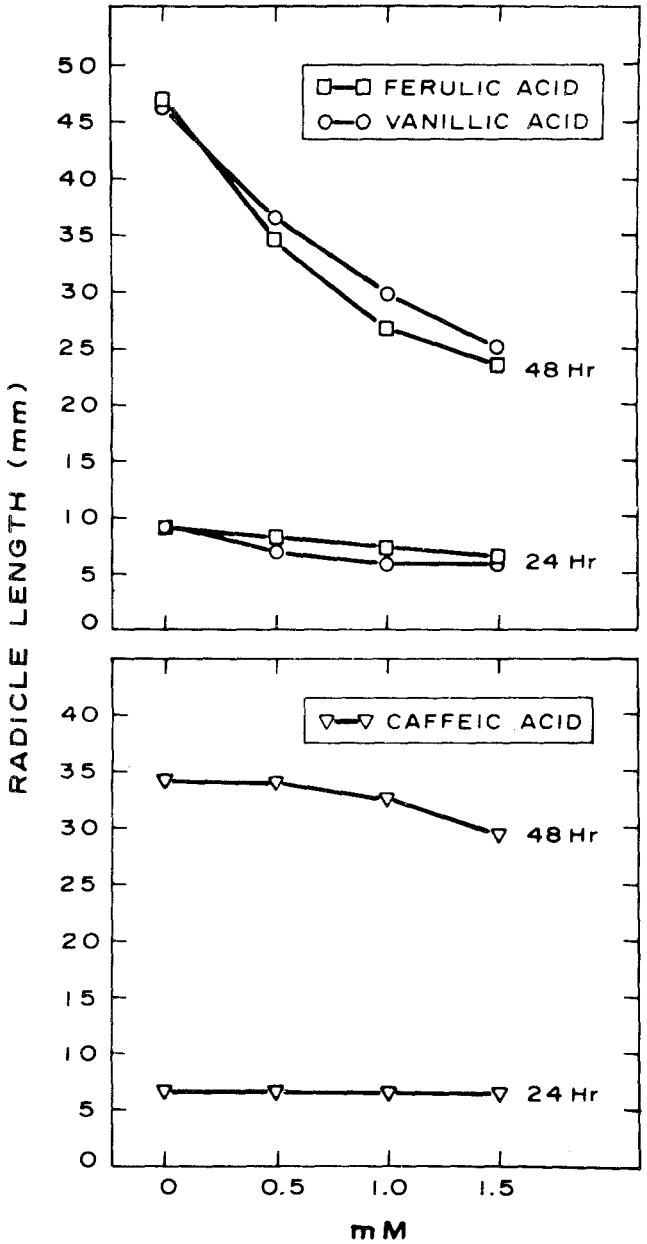


FIG. 5. Cucumber radicle lengths at 24 and 48 hr in Petri dishes containing 15 mM MES buffer and various concentrations of ferulic, vanillic, and caffeic acids. Lines were determined from the appropriate models given in the text. $N = 4$. Seeds were germinated at 25–27°C. The pHs of the solutions were 5.8 at start and 5.5 at 48 hr. Each Petri dish contained 25 seeds.

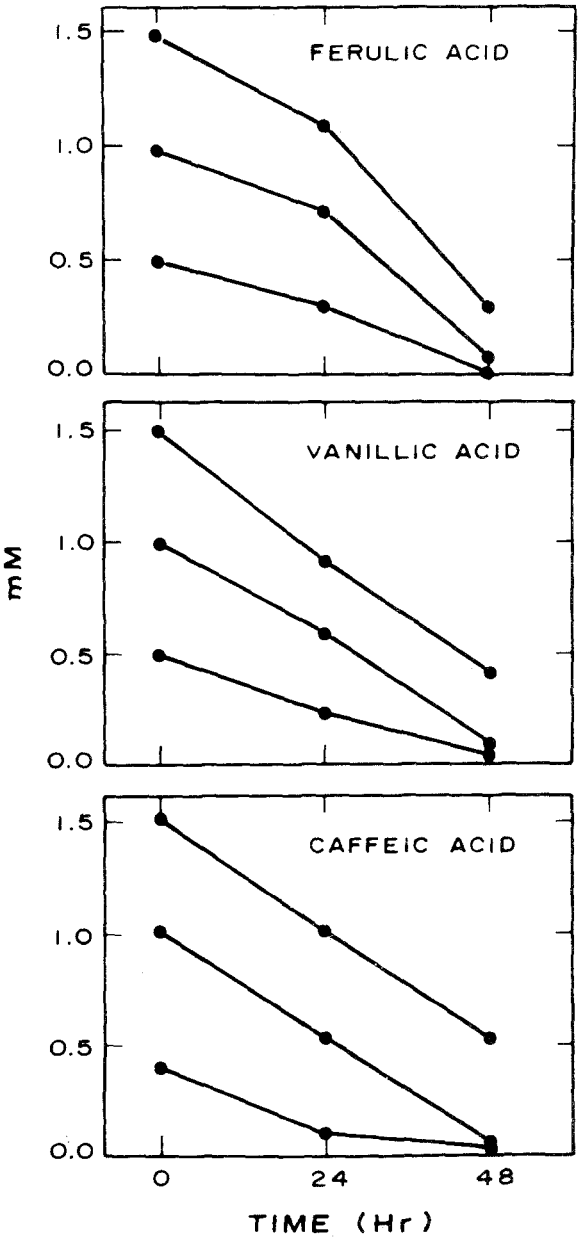


FIG. 6. The mM concentrations of ferulic, vanillic, and caffeic acids at 0, 24, and 48 hr in Petri dishes containing cucumber seeds, 15 mM MES buffer, and various concentrations of phenolic acids. $N = 2$. Seeds were germinated at 25–27°C. Each Petri dish contained 25 seeds. The pHs of the solutions were 5.8 at start and 5.5 at 48 hr.

Effects of Phenolic Acids. To determine how some of the identified microbial metabolic products might affect the radicle length of cucumber, these were also tested. Gallic and *p*-hydroxybenzoic acid did not significantly affect radicle length at the concentrations tested. Results for cinnamic and *p*-coumaric acid were described by the following models:

$$\text{Radicle length} = 32.18 - 6.84(\text{CI})$$

$$\alpha = 0.0001 \qquad R^2 = 0.91$$

$$\text{Radicle length} = 32.34 + 3.03(\text{PC}) - 3.14(\text{PC}^2)$$

$$\alpha = 0.0001 \qquad R^2 = 0.85$$

where radicle lengths is in mm and CI and PC represent the cinnamic and *p*-coumaric acid concentrations in mM, respectively. Of the two acids, cinnamic acid was the most inhibitory to cucumber radicle growth.

DISCUSSION

The initial species survey and subsequent tests to determine species susceptible to ferulic acid and several of its microbial metabolic products led us to identify cucumber (*Cucumis sativus* cv Early Green Cluster) as a sensitive species with several important characteristics. These were: (1) germination occurs in less than 24 hr at 25–27° C, (2) radicle emergence and growth are predictable, (3) radicle growth is rapid, and (4) radicles are of sufficient size within 24–48 hr for rapid and precise measurements.

Normally, germination studies are conducted in Petri dishes containing filter paper, distilled water, the compound to be tested, and seeds. The pH of the solutions is frequently not adjusted or monitored. We noted, however, that the pH of the various solutions containing the phenolic acids was approximately 3.2–3.6. The pK for ferulic acid, the compound of primary interest, is approxiamtely 4.8. The main effect of pH would be on ionization and solubility of phenolic acid. The pH of the solution could thus have a substantial impact on the uptake and activity of ferulic acid. We therefore adjusted the pH of our various solutions. However, as noted in the Results section, the pH of the aqueous solutions changed almost immediately once the solutions were added to the Petri dishes. The pH of the various solutions changed from 4.5 (initial pH) to 6.6 (pH at 48 hr), 6.0 to 6.8, and 7.5 to 7.1. There were, however, significant pH–phenolic acid concentration interactions for several species. The addition of some type of buffer appeared necessary to stabilize the pH of the solutions. A stable pH is of particular importance when comparing the action of phenolic acids at different concentrations and when comparing the action of phenolic acids individually or in mixtures on radicle growth.

The pH values of the soil systems under study in our laboratory range

from 5.2 to 6.6. We therefore chose MES [2-(*N*-morpholino)ethanesulfonic acid] buffer which has a buffering range of 5.5–6.5 (Good et al., 1966). We found that a 15 mM solution of MES buffer was sufficient to maintain the pH values desired for the concentrations of the phenolic acids used. Addition of the various phenolic acid concentrations to the 15 mM buffer did not modify the water potential sufficiently to significantly reduce radicle lengths of cucumber. MES buffer also did not significantly modify the inhibitory effects of ferulic and vanillic acid on cucumber radicles.

Physiochemical degradation and microbial degradation of the phenolic acids were monitored over time. Physical degradation was minimized by keeping the Petri dishes containing the phenolic acids and seeds in the dark. Cucumber seeds frequently have associated with them substantial microbial populations which are found both externally and internally (Mundt and Hinkle, 1976; Leben, 1961). Since microbial metabolism of ferulic acid in the Petri dishes could have a substantial impact on its inhibitory potential, an attempt was made to sterilize the cucumber seeds. However, various sodium hypochloride treatments were ineffective, since bacterial colonies were found on agar plates when water used to rinse treated seeds was plated on Trypticase Soy agar. Therefore, seeds for all experiments were not surface-sterilized. Sterile techniques were employed in setting up the germination experiments. All solutions were filter sterilized, and all other materials (filter paper, pipets, etc.) were autoclaved. Since the seeds were not sterilized, it was not possible to determine whether the decline of the phenolic acids in the Petri dish was mainly due to adsorption/absorption by seeds or microbial metabolism.

The phenolic acid concentrations in the Petri dishes declined rapidly. Based on the concomitant appearance of a variety of phenolic acids, some of which had been previously identified as microbial breakdown products of ferulic acid (Evans, 1963; Flaig, 1964; Dagley, 1971; Turner and Rice, 1975; Martin and Haider, 1976), we concluded that the reduction in concentration and the introduction of other phenolic acids by microbial activity could play an important role in the magnitude of inhibition on cucumber radicles, the former reducing inhibition and the latter possibly increasing and/or decreasing inhibition. We noted, however, that the inhibition of cucumber radicle growth increased with time. Unfortunately, due to the size of the Petri dishes used, we could not go beyond 48 hr because the germinating cucumber seedlings became very crowded in the Petri dishes and water became a limiting factor.

Effects of Phenolic Acids. Inhibition of cucumber radicles could best be described as a curvilinear response, although in a few instances it was linear over the concentration range used. Since radicle emergence was not affected by any of the concentrations used in these experiments and initial radicle expansion (first 24 hr) was little affected by the phenolic acids, it

appears that the effects of the phenolic acids tested were on cell division and subsequent cell expansion. If true, this could explain why inhibition of radicle length increased with time and declined in a curvilinear manner with increasing phenolic acid concentrations. In the latter case, the radicle lengths of cucumber could not be suppressed below that produced by initial radicle expansion.

Although uptake of phenolic acids by cucumber seeds has not been demonstrated, radicle lengths were reduced 7% at 0.125 mM concentration of ferulic and vanillic acid. At 0.25 mM, reductions of radicle lengths ranged from 13 to 16% for these two acids, while inhibition by caffeic acid ranged from 0 to 8% for the same concentration. The number of caffeic acid microbial metabolic products observed in the Petri dish at 24 and 48 hr indicated that it may be the least stable of the three phenolic acids tested. Cinnamic acid was one of the more inhibitory compounds of the metabolic products. At 0.25 mM, cinnamic acid reduced the radicle lengths of cucumber by 5%. Thus, except for vanillic acid, all other microbial metabolic products produced from ferulic acid were less inhibitory than ferulic acid.

Effects of Phenolic Acid Mixtures. Rasmussen and Einhellig (1977) and Williams and Hoagland (1982) noted in their germination studies that in some instances phenolic acids in mixtures acted differently than expected in inhibiting germination (i.e., radicle emergence). They observed additive effects (expected) and greater than additive effects (unexpected) for mixtures of phenolic acids. We chose to test combinations of ferulic acid (4-hydroxy-3-methoxycinnamic acid), vanillic acid (4-hydroxy-3-methoxybenzoic acid), and caffeic acid (3,4-dihydroxycinnamic acid). Vanillic and caffeic acid are two initial microbial metabolic products of ferulic acid (Turner and Rice, 1975; Martin and Haider, 1976). For each mixture tested, we noted that the inhibition of the mixture of phenolic acids was lower than the sum of the inhibition by the individual compounds. Inhibition ranged from 3 to 35% below that expected. Rasmussen and Einhellig (1977) also worked with compounds of similar structure [(ferulic and *p*-coumaric (*p*-hydroxycinnamic) acid]. Thus structure and associated competition between molecules for sites could not explain the differences in response for these two studies. More likely, it had to do with the processes under study (i.e., radicle emergence vs. radicle growth) and the bioassay species utilized. Finally, one might expect that if increasing concentrations of phenolic acids bring about a curvilinear response of inhibition of radicle lengths (i.e., a decline of the magnitude of inhibition) that the total concentration of a mixture would affect the relationship between expected and actual inhibition observed. In other words, as the total concentration of the mixture increases, the observed inhibition of cucumber radicle length for the mixture treatments would fall farther and farther from the expected inhibition. This appeared to be the case at least for ferulic and vanillic acid, the only data set appropriate

for such a comparison. The inhibition was 5.3 ± 1.3 ($\bar{X}$ ± SE), 13.0 ± 3.6, and 22.7 ± 4.5% below expected values for 0.5, 1.0, and 2.0 mM mixtures, respectively. This would suggest that at low enough concentrations the effects of ferulic and vanillic acid on cucumber radicle length could be additive.

Summary. Of the ferulic acid microbial metabolic products observed in Petri dishes containing germinating cucumber seeds, only vanillic acid was approximately of equivalent toxicity to ferulic acid. The remaining phenolic acids ranged from considerably less toxic to nontoxic over the concentration range of 0.25–2 mM. Concentrations in Petri dishes ranged from 1 to 10 ppm. In soils where concentrations of some of these compounds may be higher, they could play a significant role in determining radicle growth of cucumber. Whitehead and Hartley (1982), for example, observed in soil solutions from soils in which *Agropyron repens* was growing concentrations equivalent to 0.058 mM *p*-hydroxybenzoic acid, 0.016 mM vanillic acid, 0.064 mM *p*-coumaric acid, and 0.04 mM ferulic acid. In soils in which *Campanula rapunculoides* was growing, they observed the equivalent of 0.18 mM *p*-coumaric acid. Soils in all these instances were extracted with 0.5% $Ca(OH)_2$. The impact of various mixtures on germination can be additive, greater than additive (Rasmussen and Einhellig 1977; Williams and Hoagland, 1982) or, as in our case on radicle growth, antagonistic. Depending on the initial total concentration of the mixture of phenolic acids in the Petri dishes, antagonistic effects ranged from 5 to 35%. By that we mean that the inhibition of radicle length by the mixtures was 5 to 35% lower than the sum of the inhibition of each phenolic acid tested separately. On the linear portion of the response curve for cucumber radicles, the antagonistic effects of ferulic and vanillic acid averaged less than 5%.

Germination bioassays are a very important tool in the study of allelopathic phenomena. Up to this point, however, little progress has been made in standardizing bioassay procedures. Based on observations while conducting these experiments, we recommend the following for future germination studies: (1) that the pH of the various phenolic acid solutions being tested be stabilized with an appropriate buffer which will not interfere with normal growth and will not interact with the phenolic acid, (2) that seeds be sterilized if possible, (3) that germination bioassays be carried out in the dark since many phenolic acids are photolabile, (4) that the response curves of the biological variables being measured be determined before extensive experimentation, so that responses to phenolic acids can be determined on the linear portion of the response curve, (5) that the loss of phenolic acids and the occurrence of microbial products in Petri dishes be determined, and (6) that an adequate water supply be maintained over the entire study period.

General applications of the above recommendations could lead to more uniform germination studies in the future. This would allow more direct comparisons and wider application of resulting germination bioassay data.

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SEX PHEROMONE SOURCE LOCATION BY GARTER SNAKES: A Mechanism for Detection of Direction in Nonvolatile Trails

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Abstract—Male plains garter snakes, *Thamnophis radix*, tested in a 240-cm-long arena can detect directional information from a female pheromone trail only when the female is allowed to push against pegs while laying the trail. The female's normal locomotor activity apparently deposits pheromone on the anterolateral surfaces of vertical structures in her environment. The male sensorily assays the sides of these objects and from this information determines the female's direction of travel.

Key Words—Sex pheromone trail, direction determining, plains garter snake, *Thamnophis radix*.

INTRODUCTION

Snake sex pheromones allow for sex and species recognition and stimulate male courtship behavior (Devine, 1977; Noble, 1937). Recent evidence indicates that the sex pheromone eliciting reproductive activity in the garter snakes, genus *Thamnophis*, is a lipoprotein exuded from the skin (Garstka and Crews, 1981). This pheromone is synthesized in the liver, transported via the dermal vascular bed to the epidermis (Rauch, 1978) and, upon movement of the female, is forced through the hinge region of the scales (Garstka and Crews, 1981).

This skin pheromone is also believed to be deposited as a trail (Garstka and Crews, 1981; Noble, 1937) which guides males to females during the breeding season (Ford, 1981). Trailing activity in garter snakes is ac-

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complished utilizing the vomeronasal system (Heller and Halpern, 1981). The tongue of a trailing snake picks up substrate odorants deposited by the preceding snake, and this pheromone is delivered to the vomeronasal epithelium which mediates its analysis (Gillingham and Clark, 1981; Halpern and Kubie, 1983). Several studies suggest that the trail pheromone also contains information concerning the sex, receptivity, and species of the trail-laying animal (Ford, 1978, 1982; Ford and Schofield, 1984; Noble, 1937). However, the mechanism by which the male recognizes which way to go on the trail, in order to locate the female, has not been examined.

Any species that utilizes pheromones as trails to locate resources must have some mechanism to communicate information concerning the direction of the resource. If the trail pheromone is volatile, a longitudinal gradient would occur and an animal encountering a trail could follow it in the direction of increasing concentration. However, this mechanism would not be available to snakes as the lipoprotein suggested for the sex pheromone, vitellogenin, is a large, complex molecule (Gibson et al., 1983) and would likely produce a trail of low volatility. This is supported by evidence that snake trails can last from a few hours to several days (Ford, 1976). It appears that some other mechanism imparts directional information.

There are several clues as to how directionality can be specified in snake sex pheromone trails. First, the pheromone is released predominantly from the dorsal and lateral skin (Gillingham and Dickinson, 1980; Noble, 1937), even though a ventral secretion would seem to be more efficient in laying a terrestrial trail. Secondly, males tend to tongue-flick objects the female has touched as she moves through the environment (personal observation), implying significant chemosensory processing. Thirdly, colubrid snakes achieve locomotion by pushing against the anterolateral surface of environmental structures (rocks, sticks, etc.) they encounter. The lateral forces cancel each other and forward motion is achieved (Gans, 1962). The sex pheromone in the skin could be exuded during this activity and deposited not only on the substrate as a trail, but also on the anterior and lateral surfaces of the vertical objects that the female uses for locomotion (Figure 1A). We hypothesize that male snakes sensorily assay the pheromone distribution on these objects and from this information determine the direction to travel to locate the female (Figure 1B).

METHODS AND MATERIALS

The ability of male plains garter snakes, *Thamnophis radix,* to follow female pheromone trails, produced under three different physical conditions, was tested. Animals were collected in May of 1981 and 1982 from Illinois and Colorado, maintained individually in an animal room at the University of

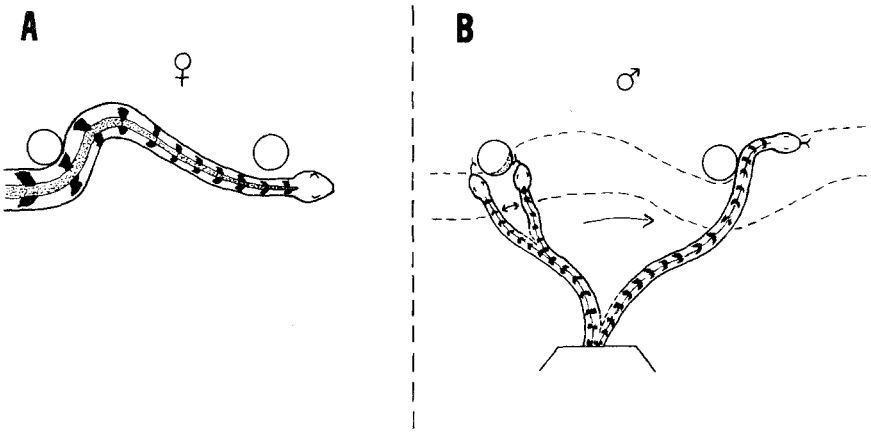

FIG. 1. Hypothesized mechanism for male garter snakes to determine direction from a female snake's trail. (A) Female garter snake laying a trail. Note she pushes against the anterolateral surface of the ojbects in her environment (pegs). (B) Behavior of a male garter snake when he encounters a female trail. Stipling on the first peg represents the probable deposition of the pheromone there, whereas the dashed lines represent the deposition of the pheromone on the substrate as a trail. The male tongue-flicks both the anterior and posterior sides of the pegs, and with this sensory assay detects the pheromone on the anterior surface. He then proceeds in this direction, using chemotactic orientation on the trail.

Texas at Tyler under a 14 : 10 light cycle at 25° C, and were tested within three weeks of their capture. This is the breeding period for this species, and previous observations indicated that strong trailing behavior would occur during this time period (Ford, 1981).

Three tests were set up for each male in a 240 × 75-cm arena. In each, the male was first held for 1 hr in a small box with a trap door. Then a pheromone trail was laid by inducing a female from the same locality to move down the length of the arena between two boards spaced 10 cm apart (Figure 2A). The boards were removed and the male released by placing the holding box at the midpoint of the trail and opening the trap door (Figure 2B). When the male left the box and followed the trail, his direction was recorded (scored after he went one half the remaining distance to the end of the trail, although males usually traveled the complete distance). In the first test, a single row of pegs, each firmly covered with a plastic soda straw and spaced 5 cm apart, was present between the two boards (Figure 2A). The female utilized the pegs to achieve locomotion while laying the trail and thus touched both the floor and the pegs. In the second test, two rows of pegs were placed in the arena (as in Figure 2B), but the boards (still 10 cm apart) were placed inside the pegs. The female pushed against the boards to achieve locomotion (concertina move-

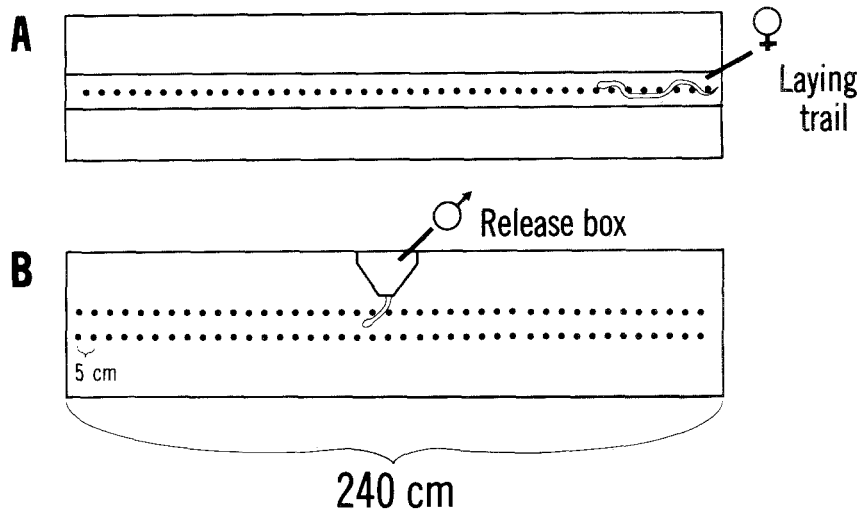

FIG. 2. Arena used to test direction determining ability of male plains garter snakes, *Thamnophis radix*, on female phermone trails. (A) Arena set up with a single row of pegs (tests 1 and 3) and showing a female laying a trail between the two removable boards. (B) The arena showing the male release. The boards had been placed inside the two rows of pegs when the female's trail was laid (test 2).

ment; Gans, 1962) and, when the boards were removed, the trail was present only on the substrate. In the third test, the arena contained one row of pegs as in test 1 and, after the female laid the trail, the straws were turned around 180 degrees so that, hypothetically, the substrate trail "indicated" one direction and the pegs "indicated" the reverse.

The floor of the arena in all tests was covered with brown wrapping paper which the pheromone did not penetrate (Ford, 1979), and the paper was removed and replaced after each test as were the straws covering the pegs. The order of testing for each male was randomized, as was the direction in which the female trail was laid and which female was used to lay the trail. Each male was run in only one of his three tests on any given day. If a male did not trail during a test, i.e., he did not follow the pegs in either direction (typically, nontrailing males exhibited wall-seeking behavior) or he did not leave the holding box within 1 hr, the test was repeated at a later time. If he still did not trail, a new male was substituted in his remaining tests. Each male was therefore run only once on a test and, for analysis, we assumed independence of the three experiments. A one-tailed binomial test of the hypothesis that the male would follow the trail in the direction of the female was conducted for experiments 1 and 2. The hypothesis for test 3 was that the male would go the direction the pegs "indicated."

RESULTS AND DISCUSSION

Male *Thamnophis radix* can detect directionality in a female's pheromone trail when the female utilizes pegs for locomotion (test 1), but cannot do so when the trail is only on the floor of the arena (test 2) (Table 1). These results indicate the pegs (environmental objects) are necessary for direction determination. The third experiment further supports this conclusion (although P was only 0.06) as the males tended to follow the trail in the direction the pegs "indicated" without regard for the trail direction on the paper substrate.

The only directional information that the males could receive from the pegs relates to the deposition of the female's pheromone on the anterior surface of objects in their environment (Figure 1A). The normal locomotion of the female requires structures to push against, and so the secretion of a nonvolatile pheromone from the lateral skin provides a mechanism to impart directionality in the trail. When a male encounters a female's trail, a chemosensory analysis of these objects would allow him to detect a greater quantity of pheromone on the side of the female's direction of travel. We noted an increase in tongue-flick rate when the trail was encountered by the males, and much of this activity was concentrated at the first few pegs approached (Figure 1B). In particular, we observed that more than one side of the peg was touched by the male's tongue. This sensory activity is apparently involved not only in assaying the chemical composition of the pheromone (i.e., to allow species identification: Ford, 1978, 1982; Ford and Schofield, 1984) but also in evaluating the proper direction to proceed to find the female. The male then utilizes the tongue and vomeronasal organ in chemotactic

TABLE 1. ABILITY OF MALE PLAINS GARTER SNAKES, *Thamnophis radix*, TO DETERMINE DIRECTION OF FEMALE PHEROMONE TRAIL UNDER THREE DIFFERENT PHYSICAL CONDITIONS[a]

Test	N	Number of males going correct direction	P
1. Pheromone on substrate and vertical pegs	25	17	0.05
2. Pheromone on substrate only	20	9	NS[b]
3. Pheromone on substrate and vertical pegs reversed	20	14	0.06

[a] For experiments 1 and 2, a one-tailed binomial test of the hypothesis that the males would follow the trail in the direction of the female was conducted. For experiment 3 the hypothesis tested was that the males would go the direction the pegs "indicated."
[b] Not significant.

orientation to follow the substrate trail (Ford, 1979), weaving his head in a sinusoidal manner that resembles the corrective steering reactions used by ants on food-recruiting pheromone trails (Hangartner, 1967; Leuthold, 1968).

The use of a pheromone trail to locate a resource, whether it be a female, food, nesting site, or other necessity, requires some mechanism to detect the direction of this resource. The food-recruiting trails of social insects are produced with highly volatile pheromones (Bossert and Wilson, 1963) and could be expected to produce a polarized trail with the odor gradient. However, because of constant new pheromone deposition by the many insects on the trail, a nonpolarized longitundinal trail is actually produced (Farkas and Shorey, 1974). It is therefore generally believed that chemotaxis is not involved in orienting social insects in the proper direction on pheromone trails, but that either object orientation or photomenotaxis is utilized (Hölldobler, 1971). A polarized trail is produced by some snails, but whether this involves a longitudinal concentration gradient or some tactile directional cue in the mucus is still unclear (Raftery, 1983; Wells and Buckley, 1972). Because snake sex pheromone trails are produced with a high-molecular-weight substance with low volatility, a chemical gradient in the trail is unlikely. It is also unlikely that photomenotaxis or object orientation could be utilized, as the female's choice of direction is variable. We therefore suggest that female snakes pheromonally mark the anterior surface of environmental objects they utilize for locomotion and male snakes chemosensorily assay those objects to obtain directional information from the female trails.

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STRUCTURE-ACTIVITY RELATIONSHIPS AMONG AROMATIC ANALOGS OF TRAIL-FOLLOWING PHEROMONE OF SUBTERRANEAN TERMITES

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Abstract—A series of 12 substituted (*Z*)-4-phenyl-3-buten-1-ol (PBO) derivatives were synthesized and evaluated for trail-following activity in five species of subterranean termites in the genera *Coptotermes, Prorhinotermes, Reticulitermes,* and *Schedorhinotermes* (Isoptera:Rhinotermitidae). The unsubstituted parent PBO was the most active for all species, and electron-withdrawing and electron-donating groups both reduced potency. Sensitivity to substitution in the ortho position suggests steric inhibition of binding by the 2′-substituted analogs. Different sensitivities to these pheromone analogs were found among the five species, with *R. flavipes* and *S. lamanianus* showing the highest level of trail-following activity for the PBO analogs.

Key Words—Termites, *Coptotermes, Prorhinotermes, Reticulitermes Schedorhinotermes,* Isoptera, Rhinotermitidae, structure–activity, pheromone analogs, trail-following pheromone.

INTRODUCTION

The trail-following pheromone of *Reticulitermes virginicus,* (*Z,Z,E*)-3,6,8-dodecatrienol (Tai et al., 1969), elicits trail following at 0.01 pg/cm. This material is also produced by a brown rot fungus *Gloeophyllum trabeum*

[1]Fellow of the Alfred P. Sloan Foundation (1981–85) and Camille and Henry Dreyfus Teacher-Scholar (1981–86).

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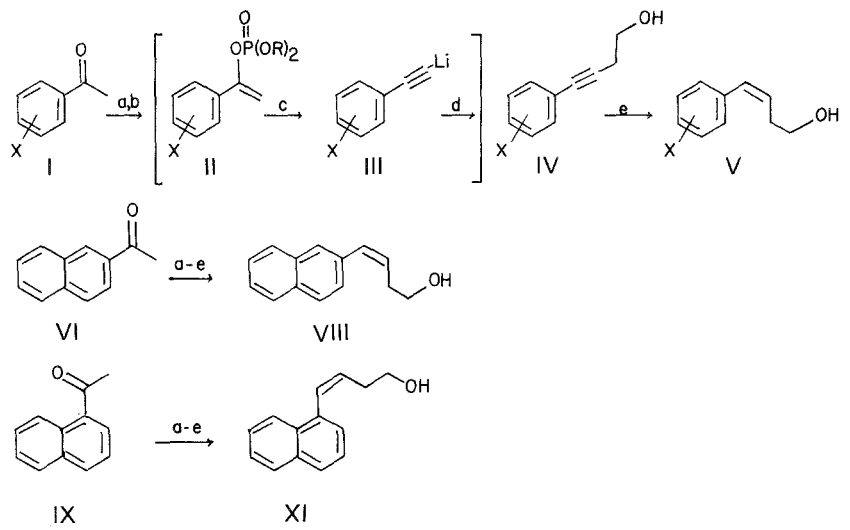
(formerly *Lenzites trabea*), which renders wood highly desirable to these subterranean termites (Matsumura et al., 1976). Other *Reticulitermes* species will follow this dodecatrienol, although it may not be the bona fide trail pheromone of those species (Howard et al., 1976). Interspecific responses to this material and to natural *Reticulitermes* trails have been reported for *Coptotermes* (Matsumura et al., 1972) *Trinervitermes, Amitermes,* and *Schedorhinotermes* (Kaib et al., 1982; see also review by Prestwich, 1983). A number of positional and geometrical dodecatrienol isomers were prepared and evaluated in vivo with *R. lucifugus santonensis* (Akkerboom, 1974; Ritter et al., 1977; Ritter and Persoons, 1975, 1977). In addition, a number of aromatic, monoene and diene analogs have been prepared (Tai et al., 1971; Kajiwara et al., 1978) and evaluated in vivo. A common feature for the most active isomers is the (*Z,Z*)-3,6-alkadien-1-ol, a concave functionality apparently required for the proper fit to the receptor site.

One of these analogs, (*Z*)-4-phenyl-3-buten-1-ol (PBO), seemed ideal for the determination of structure–activity relationships. We reasoned that through the systematic substitution of sterically small electron-donating and electron-withdrawing groups of the phenyl ring, useful information on the stereoelectronic requirements of the pheromone receptor protein could be obtained. With this information, we planned the design of improved, economical attractants for bait-block control schemes using latent poisons (Prestwich et al., 1981, 1983). Moreover, we hoped to design a photoaffinity label (e.g., an aryl azide) to further probe the receptor protein structure. In this paper, we report the synthesis of 12 PBO analogs and their biological activity in five different termite species.

METHODS AND MATERIALS

Synthesis. The general scheme used to prepare PBO analogs is shown in Scheme 1. Substituted acetophenones were purchased from Aldrich Chemical Co. and converted to the corresponding alkynes by the method of Negishi et al. (1980). Each acetophenone I was first converted to the diethyl enol phosphonate II with lithium diisopropylamide (LDA) followed by diethylchlorophosphate. The acetylide anion III was generated in situ by treatment with excess LDA and then condensed with ethylene oxide to give the arylalkynol IV. Partial reduction over 5% Pd/$BaSO_4$ with pyridine as solvent gave the PBO V which was purified by preparative GLC prior to bioassay. Experimental conditions are given in detail for the conversion of acetophenone (Ia) to (*Z*)-4-phenyl-3-buten-1-ol (Va); key analytical data are then reported for the substituted PBO analogs.

General. All reactions were performed under a nitrogen atmosphere. Tetrahydrofuran (THF) was purified by distillation from benzophenone ketyl



SCHEME 1. Synthesis of PBO analogs. Reagents: (a) 1.1 equiv. $LiN(iPr)_2$, THF, −78°; (b) $ClP(O)(OC_2H_5)_2$, THF, −78° to 0°; (c) 2.5 equiv. $LiN(iPr)_2$, THF, −78°; (d) ethylene oxide, THF; (e) H_2, 5% $Pd/BaSO_4$, pyridine.

immediately before use. Diisopropylamine was distilled from CaH_2. The *n*-butyllithium was obtained as a 1.6 M solution in hexane and was regularly standardized by the method of Kofran and Baclawski (1976). A 5 M solution of ethylene oxide was prepared by combining THF with ethylene oxide, condensing the latter with a cold finger into a graduated dropping funnel set atop a flask containing the premeasured THF.

[^{1}H]NMR and [^{13}C]NMR were obtained using a Varian CFT-20 NMR spectrometer operating at 80 MHz and 20 MHz, respectively. Infrared spectra were obtained using a Perkin-Elmer 727 model infrared spectrometer, in conjunction with standard NaCl plates.

Gas chromatography (GC) was carried out with a Varian 3700 gas chromatograph in conjunction with a Varian Vista series 401 data system and either (1) 2-mm ID × 2-m glass column packed with a 3% OV-17 on 100/120 Gas Chrom Q, or (2) Durawax DX-4 30-m × 0.25-mm capillary column (180° isothermal). A standard temperature program was used for OV-17 analyses: T_i = 100° C (2 min), T_p = 5°/min, T_f = 200° C (2 min).

Thin-layer chromatography (TLC) analyses were performed using Polygram Sil G/UV_{254} precoated silica gel plates, developed with ethyl acetate–hexane mixtures, and visualized by dipping in ethanolic–vanillin–H_2SO_4 solution (9 g vanillin, 1.5 ml/H_2SO_4, 300 ml EtOH) followed by heating. Flash chromatography on 230–400 mesh silica under N_2 pressure was used to purify all product mixtures.

Preparation of Lithium Diisopropylamide (LDA) (Illustrated for 5.25 mmol). To 15 ml dry THF was added 0.74 ml (0.53 g; 5.25 mmol) diisopropylamine. The solution was cooled to 0° C and 5.25 mmol of *n*-butyllithium in hexane was added dropwise. The solution was stirred for 30 min at 0° C.

4-Phenyl-3-butyn-1-ol (IVa). A solution of 5.25 mmol of LDA was prepared as above. After cooling to −78° C, 0.58 ml acetophenone (Ia) (0.60 g; 5.00 mmol) was added dropwise. After 1 hr stirring, 0.83 ml (5.75 mmol) of diethyl chlorophosphate was added dropwise. After 1 hr stirring, the mixture was removed from the cooling bath and stirred 14 hr with concurrent warming to ambient temperature. A solution of 11.25 mmol LDA was prepared as above. The reaction mixture was then cooled to −78° C, and added dropwise to LDA solution, also at −78° C. After 30 min stirring at this temperature, the mixture was removed from bath and allowed to come to ambient temperature over 30 min. The mixture was then cooled to 0° C and 2.11 ml ethylene oxide in THF (11.6 mmol; 5 M solution) was added dropwise. The mixture was stirred 16 hr with concurrent warming to ambient temperature, as the ice bath melted. The reaction was quenched with 3 M HCl and extracted 4× with ether. The combined organics were washed (2 N H_2SO_4, H_2O, satd. $NaHCO_3$, satd. NaCl), dried ($MgSO_4$), and concentrated in vacuo to give 710 mg of a brown oil. Chromatography afforded 310 mg of a yellow liquid, which was analyzed by TLC (one orange spot) and GLC (OV-17, t_R = 18.70 min); the calculated yield from GC was 22.1% based on starting acetophenone. This material was identical with a sample of IVa prepared from commercial phenylacetylene. [^{1}H]NMR ($CDCl_3$), δ2.15 (s, OH), 2.68 (t, 7 Hz, H-2), 3.80 (t, 7 Hz, H-1), 7.22-7.46 (m, 5 H); [^{13}C]NMR ($CDCl_3$) δ23.44 (C-2), 60.83 (C-1), 78.72 (C-3), 81.95 (C-4), 123.35-131.91 (phenyl); IR (neat) 3375 (b,s), 3060 (s,w), 2850 (s,m), 2620, 2240 (s,w), 1610, 1500 (s,m), 761, 697 (s,s) cm^{-1}

4-(2'-Methylphenyl)-3-butyn-1-ol (IVb). From 0.67 g *o*-methylacetophenone (5.0 mmol) was obtained 120 mg of chromatographed IV*b*; GLC (OV-17), t_R = 18.67 min; [^{1}H]NMR, δ1.96 (s, OH), 2.47 (s, CH_3), 2.76 (t, 7 Hz, H-2), 3.84 (t, 7 Hz, H-1), 7.11-7.44 (m, 4H).

4-(3'-Methylphenyl)-3-butyn-1-ol (IVc). From 0.67 g *m*-methylacetophenone was obtained 108 mg of chromatographed IVc. GLC (OV-17), t_R = 18.20 min; [^{1}H]NMR, δ2.40 (s, CH_3), 2.64 (t, 7 Hz, H-2), 3.76 (t, 7 Hz, H-1), 7.05-7.85 (m, 4H).

4-(4'-Methylphenyl)-3-butyn-1-ol (IVd). From 0.67 g *p*-methylacetophenone was obtained 156 mg chromatographed IVd; GLC (OV-17, t_R = 18.30 min); [^{1}H]NMR, δ2.41 (t, 7 Hz, H-2), 3.97 (t, 7 Hz, H-1), 6.80-7.25 (m, 4 H).

4-(2'-Fluorophenyl)-3-butyn-1-ol (IVe). From 0.82 g (5 mmol) of *o*-fluoroacetophenone was obtained 115 mg chromatographed IVe; GLC (OV-17, t_R = 19.9 min); [^{1}H]NMR, δ2.63 (t, 7 Hz, H-2), 3.79 (t, 7 Hz, H-1), 6.74-7.32 (m, 4 H).

4-(3'-Fluorophenyl)-3-butyn-1-ol (IVf). From 0.82 g *m*-fluoroacetophenone was obtained 106 mg chromatographed IVf; GLC (OV-17, t_R = 16.5 min); [^{1}H]NMR, δ2.67 (t, 7 Hz, H-2), 3.78 (t, 7 Hz, H-1), 6.80–7.41 (m, 4H).

4-(4'-Fluorophenyl)-3-butyn-1-ol (IVg). From 0.82 g *p*-fluoroacetophenone was obtained 232 mg chromatographed IVg, GLC (OV-17, t_R = 15.6 min); [^{1}H]NMR, δ2.66 (t, 7 Hz, H-2), 3.77 (t, 7 Hz, H-1), 6.87–7.03 (m, 2 H), 7.21–7.43 (m, 2 H).

4-(2'-Methoxyphenyl-3-butyn-1-ol (IVh). From 0.88 g of *o*-methoxyacetophenone was obtained 360 mg chromatographed IVh, GLC (DX-4, 210°, t_R = 6.54); [^{1}H]NMR, δ1.25 (t, 7 Hz, OH), 2.70 (t, 7 Hz, H-2), 3.7 (m, H-1), 3.85 (s, OCH_3), 6.7–7.4 (m, 4H).

4-(3'-Methoxyphenyl)-3-butyn-1-ol (IVi). From 0.88 g of *m*-methoxyacetophenone was obtained 400 mg chromatographed IVi, GLC (DX-4, 210°, t_R = 8.56); [^{1}H]NMR, δ1.25 (t, 7 Hz, OH), 2.65 (t, 6 Hz, H-2), 3.75 (m, H-1), 3.75 (s, OCH_3), 6.7–7.3 (m, 4 H).

4-(4'-Methoxyphenyl)-3-butyn-1-ol (IVj). From 0.88 g of *p*-methoxyacetophenone was obtained 300 mg chromatographed IVj, GLC (DX-4, 210°, t_R = 8.51); [^{1}H]NMR, δ1.25 (t, 7 Hz, OH), 2.65 (t, Hz, H-2), 3.75 (m, H-1), 3.77 (s, OCH_3), 6.6–7.4 (m, 4H).

4-(2'-Naphthyl)-3-butyn-1-ol (VII). From 850 mg of 2-acetonaphthone was obtained 195 mg orange–yellow crystals after chromatography. [^{1}H]NMR, δ1.84 (s, OH), 2.70 (t, 7 Hz, H-2), 3.80 (t, 7 Hz, H-1), 7.20–7.47 (m, 3 H), 7.65–7.87 (m, 4 H).

4-(1'-Naphthyl)-3-butyn-1-ol (X). From 450 mg of 1-acetonaphthone (IX) (Aldrich, 70%, containing ca. 30% of 2-isomer) was obtained 100 mg of crystals after chromatography. [^{1}H]NMR, δ3.8 (m, H-1), 2.85 (t, 6 Hz, H-2), 7.0–8.8 (m, aromatic H).

(Z)-*4-Phenyl-3-buten-1-ol (Va).* Overreduction exceeded 25% when using Lindlar catalyst or 5% Pd/$BaSO_4$ poisoned with quinoline. However, <5% overreduction was obtained when the reduction was performed in pyridine with 5% Pd/$BaSO_4$. This preparation is typical. A solution of 100 mg of alkynol IVa in 1 ml pyridine is added to a solution of 50 mg 5% Pd/$BaSO_4$ catalyst in 5 ml pyridine after presaturation with H_2 (1 atm, 25° C). After uptake of 1 equivalent of H_2, the reaction slows abruptly and is worked up by pouring into 25 ml ethyl acetate, filtration through Celite, washing (3 × 2 N HCl, 2 × $CuSO_4$, H_2O), drying ($MgSO_4$), and solvent removal to give a quantitative recovery of crude alkenol. A portion (20 mg or more) was purified by preparative GLC (Varian 920, $\frac{3}{8}$ in. × 10 ft 20% Carbowax 20 M on 45/60 Chromosorb W (AW, DMCS), 180° C (210° for methoxy derivatives) for spectral data and trail-following assays. Each compound purified by preparative GC was >90% pure by GLC (DX-4, 180°), with the impurities consisting solely of small peaks of early-retention-time material. Naphthyl analogs VIII and XI were not readily purified by preparative GC; thus, they

were purified on 20% $AgNO_3$–SiO_2 pipet flash columns by elution with 20% ethyl acetate in hexane to free them of saturated and/or alkynyl impurities. The NMR, MS, and GC data for the 12 PBO analogs Va–Vj, VIII, and XI are summarized in Table I.

Bioassays. The trail-following assay was modified from the open-field assay (Howard et al., 1976) employed previously for *Reticulitermes* species. The assay described below was also used to determine biological activity in a series of ω-fluoroalcohols prepared as attractant–poisons (Carvalho and Prestwich 1984). Five microliters of test solution was applied to a 10-cm semicircular pencil trail on nonabsorbent bond paper, and the solvent was allowed to evaporate (at least 10 sec). The tests were performed with single termites under ambient light, temperature, and humidity conditions.

Termites were collected from the field and maintained in laboratory colonies prior to use. *Coptotermes formosanus* was obtained from a Gulfport, Mississippi, colony collected originally near Lake Charles, Louisiana. *Prorhinotermes simplex* were from a Stony Brook laboratory colony collected in 1979 from Miami, Florida. *Reticulitermes flavipes* were obtained from Gulfport, Mississippi, and from the Stony Brook campus and *R. virginicus* were obtained from Gulfport, Mississippi. *Schedorhinotermes lamanianus* were from a Stony Brook laboratory colony originally collected in 1980 from the coastal forests near Mombasa, Kenya. Workers were held on agar–cellulose slants for at least 24 hr to minimize feeding and colony odor contamination. A worker (undifferentiated larva past the third instar) was placed under an inverted vial at one end of the trail. After 10 sec, the vial was removed, and the termite's behavior was monitored for 2 min. A negative score (−) was recorded if no orientation or following occurred during this period. If a worker followed the trail from one end to the other, turned around, and came back to the starting point without backtracking or straying more than 1 cm, a positive score (+) was recorded. Each termite was timed during this process and scored 1–14 based on following time. Scoring system: 1 (5–10.5 sec), 2 (10.6–15.5 sec), 3 (15.6–20.5 sec), . . .11 (55.6–60.5 sec), 12 (>60.5 sec), 13 (one-way following only), 14 (no following).

Five termites were tested on each piece of paper, constituting one replicate. No sternal gland dragging behavior (trail deposition) was observed during these assays. Four replicates (five termites per replicate) were performed for each compound at each concentration. A net (+) score for a replicate required 3/5 termites scoring (+). A net of three out of four replicates must show (+) scores for a given compound/concentration to be scored (+).

A stock solution (1.0 mg/ml in hexane) of each compound was prepared and serially diluted in decades (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). The lowest concentration followed with a (+) score was rediluted 1:3 to assess the threshold level for the (+) response. Solutions were stored at −17° C in glass with Teflon-clad lids.

TABLE 1. PHYSICAL AND SPECTRAL DATA FOR PBO ANALOGS

PBO analog	GLC (DX-4) retention time (180°)	[^{1}H]NMR (splitting pattern, J, Hz)							HRMS (M+)	Mass spectrum low resolution, m/z (rel int), largest four peaks
		OH	H-1	H-2	H-3	H-4	Ph	R-Ph		
Va	3.71	1.37	3.70	2.57	5.64	6.52	7.23		148.0877	148 (32), 118 (23),
		(bs)	(t,7)	(dt,7,11)	(dt,7,11)	(d,11)	(s)			117 (100), 115 (59)
Vb	4.20	1.65	3.70	2.45	5.75	6.59	6.9–7.4	2.29	162.1060	162 (35), 131 (100),
o-Me		(bs)	(t,7)	(dt,7,11)	(dt,7,11)	(d,11)	(m)	(s)		115 (27), 91 (28)
Vc	4.65	1.35	3.75	2.63	5.68	6.57	6.9–7.4	2.37	162.1054	131 (100), 116 (33.4)
m-Me		(t,5)	(dt,6,11)	(dt,6,11)	(dt,6,11)	(d,11)	(m)	(s)		115 (33), 91 (33)
Vd	5.26	1.30	3.68	2.60	5.63	6.52	6.9–7.3	2.35	162.1039	162 (28), 131 (100),
p-Me		(t,5)	(dt,6,11)	(dt,6,11)	(dt,6,11)	(d,11)	(m)	(s)		116 (25), 91 (28)
Ve	3.96	1.36	3.69	2.48	5.75	6.50	6.8–7.4		166.0783	135 (100), 133 (27),
o-F		(bt)	(dt,7,11)	(dt,7,11)	(dt,7,11)	(d,11)	(m)			115 (20), 109 (20)
Vf	4.21	1.32	3.70	2.56	5.65	6.45	6.7–7.3		166.0801	166 (32), 136 (37),
m-F		(bs)	(m)	(dt,7,11)	(dt,7,11)	(d,11)	(m)			135 (100), 133 (39)
Vg	4.18	1.24	3.72	2.58	5.65	6.53	6.7–7.3		166.0788	136 (35), 135 (100),
p-F		(bt)	(dt,7,11)	(dt,7,11)	(dt,7,11)	(d,11)	(m)			133 (32), 115 (28)
Vh	8.75	1.50	3.70	2.48	5.70	6.63	6.7–7.4	3.86	178.0991	147 (100), 131 (22),
o-OMe		(bs)	(m)	(dt,7,11)	(dt,7,11)	(d,11)	(m)	(s)		115 (39), 91 (43)
Vi	11.81	1.32	3.70	2.58	5.65	6.56	6.7–7.3	3.78	178.1000	178 (63), 147 (100),
m-OMe		(bt)	(m)	(dt,7,11)	(dt,7,11)	(d,11)	(m)	(s)		115 (47), 91 (41)
Vj	13.23	1.27	3.70	2.60	5.50	6.45	6.6–7.3	3.80	178.1015	178 (20), 147 (100),
p-OMe		(bt)	(m)	(dt,7,11)	(dt,7,11)	(d,11)	(m)	(s)		115 (22), 91 (21)
VIII	7.21		3.79	2.71	5.78	6.75	7.4–8.9		198.1054	167 (100), 166 (16),
	(240°)		(t,7)	(dt,7,11)	(dt,7,11)	(d,11)	(m)			165 (45), 152 (36)
X	5.57		3.68	2.33	5.99	7.05	6.9–8.6		198.1050	167 (100), 165 (73),
	(240°)		(t,7)	(dt,7,10)	(dt,7,10)	(d,10)	(m)			153 (32), 152 (63)

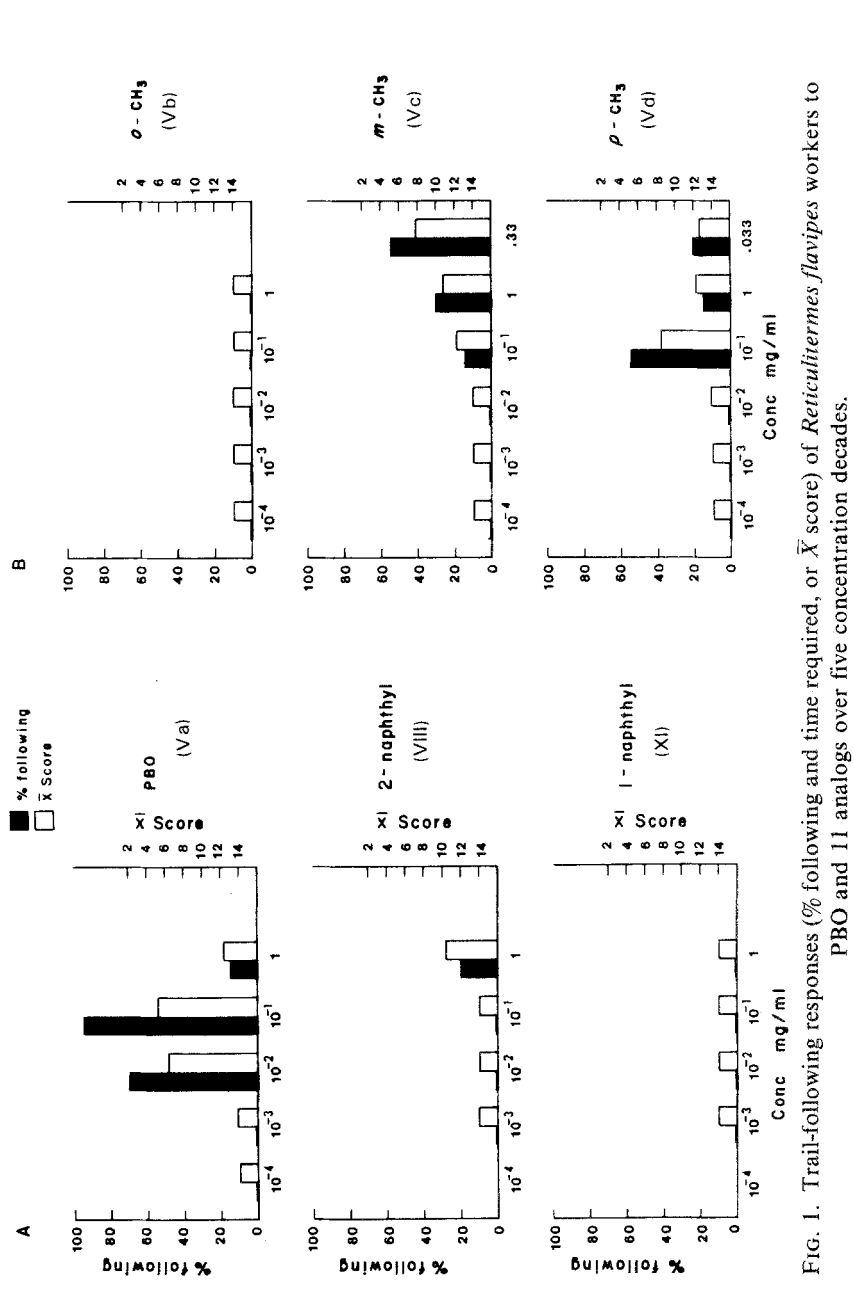


FIG. 1. Trail-following responses (% following and time required, or $\bar{X}$ score) of *Reticulitermes flavipes* workers to PBO and 11 analogs over five concentration decades.

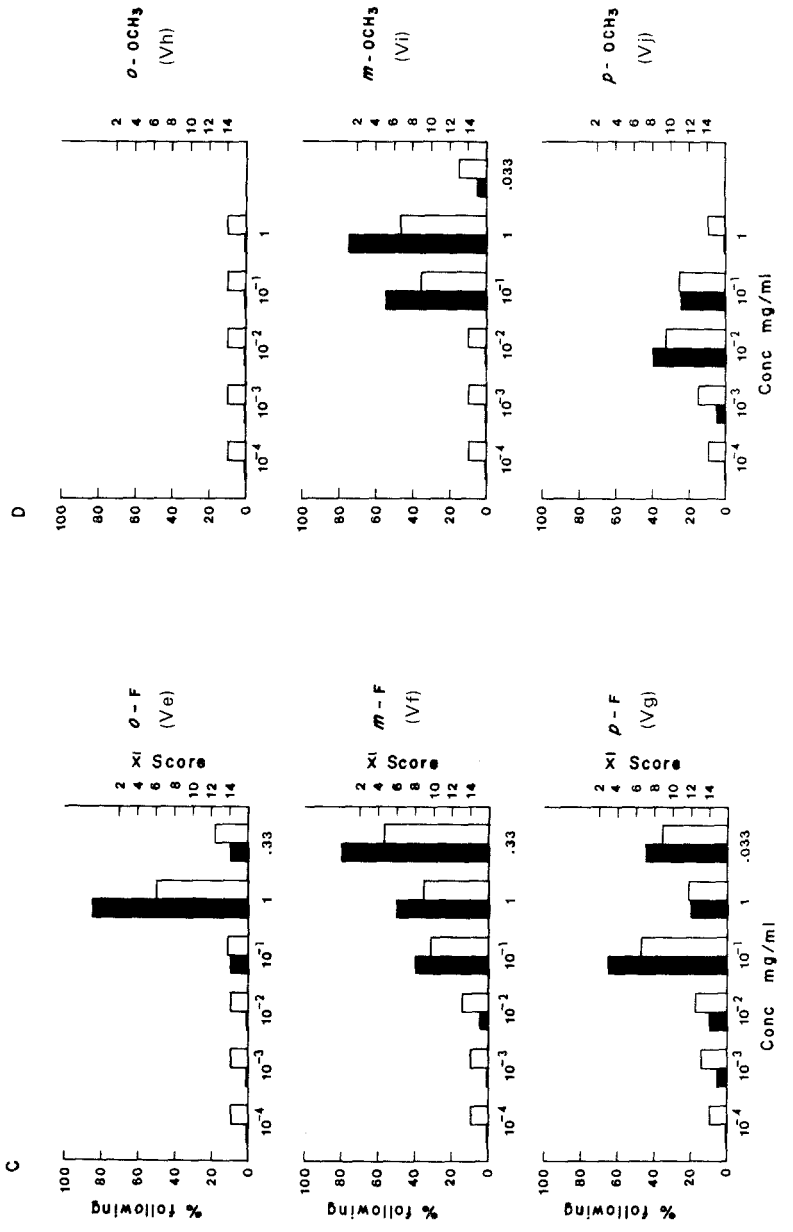


FIG. 1. Continued.

TABLE 2. THRESHOLD TRAIL-FOLLOWING RESPONSES OF THREE TERMITE SPECIES TO PBO ANALOGS[a]

Compound	*R. flavipes*				*R. virginicus*				*S. lamanianus*			
	[PBO]	TFR	%F	$\bar{X}$	[PBO]	TFR	%F	$\bar{X}$	[PBO]	TFR	%F	$\bar{X}$
V_a,X = H	0.01	+	70	6.3 ± 4.8	0.01	+	60	8.6 ± 4.3	0.01	+	80	7.1 ± 4.1
V_b,X = *o*-CH_3	1.0	−	0	14.0	1.0	−	0	14.0	1.0	−	0	14.0
V_c,X = *m*-CH_3	0.3	±	55	8.0 ± 4.2	1.0	±	25	12.2	0.1	+	85	7.8 ± 3.2
V_d,X = *p*-CH_3	0.1	+	55	8.4 ± 4.8	0.1	±	40	10.0 ± 4.8	0.01	+	85	7.8 ± 3.2
V_e,X = *o*-F	1.0	+	85	6.2 ± 4.2	1.0	−	0	13.8	0.1	±	55	9.1 ± 4.8
V_f,X = *m*-F	0.3	+	80	4.9 ± 3.8	0.1	+	65	8.8 ± 4.1	0.01	+	90	6.8 ± 3.1
V_g,X = *p*-F	0.1	+	65	6.6 ± 5.4	0.1	±	55	9.75 ± 5.0	0.01	+	95	7.7 ± 2.6
V_h,X = *o*-OCH_3	1.0	−	0	14.0	1.0	−	0	14.0	1.0	−	0	14.0
V_i,X = *m*-OCH_3	0.1	+	55	9.0 ± 4.8	0.1	±	35	10.0 ± 4.6	0.1	+	90	5.1 ± 2.6
V_j,X = *p*-OCH_3	0.01	±	40	9.7 ± 5.1	0.01	±	20	11.6 ± 4.1	0.001	+	70	7.3 ± 4.5
VIII,2-naphthyl	1.0	±	20	10.6 ± 5.5	1.0	−	0	13.8	1.0	−	0	14.0
XI,1-naphthyl	1.0	−	0	14.0	1.0	−	0	14.0	1.0	−	0	14.0

[a]Bioassays were modified (single worker) open field assays with 5 μl of test solution applied to a 10-cm arc. Values for positive responses are shown for four replicates of five termites each; one or two replicates only were performed when no following was detected. Following responses (TFR): (−), (±), (+), respectively, for no response, insufficient response, positive response; Percentage following (%F) at threshold concentration; and time score ($\bar{X}$) for following. See text for details.

The (+) response thresholds, rates of following, and percentage following for *Reticulitermes flavipes* are illustrated for 12 PBO analogs over five decades of concentration in Figure 1. The data for the three *Schedorhinotermes* and *Reticulitermes* species are summarized in Table 2. *Prorhinotermes* and *Coptotermes* did not exhibit any trail-following responses to the PBO analogs, and no more data are illustrated herein.

RESULTS AND DISCUSSION

The conversion of commercially available substituted acetophenones to the corresponding acetylenic alcohols could be achieved in a straightforward one-pot procedure, modified from Negishi et al. (1980) (Scheme 1). The more hindered ortho-substituted acetophenones generally required longer reaction times and gave lower yields. Attempts to prepare acetamido- or nitro-substituted analogs were unsuccessful by this route, leading only to intractable black material and no product during the attempted phosphonic acid elimination to the acetylene. Thus, our goal of aryl azide synthesis remains as yet unfulfilled.

Purification of the (*Z*)-3-alkenols by flash chromatography on $AgNO_3$–silica gel or by preparative GC was essential to remove unreacted starting alkynol and saturated alkanol (up to 30%). Trail-following responses were found to be inhibited by traces of pyridine and saturated analogs. However, the alkynols did not interfere; in the unsubstituted 4-phenyl-3-butyn-1-ol, weak trail-following responses were observed for *R. flavipes*.

The five termites species examined in this study represent four different subfamilies of the subterranean Rhinotermitidae. The two *Reticulitermes* species are from North America. In the southern states *R. flavipes* and *R. virginicus* (Heterotermitinae) are sympatric, and *R. flavipes* extends into the northeastern and midwestern states. *Coptotermes formosanus* (Coptotermitinae) is an introduced representative of a highly destructive genus of tropical and subtropical termites. *Prorhinotermes simplex* (Prorhinotermitinae) is restricted to Florida and is more closely related to the highly evolved Rhinotermitinae to which the African *Schedorhinotermes lamanianus* belongs. The evolutionary and systematic aspects of the exocrine chemistry (defense, trail following) of this subfamily has been reviewed recently (Prestwich, 1983).

For *Reticulitermes flavipes,* the following responses ($\bar{X}$) to the substituted (*Z*)-4-phenyl-3-buten-1-ol analogs at their maximum response levels were in all cases significantly different (lower) from the parent PBO (0.05 mg/ml) at the 0.05 level, as determined by a Student-Newman-Keuls LSR based on ANOVA of positive responses only (Sokal and Rohlf, 1981). The response to PBO at 0.01 mg/ml was also significantly lower than at 0.05

mg/ml (Figure 1, Table 2). All substituents, therefore, reduced the presumed receptor interactions which release behavioral responses (Table 2). These results discouraged us from embarking on a more thorough survey of halogen, amine, and alkyl substituents on the aromatic nucleus to determine structure–activity relationships.

We rationalize our observations as follows (Figure 2): (1) Steric crowding at the ortho position removes activity. Only the *o*-fluoro PBO Ve retained activity at 1 mg/ml equivalent to PBO Va at 0.01 mg/ml. Fluorine has a van der Waals radius of 1.38 Å relative to 1.04 Å for hydrogen. Methyl, methoxy, and fused aryl moieties prevent binding to the receptor (Figure 2). (2) Steric crowding at the meta position reduces activity 10- to 100-fold. Thus, compared to PBO Va at 0.01 mg/ml, equivalent following responses occurred at 0.3 mg/ml for *m*-fluoro PBO Vf and 1.0 mg/ml for *m*-methyl PBO Vc and *m*-methoxy PBO Vi. (3) Stereoelectronic effects at the para position reduce activity. Again, the steric bulk of the substituent is probably the major contributor to loss of activity, although electron-withdrawing and -releasing properties may also be important. Insufficient data are available from this approach to evaluate σ and π contributions to the observed behavior.

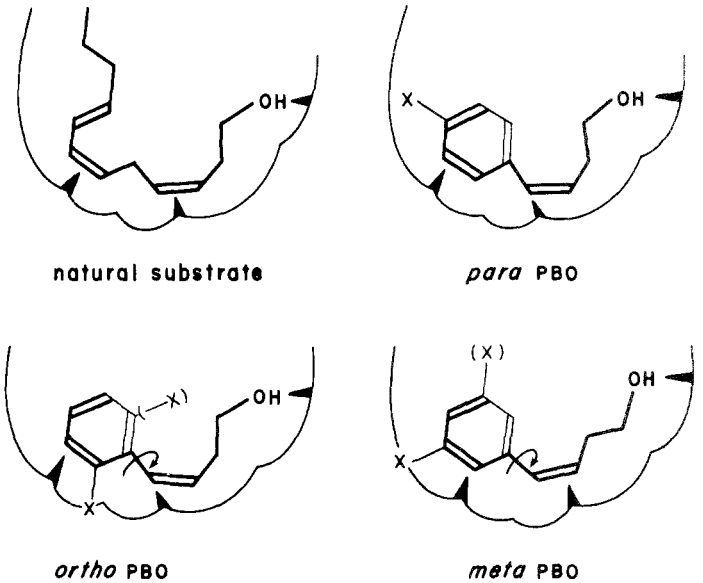


FIG. 2. Schematic depiction of the interactions of the natural trail pheromone and PBO analogs with the putative receptor site. All substituted PBOs possess rotamers which potentially interfere with binding to the lipophilic U-shaped pocket of the receptor.

The patterns of relative effects of substituents seen in *R. flavipes* are mirrored in *R. virginicus* and *S. lamanianus*. In general, *R. virginicus* showed reduced following responses, with slower, more ambiguous ("indecisive") behaviors. *Schedorhinotermes lamanianus* workers were three- to tenfold more sensitive to these analogs, with an unexpected tenfold greater response to the *p*-methyl PBO Vj relative to PBO Va. *Coptotermes formosanus* and *Prorhinotermes simplex* workers were essentially unresponsive to all PBO analogs at all concentrations.

The values for detection and following thresholds reported here are, in general, higher than those previously found for PBO (Tai et al., 1971; Matsumura et al., 1972). Similarly, our results (Carvalho and Prestwich, 1984; J. Carvalho, G. Prestwich, E. Deaton, unpublished results) with unsaturated C_{12} and C_{14} alcohols also indicate higher concentrations are required than those reported by Tai et al. (1971), Matsumura et al. (1972), or Ritter et al. (1977). Our values are more in agreement with those reported by Kajiwara et al. (1978). We believe several factors are responsible for these differences, as noted by the above workers and by Howard et al. (1976): (1) laboratory conditions (temperature, humidity, light levels) for assays; (2) developmental states of colony when collected, e.g., preflight, midseason, dormant; (3) physiological state and age of colony; (4) physiological state of individual workers; (5) "hunger" of workers, i.e., starved, held on wood, held in cellulose medium; (6) effects of crowding; (7) presence of food-related odorants; (8) interspecific differences in responsiveness; and most importantly, (9) criteria for positive responses and nature of bioassay used.

The assay employed here is a more stringent version of the open-field assay devised by Howard et al., (1976). We chose to test termites individually rather than in groups of five, in order to rule out any "group effects" or mutual following. We required the same "round-trip" following described in their paper. In addition to (+) and (−) responses, we scored percent following and the time required for following, which allows somewhat finer tuning of the numbers obtained for the 12 analogs tested in five species. We observed little quantitative difference between these qualitatively dissimilar measurements (Figure 1). Apparently, the speed of trail following is closely related to the perception of the chemical as a trail-following stimulus. In our opinion, no one assay is perfect or entirely correct, since each is performed under incredibly stressful circumstances for the termites. The absolute numbers are less important than the relative potency of the compounds as tested by a research group using a consistent assay technique.

Relatively few insect pheromone perception systems have been examined by SAR (structure–activity relationship) methods, in particular by varying substituents on aromatic or aliphatic skeleton in a systematic fashion (Hansch and Leo, 1979). Recently, an attempt was made to correlate structure with behavioral and EAG activity of substituted analogs of the

bark beetle pheromone 4-methyl-3-heptanol (Pignatello and Grant, 1983). While field and EAG results were not valuable in deducing correlations, behavioral assays indicated the importance of hydrogen-bonding to the 3-oxygen in chemoreception.

A second example is that of the molecular parameters of phenylpropanoids affecting olfactory responses of melon flies and Oriental fruit flies (Metcalf et al., 1983). The melon fly (*Dacus curcurbitae*) was attracted to *p*-hydroxyphenylpropanoids, while *D. dorsalis* preferred the 3,4-dimethoxyphenylpropanoids. These are analogs of the raspberry ketone and methyl eugenol, known attractants and feeding stimulants of host plants. It appears that two discreet groups of Dacini evolved specific antennal receptors complementary to the methyl eugenol or raspberry ketone stimuli. For *D. curcurbitae,* the primary receptor site has a site complementary to the aliphatic carbonyl at a distance of two atomic diameters from a para-hydroxylated phenyl ring. Moving the carbonyl, moving the hydroxyl, or increasing lipophilicity all lower activity.

We feel that our data provide insufficient information to allow an extended discussion of molecular forces involved at the receptor site. It appears that steric considerations dominate the structure–activity profile of these aromatic analogs of trail-following pheromones. Attempts to manipulate the polarity, electronic properties, or H-bonding properties in this model are doomed to yield disappointingly negative information (i.e., no following). It will be necessary to identify a different analog model for a more rigorous SAR approach. The U-shaped model for the putative receptor site as proposed by Tai et al. (1969) and reinforced by Kajiwara et al. (1978) is further supported here. However, it now appears that any steric interference with the convex side of the concave (Z,Z)-3,6-dienol system adversely affects receptor binding and thus behavioral activity (Figure 2).

No receptors, protein or otherwise, have been unambiguously characterized for insect pheromones, although the specificity and sensitivity of insect olfaction is compelling evidence for their existence. Nonetheless, the differences in the responses of the five termite species to the same set of pheromone analogs provides strong evidence for distinct receptor proteins which are complementary to different natural trail-following pheromones in these termite species. This conclusion is analogous to the perception of food attractants by two fruit flies in the genus *Dacus* as mentioned above (Metcalf et al., 1983).

The chemical systematics of termite exocrine secretions has been recently reviewed (Prestwich, 1983). Among different genera of the same species, there exists ample precedent for high species specificity in perception of behavioral chemicals. Interspecific responses of subterranean termites to the *R. virginicus* compounds (Matsumura et al., 1972) and the interspecific responses of numerous African termites representing four subfamilies to *R.*

lucifugus trails appear to suggest surprising conservation of receptor types for eliciting trail-following responses.

On the other hand, Howard et al. (1976) find that when offered a choice between a conspecific trail and a heterospecific trail, the conspecific trail is chosen if the concentration of pheromone is equivalent. They propose that isomeric or homologous blends may explain the species specificity in the choice experiments. Alternatively, the interspecific following could reflect a response to a fundamental scent which is modulated by species-specific cues from the sternal gland or the cuticle (Howard et al., 1982).

The African grass-feeding termites in the genus *Trinervitermes* also exhibit interspecific following (Oloo and McDowell, 1982; Kaib et al., 1982). Kaib et al. (1982) found species specificity in choice tests between *T. bettonianus* and *T. gratious* extracts, while Oloo and McDowell (1982) report no specificity for the worker-laid trails of these or other *Trinervitermes* species. Extracted cuticular components are logical candidates to explain this difference. Kaib et al. (1982) also report interspecific following in no-choice assays among termites of three different subfamilies: *Amitermes* (Termitinae), *Schedorhinotermes* (Rhinotermitinae), and *Trinervitermes* (Nasutitermitinae). Species specificity is observed in choice tests, but this effect is removed by a 1 : 10 dilution of the conspecific extract. These workers postulate the existence of a generally active trail pheromone possessing the (Z)-3-enol system as a minimum requirement, with other species-specific cues present in the trails (or extracts). The identities of the trail pheromones in these interspecifically following species, however, are different (cf. Prestwich, 1983): *Trinervitermes* and some *Nasutitermes* species use cembrene-A as a trail pheromone, *Reticulitermes* species use dodecatrienols, *Schedorhinotermes* seem to have tetradecenols, and the *Amitermes* trail pheromones have yet to be determined. The specificity of responses among *Reticulitermes* species to the dodecatrienols, the specificity observed among rhinotermitid species and genera in our study to the PBO analogs, and the responses of *Trinervitermes* species to cembrene-A all converge on a consistent theme. Receptors for trail pheromones are specific for each species, but a certain latitude exists for analogs to produce trail-following responses. The trans-subfamily responses of Kaib et al. (1982) are disconcerting in this regard, since they suggest, in contrast, that a much wider latitude is tolerated (or multiple receptors are present) than one would expect from the specificity implied above. Two directions are clear for future work: (1) pheromone components need to be isolated from sternal glands and the total composition of the pheromone must be rigorously determined, and (2) pheromone receptors need to be characterized using radiolabeled pheromones. Also, the development of bioactive pheromone analogs will help in the elucidation of structure–activity and species–specificity questions.

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INTERSPECIFIC VARIATION OF DITERPENE COMPOSITION OF *Cubitermes* SOLDIER DEFENSE SECRETIONS

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Abstract—Soldiers of the humivorous termite genera *Cubitermes* and *Crenetermes* (Isoptera: Termitidae: Termitinae) secrete a mixture of unusual diterpene hydrocarbons from the fontanellar opening of the frontal gland. The defense secretions of six *Cubitermes* species and one *Crenetermes* species show species-specific distributions of the five chemically identified major components and 12 unidentified minor components. The secretion is also present with its characteric terpene distribution in white presoldiers and newly molted soldiers, but is completely absent in workers.

Key Words—*Cubitermes*, *Crenetermes*, Isoptera, Termitidae, termite soldier, defense secretion, interspecific variation, diterpene hydrocarbon.

INTRODUCTION

Combined mechanical and chemical defense is common among termite soldiers in the subfamily Termitinae (Prestwich, 1979a,b; Deligne et al., 1982). The mechanical aspects of biting and snapping in this subfamily have been described by Deligne (1971) and Deligne et al. (1982), and the chemical nature of the secretions of several genera has been reviewed by Prestwich (1979) and Deligne et al. (1982). The majority of secretions are terpenoid, consisting of mono-, sesqui-, and diterpene hydrocarbons. *Noditermes* contains β-elemene and two eudesmanes (Naya et al., 1982), while *Amitermes* species from East Africa (Prestwich, 1979a), West Africa (Baker et al., 1978), and South

[1]Fellow of the Alfred P. Sloan Foundation (1981–85) and Camille and Henry Dreyfus Teacher-Scholar (1981–86).

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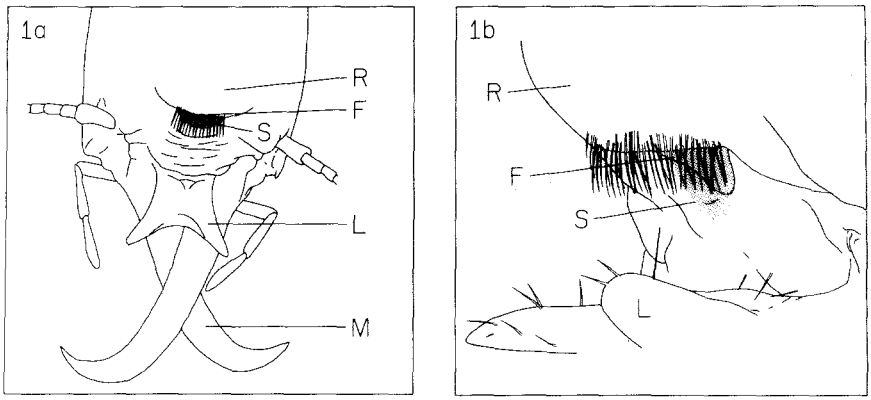

FIG. 1. Soldiers of the higher termite subfamily Termitinae often combine mechanical and chemical defense mechanisms. Left (A), a dorsal view of the head of the soldier of *Cubitermes umbratus*, showing the secretion (S) emerging from the fontanellar opening (F) of the frontal gland located within the slightly protruding rostrum (R). The labrum (L) guides the oily secretion into wounds made by the mandibles (M). Right (B); an anteriolateral close-up of the fontanelle region, showing numerous long chemo- and mechanosensory hairs surrounding the gland opening and emphasizing the downward flow of the secretion from the overhanging rostrum. Drawings by J. Schirmer from SEM photographs by M. Kaib.

America (Naya et al., 1982) contain a rich diversity of sesquiterpene alcohols and cyclic ethers in addition to their hydrocarbon constituents.

The only diterpene-producing genera of the Termitinae found to date are *Cubitermes* and *Crenetermes* (Quennedey, 1975; Prestwich, 1979a,b). Soldiers of *Cubitermes umbratus* possess biting-reaping mandibles and a cephalic frontal gland which opens on the rostrum through the fontanelle, which is surrounded by sensory hairs (Figure 1) (Deligne et al., 1982). The gland contains an irregular 12-membered ring diterpene, cubitene I (Prestwich et al., 1978), (3*E*) and (3*Z*) isomers of cembrene-A (II and III) (Wiemer et al., 1979), and the novel bicyclic bifloratriene (IV) (Wiemer et al., 1980). I had earlier noted the presence of these diterpenes (Figure 2) in other East African *Cubitermes* species. One of these, *C. ugandensis*, was found to have still a fourth different diterpene skeleton, cubugene (V) (Tempesta et al., 1984), formally related to the irregular diterpene cubitene (I). I report here the occurrence of these four diterpenes in six species of *Cubitermes* and one species of the *Cubitermes* guest species *Crenetermes mixtus*, and I document the interspecific variation of the secretion composition. I also report the first example of cephalic secretion synthesis in presoldiers. Finally, I suggest a biogenetic scheme which interrelates the four varied diterpene skeletal types.

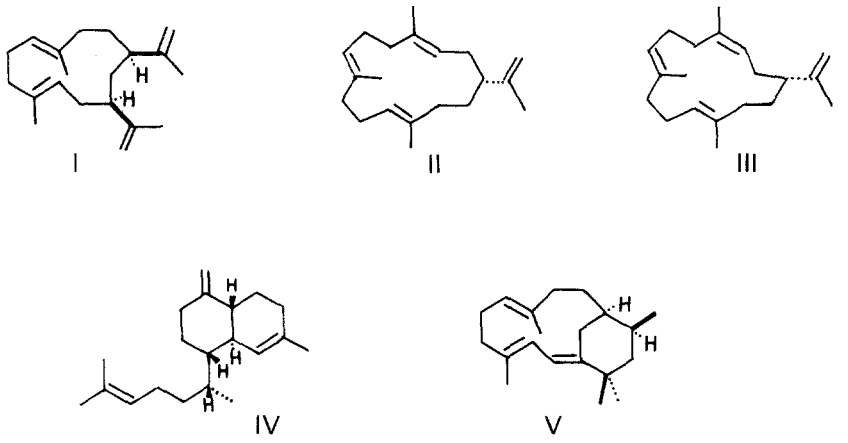

FIG. 2. Diterpenes identified from *C. umbratus* and *C. ugandensis* secretions.

METHODS AND MATERIALS

Materials. Cubitermes umbratus (Williams) was collected from over 20 0.3 to 1-m high columnar mounds in shaded areas of rich humus soil in the Makadara Forest of the Shimba Hills Forest Reserve near Kwale, Kenya. *Cubitermes muneris* (Sjöstedt) was obtained from eight 0.3 to 0.6-m-diameter dome-shaped mounds in semiarid acacia grassland in sandy soil a few kilometers south of Olorgesaille, Kenya. *Cubitermes ugandensis* (Fuller) was collected on three occasions near Eldama Ravine from the Timboroa Forest, Kenya, and was found on a grassy hillside (elevation 2500 m) densely populated with mounds of this species at the edge of an evergreen coniferous forest. *Cubitermes tenuiceps* (Sjöstedt) was collected on two different occasions as a guest species in a mound of *Trinervitermes bettonianus* in Ruiru, Kenya. *Cubitermes* sp. N.D. is an undescribed species obtained repeatedly as a guest species in mounds of *Macrotermes michaelseni* in Kajiado, Kenya; only two collections were suitable for GC analysis. *Cubitermes glebae* was collected from two soil mounds in the Jadini Forest near Ukunda, Kenya. Finally, *Crenetermes mixtus* (Williams) was obtained once as a guest species in the basal nest area of the mound of *Cubitermes umbratus* in Kwale, Kenya. When this mound was opened, the morphologically similar soldiers of the two humivorous genera exhibited considerable antagonism to one another, suggesting segregation of the species in the undisturbed state.

For each species, several mounds were sacrificed in the field and the soldiers ($<0.1\%$ of the total population) were picked out and kept alive on moist filter paper until they could be returned to the laboratory. In each case

workers were examined for diterpenes and in one *C. umbratus* mound, presoldiers and recently molted adult soldiers were collected and grouped for diterpene analysis. Termites were frozen and decapitated; the heads were macerated in distilled hexanes and the crude extract was purified by passage through a disposable pipet packed with 2 cm of 100–200 mesh Florisil. For preparative purposes, several hundred soldiers were employed, yielding 300 μg secretion per soldier in the case of *C. umbratus* and 160 μg secretion per soldier for *C. ugandensis*. For analytical purposes, 6–60 heads were used. Secretions were stored at −17°C in the dark between analyses.

The purified extract was analyzed by gas chromatography using three packed columns: (1) 6% Carbowax 20 M (2 m × 2 mm ID, 150°), (2) 3% FFAP (2 m × 2 mm ID), or (3) 10% QF-1 (2.6 m × 5 mm ID, 175°), all on 100/120 Gas Chrom Q in glass columns, and one stainless-steel capillary column (15 m × 0.5 mm ID SCOT column of FFAP, 160°). Resolution of the diterpenes was generally best using the latter system, although two individual pairs of closely eluting compounds were more clearly separated on QF-1. Most recently, we have achieved optimal resolution using a 30-m × 0.25-mm Durawax DX-4 fused silica capillary column operating at 170°C. Sample GC traces for each phase are shown in Figure 3, the DX-4 hydrocarbon "fingerprints" are shown in Figure 4, and the complete relative percent composition data are summarized in Table 1. Selective decomposition of cubugene (V) in some samples of *C. ugandensis* was attributed to air oxidation in situ. Preparative separations using medium-pressure LC on $AgNO_3$–silica gels and GLC on FFAP have been described previously (Prestwich et al., 1978; Wiemer et al., 1979, 1980; Tempesta et al., 1984).

RESULTS AND DISCUSSION

The occurrence of the irregular diterpene cubitene (I) as a major component appears to be characteristic of all *Cubitermes* species with the sole exception of *C. glebae*, which makes essentially pure cembrene-A (II). Cubitene is also the major component of the secretion of the West African species *C. sankurensis* and *C. fungifaber*, although quantitative GC data are lacking (Quennedey, 1975, 1984). Similarily, the new cubugene V is found in substantial amounts in all species but *C. umbratus* and *C. glebae*. Cembrene-A (II) is a major component only in *C. umbratus* and *C. glebae*, and bifloratriene IV is found above 5% only in *C. umbratus* and *C. ugandensis*. These diterpenes are not restricted to this genus, however. Soldiers of the guest species *Crenetermes mixtus*, found only once during the excavation of over 30 *Cubitermes* and *Noditermes* mounds in the coastal forests, also possess three of the main skeletal types (I, II, V). It is apparent from the GC data that four other diterpene hydrocarbons can be found in substantial (>5%) amounts in these

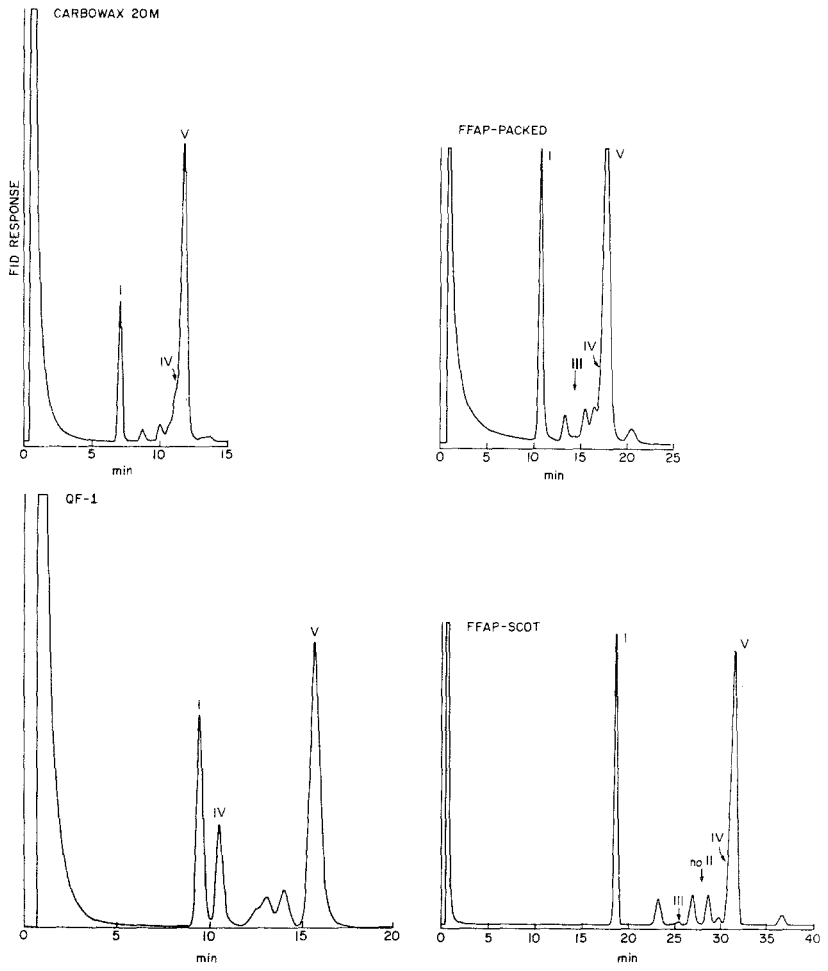

FIG. 3. Comparison of four GC columns available in 1977–1978 for resolution of *Cubitermes ugandensis* secretions; 6% Carbowax 20 M, 10% QF-1, 3% FFAP, and FFAP-SCOT column. Details given in text. Refer to Figure 4 for DX-4 trace.

secretions; *C. muneris*, for example has two major components which have not yet been determined.

Each of these GC traces appears to be a species-specific fingerprint (Figure 4). I have examined individual soldiers and over 20 individual mounds of *C. umbratus* from two locations (Makadara and Jadini forests). Two collections of the undescribed *Cubitermes* sp. N.D. from Kajiado, six colonies of *C. muneris* from Olorgesaille, and over eight mounds of *C. ugandensis* from Eldama Ravine have been analyzed. (Collection site locations are shown in

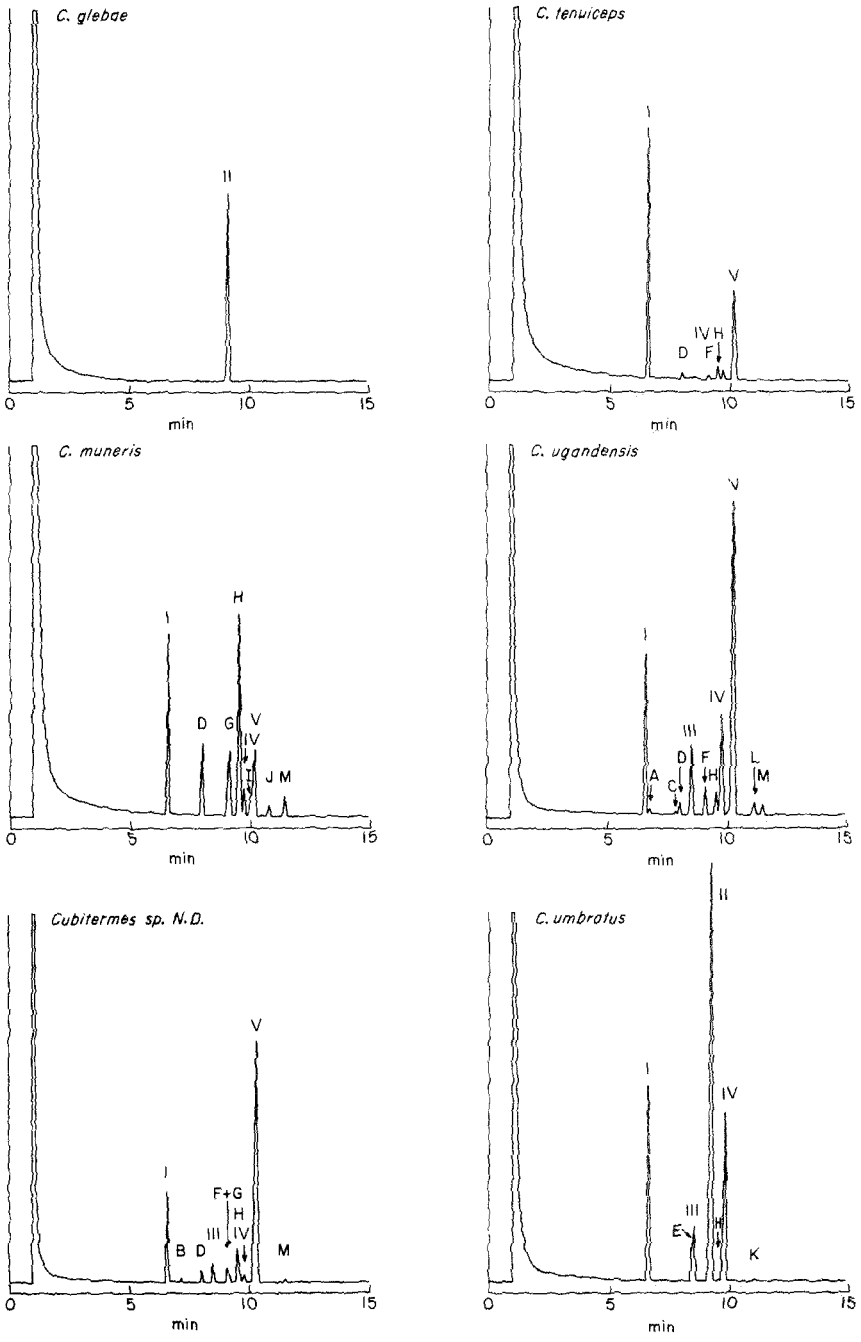

FIG. 4. Capillary GLC traces of *Cubitermes* and *Crenetermes* soldier secretions (Durawax DX-4, 30 m × 0.25 mm, 170° C). Relative percent compositions for all species are shown in Table 1. Numbers refer to structures in Figure 2.

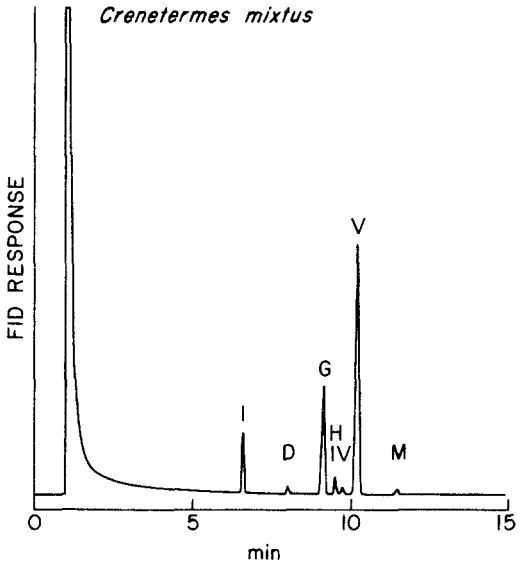

FIG. 4. Continued.

Figure 5.) Major deviations from the species-specific GC patterns presented in Figure 4 were not observed. Indeed, the taxonomy of the genus *Cubitermes* (Williams, 1959, 1966) is exceedingly difficult based on soldiers alone, and gas chromatography provides a valuable chemosystematic tool for distinguishing morphologically similar species (Prestwich, 1979b, 1983).

In one *C. umbratus* colony, a large number of immature soldiers and presoldiers was obtained. Since I did not expect chemical secretions to be present in the defenseless immatures (as is indeed the case in nasute presoldiers; G.D. Prestwich, unpublished results), I was surprised to find a substantial quantity of diterpene hydrocarbons present in (1) white presoldiers, (2) late presoldiers possessing darkened, sharpened mandibles, and (3) newly molted soldiers, with pale yellow heads and white abdomens. Diterpenes were completely absent from all worker, larval, and nymphal individuals examined. The quantity of material isolated per soldier increased from approximately 50 μg gland to over 300 μg/gland in mature soldiers. In each case the diterpene composition was indistinguishable from the mature soldier secretion (Figure 6), indicating that the four different diterpenes are produced as a fixed-composition blend, rather than arising from conversion of an early precursor in presoldiers to the final mixture in mature soldiers. This is the first report of a termite cephalic secretion occurring in an immature soldier.

Although clear chemical evidence for biosynthesis of diterpenes in *Cubitermes* soldier frontal glands is not yet available, cembrenoid diterpene

TABLE 1. RELATIVE PERCENTAGES OF DITERPENE HYDROCARBONS IN SOLDIER SECRETION OF *Cubitermes* AND *Crenetermes* SPECIES (SEE FIGURE 4) OBTAINED FROM DX-4 CAPILLARY ANALYSES[a]

	Compound																	
Species	I	II	III	IV	V	A	B	C	D	E	F	G	H	I	J	K	L	M
Crenetermes mixtus ($N = 1$)	9.9			1.4	57.1				1.3			25.4	3.4					1.3
Cubitermes glebae ($N = 1$)		100																
Cubitermes muneris ($N = 6$)	21.2			3.8	14.5 (+I)				9.9			13.8		NR, V	1.8			3.3
Cubitermes sp. N.D. ($N = 2$)	14.6		3.2	1.8	66.0		0.6		2.0		4.1 (+G)	NR,F	6.9					0.9
Cubitermes tenuiceps ($N = 2$)	60.1			2.6	30.2				1.4		1.9		2.6					
Cubitermes ugandensis ($N = 6$)	16.6		8.6	14.5	47.1	0.5		0.4	1.4		3.8		2.9				2.3	1.6
Cubitermes umbratus ($N = 12$)	15.1	44.3	10.4 (+E)	29.7	0.1					NR, III			0.1			0.3		

[a]Structures for compounds A–M are unknown. NR indicates peaks not resolved; areas are given as total for larger peak. Blanks indicate peak not detected (<0.1% total area). Values shown are means of $N = 1$–10 colonies, 6–60 soldiers per colony (see Methods and Materials). Standard deviations did not exceed ±10% of the mean relative percentage for any species component.

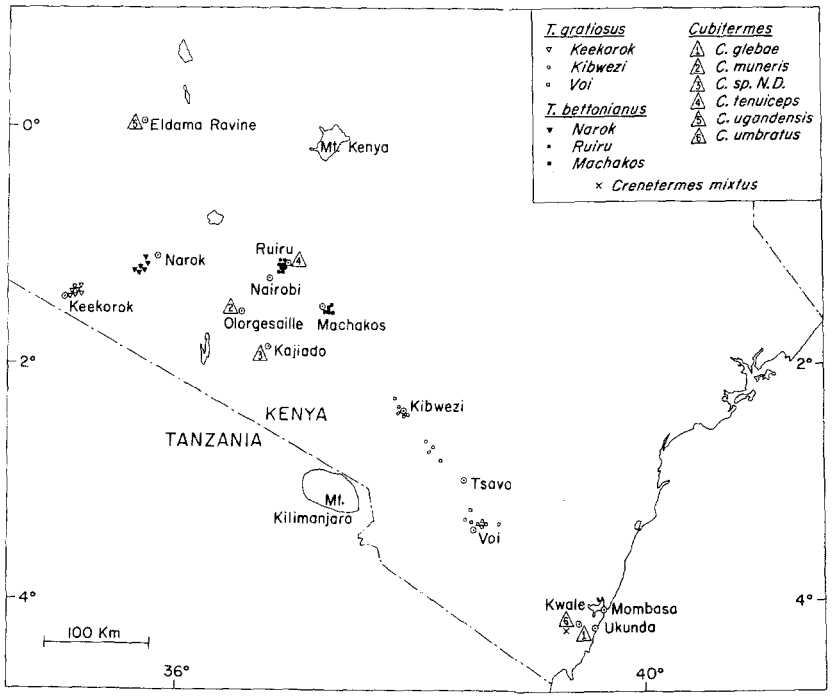

FIG. 5. Collection locations for East African *Cubitermes* species. Figure also shows locations of allopatric chemical races for two nasute species (Prestwich, 1982, 1983).

biogenesis in the soldiers of several *Nasutitermes* species has been established (Prestwich et al., 1981). The cooccurrence of the related irregular diterpenes cubitene (I) and cubugene (V) with at least three regular diterpenes in the *C. ugandensis* secretion is intriguing. One potential scheme to account for this mixture of biogenetic products is shown in Figure 7. Diterpenes II–IV are postulated to arise from *3E,6E*-farnesyl pyrophosphate (FPP) via normal head-to-tail joining of isopentenyl pyrophosphate (IPP) to give geranylgeranyl pyrophosphate (GGPP). This C_{20} compound is the precursor of regular diterpenes and could afford II or III via monocyclization of the 2*E* or 2*Z* isomers of GGPP, respectively, onto the 14 (15) double bond. Moreover, the cationic intermediate from an alternative cyclization of the 2*Z*-GGPP to the 10 (11)-double bond can undergo proton migration and cyclization to IV.

Cubitene I has two lavandulyl-type non-head-to-tail fusions, and we have postulated coupling of FPP with dimethallylpyrophosphate (DMAPP) followed by a cyclization leading ultimately to I (Prestwich, 1979b; Prestwich et al., 1978). Cubugene (V) can also be obtained from a hypothetical intermediate cation which leads to cubitene (I). Whereas the loss of a proton from

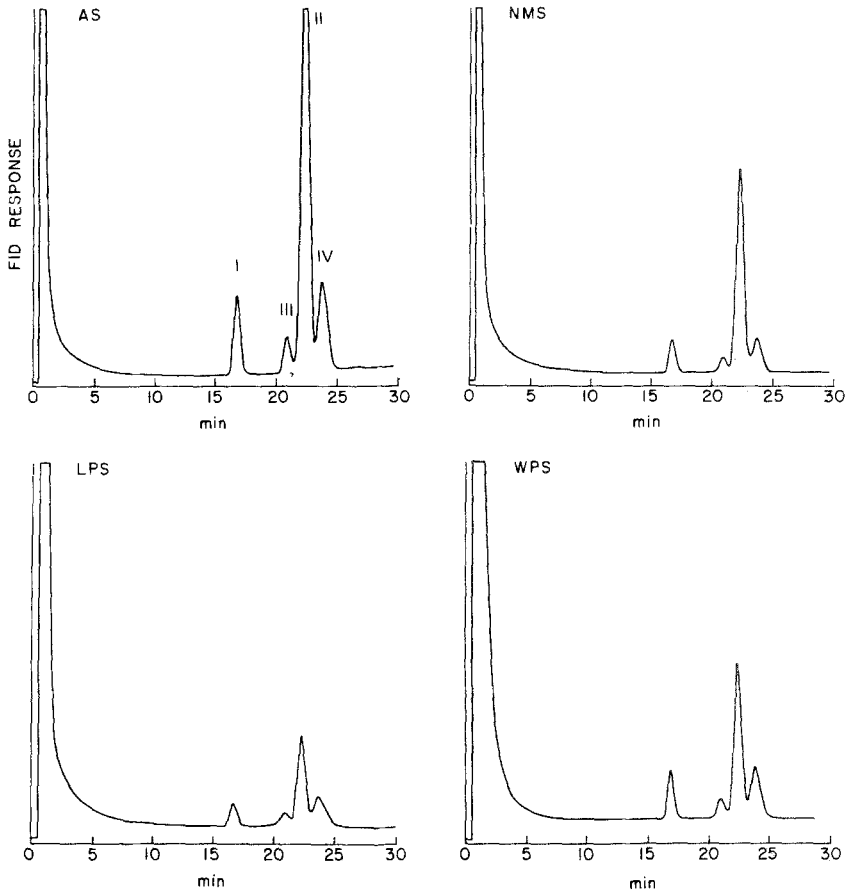

FIG. 6. GLC traces (3% FFAP, $T_i = 150°$, $T_p = 1°/\text{min}$, $T_f = 180°$) of diterpene hydrocarbons of *Cubitermes umbratus:* white presoldiers (WPS), late presoldiers (LPS) with darkened mandibles, newly molted soldiers (NMS) with slightly pigmented heads and unfed white abdomens, and mature adult soldiers (AS) with fully pigmented yellow–orange heads and soil-filled abdomens.

this cation gives cubitene, intramolecular trapping of the 3° carbocation by the isopropenyl group followed by a suprafacial 1,5 hydride migration gives an allylic carbocation which gives cubugene after proton loss. The empirical diene helicity rules suggest the (8*R*,15*S*) absolute configuration shown for cubugene (Tempesta et al., 1984). If cubitene and cubugene are derived from the same intermediate, then the absolute configuration of I can be assigned as (8*R*,10*R*), as shown. Furthermore, cubugene is unstable in air and undergoes facile epoxidation–cyclization to a tricyclic alcohol (Tempesta et al., 1984).

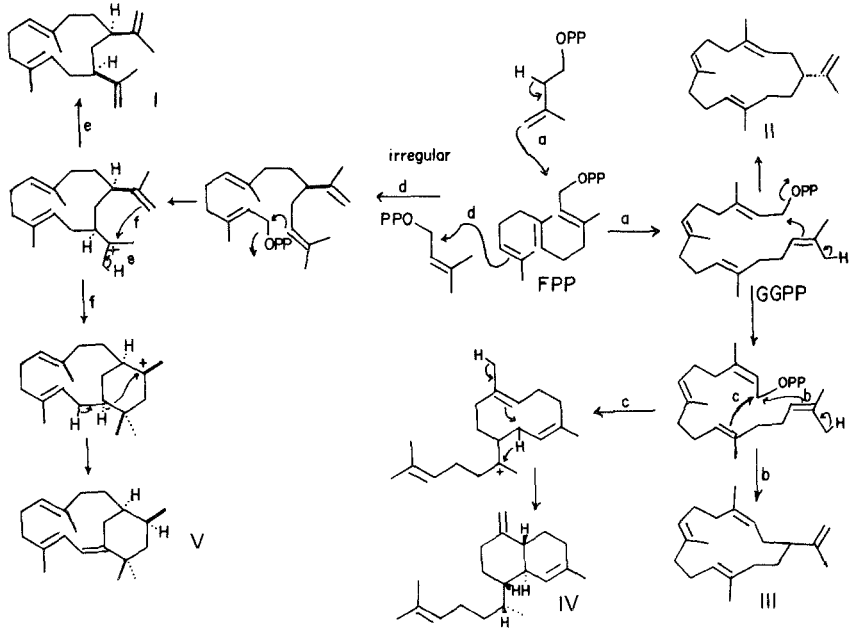

FIG. 7. Proposed biosynthetic interrelationships among the *Cubitermes* diterpenes. Refer to text for discussion.

Cubitermes are humivorous termites and live in hard soil-and-excreta nests composed of many small chambers connected by a limited number of minimally-sized holes. The connecting holes allow only one termite at a time to pass, and the hole exactly fits the maximum diameter of the soldier's head or the worker's abdomen (Deligne and Pasteels, 1982). Soldiers are scarce in *Cubitermes* and other humivores (0.02–3%) (Haverty, 1977) and are most effective in blocking strategic openings with a triple-threat: a phragmotic head, slicing mandibles, and a chemical secretion. This has been called static warfare by Deligne and Pasteels (1982), in contrast with the dynamic warfare of the more plentiful and aggressive nasute termites (Prestwich, 1982, 1983; Deligne et al., 1982).

The role of the secretion is unknown for this genus, although one may postulate repellency, irritancy, toxicity, or antihealancy as effects on an attacker. The frontal pore overhangs the clypeus and labrum in *Cubitermes* soldiers (Figure 1), and the secretion is discharged past the sensory hairs, down the indented clypeus, and onto each side of the labrum before flowing onto the swordlike mandibles (Quennedey, 1984). This is similar to the use of hydrocarbons in *Macrotermes* (Prestwich et al., 1977), macrolides in *Armitermes* (Prestwich, 1982), and sesquiterpenes in *Amitermes* and *Noditermes*

(Naya et al., 1982). Is is also analogous to the use of cibarial glands (formerly called labral glands) in the Macrotermitinae (Quennedey, 1984) to impregnate the mandibles, thus facilitating the penetration of the chemical secretion into the wound made by the mandibles.

The mode of use of the frontal weapon of *Cubitermes* thus suggest topical application to the wound, with a resulting biochemical or physical lesion. The mechanism of action of these "static warfare" defense chemicals in vivo is a fascinating topic worthy of further research. Moreover, investigations of the minor diterpene constituents will provide additional insight into the biogenetic pathways in *Cubitermes* soldiers.

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NOTE ADDED IN PROOF: An alternative biogenetic scheme is proposed by Tempesta et al. (1984) in which the irregular joining of FPP to DMAPP is not invoked. Chemical data in support of either pathway is lacking, and both remain possibilities worth considering.

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(5*Z*,9*Z*)-3-ALKYL-5-METHYLINDOLIZIDINES FROM *Solenopsis* (*Diplorhoptrum*) SPECIES

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Abstract—The alkaloidal venom components of two species of thief ants, *Solenopsis* (*Diplorhoptrum*) species AA and *S.* (*Diplorhoptrum*) *conjurata* have been found to contain (5*Z*,9*Z*)-3-hexyl-5-methylindolizidine and a mixture of (5*Z*,9*Z*)-3-ethyl-5-methylindolizidine and *cis*-2-methyl-6-nonylpiperidine, *trans*-2-methyl-6-nonylpiperidine, *cis*-2-methyl-6-undecylpiperidine, and hexadecanoic acid. *Monomorium pharaonis* was similarly investigated and found to contain the indolizidine and pyrrolidines previously described (Ritter et al., 1977b). Both indolizidines were synthesized along with their stereoisomers and separated by preparative gas chromatography. Spectral studies revealed the stereochemistry to be 5*Z*,9*Z* in both cases. The stereochemistry of 2-butyl-5-pentylpyrrolidine in *M. phaeronis* has also been established. Biosynthetic relationships are discussed.

Key Words—Ants, *Solenopsis* (*Diplorhoptrum*) species, *Monomorium pharaonis*, Hymenoptera, Formicidae, 3-alkyl-5-methylindolizidines, alkaloids, venom components, 2,5-dialkylpyrrolidines.

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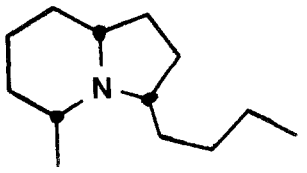
INTRODUCTION

(5*Z*,9*Z*)-3-Butyl-5-methylindolizidine (monomorine I)[4] is one of the unique and well-studied compounds among the large variety of alkaloids that have been found in the venoms of ants in the genera *Monomorium* and *Solenopsis*. It was first described as a trail pheromone component of Pharaoh's ant *M. pharaonis* (Ritter, et al., 1973) and later, along with concomitant 2,5-dialkylpyrrolidines, as being a repellent against other species of ants (Ritter et al., 1977a). The structure and stereochemistry of the natural alkaloid have been confirmed by synthesis (Ritter and Persoons, 1975a), and it has been the subject of a number of other synthetic and structural studies (Sonnet and Oliver, 1975; Sonnet et al., 1979; MacDonald, 1980; Spande et al., 1981; Stevens and Lee, 1982).

In this report, the alkaloidal venom components of two species of thief ants *Solenopsis* (*Diplorhoptrum*) which contain (5*Z*,9*Z*)-3-alkyl-5-methyl-indolizidines are described. In queens of one species, *S.* (*Diplorhoptrum*) species AA, the indolizidine is the sole alkaloid detectable, while in workers of the other species, *S.* (*Diplorhoptrum*) *conjurata*, an indolizidine occurs along with several 2-alkyl-6-methylpiperidines, compounds that are well known from this genus (Jones et al., 1980).



MONOMORINE I

METHODS AND MATERIALS

Chemical Analyses. Gas chromatographic analyses were performed on a Gow-Mac model 750P using a 2-m × 2-mm-ID glass column packed with 5% SP-1000 on 100–120 mesh Supelcoport. Generally this instrument was programed from 40°C to 200°C at 10°C/min as soon as the solvent had eluted. Retention temperatures were found to be reproducible within one degree on a given day, and all direct comparisons were performed on the same day at least twice. Preparative gas chromatography was performed on a Gow-Mac model 150 using a 2-m × 5-mm ID aluminum column packed with 5% SP-1000 on 100–150 mesh Supelcoport. Infrared spectra were obtained from neat liquid films with either a Perkin-Elmer 257 or a Perkin-Elmer 467

[4]This nomenclature refers to the configuration of the remaining methine hydrogens (5 and 9 in monomorine I) relative to the methine hydrogen at C-3 cf. Sonnet et al., 1979.

grating infrared spectrophotometer. [^{1}H]NMR were obtained at 60 MHz using a Varian A60-A or a JEOL FX-60 spectrometer and at 360 MHz with a Nicolet 360-MHz FT NMR spectrometer. [^{13}C]NMR were obtained from deuteriochloroform solutions at 15 MHz using a JEOL FX-60 spectrometer. Mass spectra were obtained using a LKB-9000 GC-MS at an ionizing voltage of 70 eV and fitted with a 2-m × 2-mm-ID glass column packed with 1% SP-1000 on Supelcoport. Melting points are uncorrected, and combustion analyses were performed by Atlantic Microlabs, Atlanta, Georgia

Ants. Collections of 50–100 individual workers of *Solenopsis conjurata* were obtained from three locations near Sirena, Costa Rica, and immediately placed in vials containing 1–2 ml of methylene chloride. Twenty-five queens of *Solenopsis* (*Diplorhoptrum*) species A were collected in Marathon Key, Florida, in the same manner. *Monomorium pharaonis* workers were taken from a laboratory colony in Athens, Georgia, and placed in methylene chloride for subsequent analysis.

5-Oxohexanal ethylene ketal (II). A solution containing 25.0 g of 5-oxohexanoic acid (Nazarov and Zav'yalov, 1952) and 0.2 g of *p*-toluenesulfonic acid in 75 ml of ethanol was heated to reflux for 2 hr using a Soxhlet extraction apparatus containing a thimble filled with anhydrous $MgSO_4$. The solvent was removed under reduced pressure, and the residue was taken up in ether, washed with saturated $NaHCO_3$, dried ($MgSO_4$), filtered, and the solvent removed to yield 25.0 g of crude ethyl-5-oxohexanoate. This ketoester was taken up in 150 ml of benzene containing 11 ml of ethylene glycol and 0.1 g of *p*-toluenesulfonic acid. The mixture was heated to reflux under a Dean-Stark apparatus for 2 hr, cooled, washed sequentially with saturated $NaHCO_3$ and brine, and dried over anhydrous $MgSO_4$. After filtration, the solution was distilled to give 25.6 g of colorless ethyl 5-oxohexanoate ethylene ketal (I), bp 70–73°C (0.1 mm Hg) [lit. 128–129°C (15 mm Hg)] (Meltzer et al., 1960): IR 1725 cm^{-1}; NMR (60 MHz) δ = 4.13 (2H, q, J = 7.0 Hz), 3.93 (4H, br s), 2.31 (2H, m), 1.63 (4 H, m), 1.28 (3H, 5), and 1.24 (3H, t, J = 7.0 Hz). A solution containing 24.0 g of I in 50 ml of anhydrous ether was added dropwise to 500 ml of ether containing 5.0 g of lithium aluminum hydride under a nitrogen atmosphere. The resulting mixture was stirred overnight and worked up in the usual manner (Fieser and Fieser, 1967) to give 14.4 g (76% yield) of 6-hydroxy-2-hexanone ethylene ketal, bp 78–82°C (0.1 mm Hg): IR 3400, 1377, 1220, 1140, 1060 cm^{-1}; NMR (60 MHz) δ = 3.93 (4 H, br s), 3.50 (3 H, m, −OH and CH_2—O, 1.60 (6H, m), and 1.30 (3H, s). This alcohol was immediately oxidized with pyridinium chlorochromate (30 g) in the presence of anhydrous sodium acetate (3 g) and worked up in the usual manner (Corey and Suggs, 1975) to give 8.6 g of 5-oxohexanal ethylene ketal (II) (60% yield), bp 65–68°C (0.2 mm Hg): IR 2720, 2710, 1720, 1370, 1220, 1140, 1070, 950, and 860 cm^{-1}; NMR (60 MHz) δ = 8.90 (1H, t, J = 1.8 Hz), 3.97 (4H, br, s), 2.47 (2H, m), 1.6 (4H, m), and 1.30 (3H, s).

2,6,9-Undecatrione (III). A solution containing 9.5 g of II (60 mmol), 5.05 g of ethyl vinyl ketone, and 1.6 g of 5-(2-hydroxyethyl)-4-methyl-3-benzylthiazolium chloride in 8.5 ml of triethylamine was heated to reflux under a nitrogen atmosphere for 16 hr. The mixture was filtered and the solvent removed in vacuo. The residue was taken up in 60 ml of 50% acetic acid and heated to reflux for 1 hr. After cooling and careful neutralization with solid $NaHCO_3$, the mixture was extracted with 3 × 50-ml portions of ether, dried over anhydrous $MgSO_4$, filtered and distilled to give 5.5 g (49% yield) of a waxy solid, bp 115–120°C (0.07 mm Hg), mp 65–66°; NMR (60) δ = 2.73 (4 H, s), 2.3–2.6 (6H, m), 2.17 (3H, s), 1.85 (2H, m), and 1.07 (3H, t, J = 7.0 Hz); MS, m/z (rel %) 198 (1, M+·), 180(16), 169(12), 160(3), 151(11), 145(6), 141(30), 128(7), 123(20), 113(100), 111(27), 108(2), 95(17), 85(60), 83(18), 71(30), 57(72), 55(31), 43(98), 42(14), and 41(13). The melting point and NMR spectra of III matched those reported in the literature (Stetter and Mertens, 1981).

3-Ethyl-5-methylindolizidine(IV). A solution containing 2.0 g of triketone III, 0.9 g of ammonium acetate, 0.05 g of potassium hydroxide, and 1.01 g of sodium cyanoborohydride in 40 ml of anhydrous methanol was stirred under a nitrogen atmosphere for 16 hr. After the usual work up involving addition of sodium borohydride, followed by acidification and extraction with ether (Jones et al., 1980), the mixture was made basic with potassium hydroxide and extracted with 3× 35-ml portions of ether. The combined ether extracts were dried (anhydrous K_2CO_3) and the solvent removed in vacuo to give 1.2 g (70% yield) of indolizidine IV. Gas chromatographic analysis on 10% SP-1000 showed four components, IVa, IVb, IVc, IVd (Figure 1, chromatograph II), which had retention temperatures of 124°C, 134°C, 145°C, and 155°C, respectively, and baseline separation for the programing conditions described previously. The four components had almost identical mass spectra: MS, m/z (rel %) 167(1, $M^{+}\cdot$), 166(3), 152(6), 139(10), 138(100), 124(2), 122(1), 110(2), 96(2), 95(5), 84(1), 82(2), 70(3), 69(3), 68(3), 67(3), 56(2), 55(5), 42(2), and 41(6). Preparative gas chromatography permitted the isolation of pure samples of IVa and IVb as well as a mixture of IVc and IVd. Anal. Calcd for $C_{11}H_{21}N$: C, 78.98; H, 12.65; N, 8.37. Found C, 78.96; H, 12.69; N, 8.34.

2-Methyl-6-(3-hydroxypentyl)pyridine (*V*). This hydroxy alkylpyridine was prepared from 2,6-lutidine by the standard method (Sonnet and Oliver, 1975) and was purified by distillation, bp 86–92°C (0.1 mm Hg): IR 3400, 1592, 1578, 1460, 1155, 1120, 1065, 1035, 985, and 935 cm^{-1}; NMR (60 MHz) δ = 7.5 (1H, d of d, J = 7.5, 7.5 Hz), 6.98 (2H, br d, J = 7.5 Hz), 5.47 (1H, br s), 3.63 (1H, quintet, J = 7.0 Hz), 2.98 (2H, 5, J = 7.0 Hz), 2.51 (3H, s), 2.0 – 1.4 (4H, m) and 0.98 (3H, t, J = 7.0 Hz); MS, m/z (rel. %) 179 (1, $M^{+}\cdot$), 178(1), 162(7), 150(30), 132(5), 120(36), 108(9), 107(100), 93(8), 92(5), 79(3), 77(4), 66(3), and 65(5).

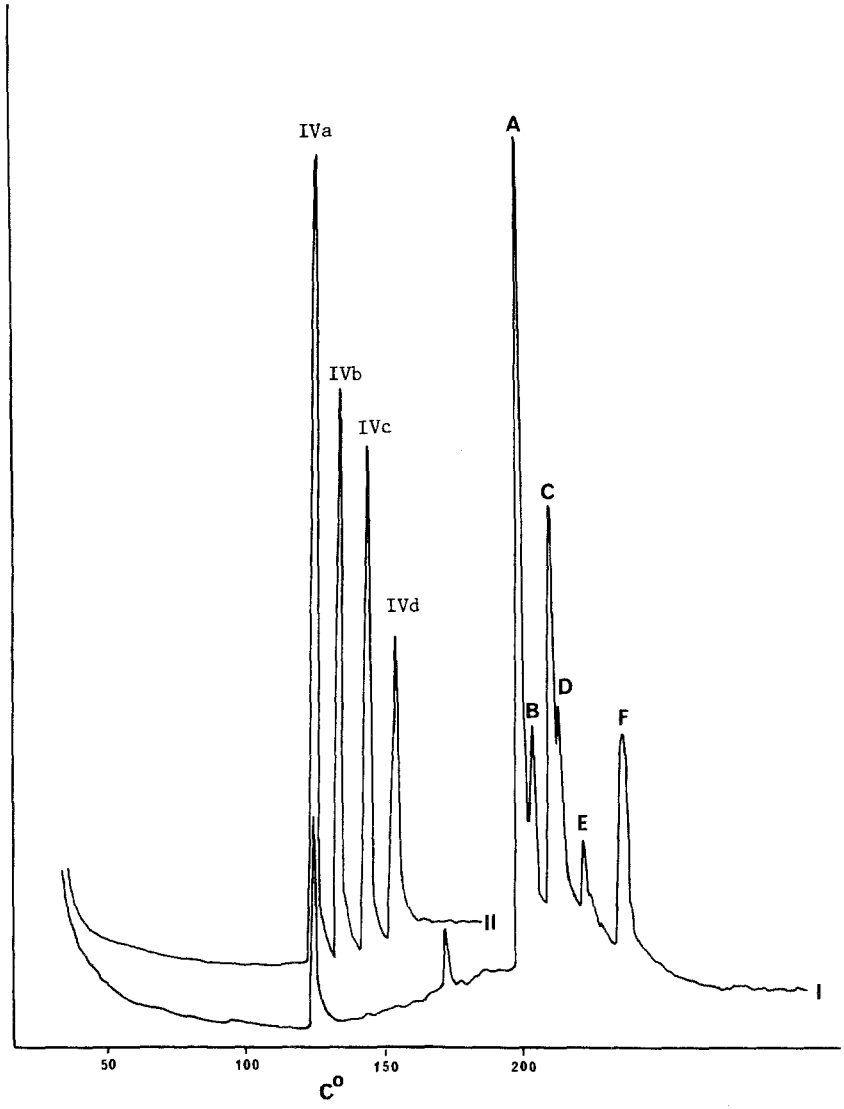


FIG. 1. Gas chromatograms on a 2m 10% SP-1000 column.
I. *Solenopsis conjurata*.
II. The reductive animation products from triketone *3*.

cis-*2-Methyl-6-(3-hydroxypentyl)piperidine* (*VI*). A solution containing 3.9 g of V and 0.25 g of PtO_2 in 35 ml of acetic acid was hydrogenated at 3 atm pressure of hydrogen overnight. After filtration, the solvent was removed in vacuo and the residue was made basic with 10% NaOH. The mixture was extracted with 3× 40-ml portions of ether, and the combined ethereal

extracts were dried over anhydrous K_2CO_3. After filtration, the solvent was removed in vacuo to produce 3.7 g of a waxy solid: a sample prepared by gas chromatography on an SP-1000 column had mp 80–84°. MS, *m/z* (rel. %) 185 (1, $M^+\cdot$), 184(1), 170(3), 156(8), 152(2), 138(1), 99(9), 98(100), 96(2), 84(2), 82(2), 81(2), 70(3), 69(2), 56(3), 55(4), 43(6), and 41(4). A 1-g sample of VI was converted to its *N*-benzyl derivative (benzoyl chloride, DMAP; $LiAlH_4$) (Hill and Chan, 1965), which was eluted as a single peak from either SE-30 or SP-1000 columns: MS, *m/z* (rel %) 275(1, $M^+\cdot$), 274(1), 260(8), 246(5), 189(25), 188(100), 174(10), and 91(80). The [^{1}H]NMR spectrum (CCl_4) showed a pair of singlets at $\delta = 3.71$ and $\delta = 3.78$, with the latter predominating in a ratio of 3:2. Upon the addition of a small amount of D_6-DMSO, only the singlet at $\delta = 3.78$ was observed.

cis-*2-Methyl-6-(3-oxopentyl)*-N-*benzylpiperidine.* A solution of 28 mg of the above *N*-benzyl derivative of VI in 0.3 ml of pyridine was treated with 50 mg pyridine dichromate (Aldrich; cf. Corey and Schmidt, 1979) in 1 ml of pyridine and allowed to stand overnight. The solvent was removed by distillation at reduced pressure, and the residue was partitioned between water and methylene chloride. The organic layer was washed with water and the solvent removed under reduced pressure. The residue was distilled (170°/0.1 mm) to provide 12 mg of a clear oil, MS, *m/z* (rel. %) 273 (0.4, $M^+\cdot$), 272(0.7), 271(0.8), 258(6), 214(92), 211(1), 190(17), 189(100), 96(3), 92(7), 91(76), 65(4), 57(5), and 55(4). [^{13}C]NMR: CH_3: 7.9, 22.1 ppm; CH_2: 24.0, 28.4, 29.4, 32.6, 35.7, 38.6, 52.6; CH: 58.2, 62.2, 126.1, 127.9 (2C), 128.1 (2C); C; 142, 211.9.

Preparation of (5Z,9Z)- and (5E, 9E)-3-Ethyl-5-methylindolizidines (IVa) and (IVc) from (VI). A solution containing 3.6 g of VI in 50 ml of acetic acid was oxidized with CrO_3 as described previously (Sonnet and Oliver, 1975). The reaction was followed by gas chromatography, and after the final work-up, distillation gave a pale yellow liquid (VII) whose IR spectrum (1710 and 1660 cm^{-1}) showed ketone and enamine forms. A benzene solution containing 2.0 g of VII was treated with approximately one equivalent of perchloric acid, and the solvent was removed in vacuo. The residue was taken up in toluene and refluxed under a Dean-Stark trap 3 hr. Following removal of the toluene, the residue was taken up in 20 ml of anhydrous methanol. Half the methanolic solution was hydrogenated overnight under 3.7 atm of hydrogen in the presence of 0.15 g of PtO_2. After filtration and removal of the solvent, the residue was made basic with 10% NaOH, extracted with ether, and the ethereal extracts were dried over anhydrous K_2CO_3. Gas chromatographic analysis showed the presence of only one component, which had an identical retention temperature with isomer IVa by coinjection and direct comparison. The second half of the methanolic solution was treated with excess sodium cyanoborohydride (N_2 atmosphere, pH = 4) and stirred

overnight. The mixture was carefully acidified, and the solvents were removed in vacuo. The residue was made basic, extracted thoroughly with ether, and the combined ether extracts were dried over anhydrous K_2CO_3. Gas chromatographic analysis showed the presence of two components in a 1:1 ratio which had identical retention temperatures with isomers IVa and IVc by coinjection and direct comparison. Isomers IVb or IVd were not detected. The mass spectra of the indolizidine isomers prepared from VI were identical with those obtained from the reductive amination of triketone III. Preparative gas chromatography provided pure samples of IVa and IVc from these experiments. In a third experiment, 1.0 g of VII in 30 ml of benzene was treated with dry HCl and heated to reflux 3 hr under a Dean-Stark trap. Upon cooling, the solution was treated with excess dry HCl, and the solvent was removed and replaced with methanol. Reduction with excess sodium cyanoborohydride again produced a mixture of isomers IVa and IVc, although in a 2:1 ratio. Again, none of IVb or IVd was detected.

The important [^{1}H]- and [^{13}C]NMR data for the four isomers are summarized below. The data for isomer IVd are those signals remaining when the signals for isomer IVc were subtracted from the spectra of the IVc-IVd mixture. The [^{1}H]NMR spectra were obtained from trifluoroacetic acid solutions at 360 MHz. The attached proton test (Patt and Schoolery, 1982) was used to determine carbon multiplicity in the [^{13}C]NMR spectra.

IVa. [^{1}H]NMR δ = 3.56 (1H, m), 3.36 (1H, m), 3.23 (1H, m), 1.58 (3H, d, J = 6.2 Hz), 1.13 (3H, t, J = 6.8 Hz); [^{13}C]NMR δ = 67.3 (CH), 64.4 (CH), 60.1 (CH), 35.8 (CH_2), 32.1 (CH_2) 30.8 (CH_2), 30.3 (CH_2), 29.2 (CH_2), 24.9 (CH_2), 22.7 (CH_3), and 11.0 (CH_3).

IVb. [^{1}H]NMR δ = 4.02 (1H, m), 3.50 (1H, m), 3.40 (1H, m), 1.45 (3H, d, J = 6.8 Hz), 1.13 (3H, t, J = 6.8 Hz); [^{13}C]NMR δ = 60.6 (CH), 55.6 (CH), 47.4 (CH), 32.2 (CH_2), 31.4 (CH_2), 29.1 (CH_2), 27.6 (CH_2), 25.1 (CH_2), 18.2 (CH_2), 10.6 (CH_3), and 7.6 (CH_3).

IVc. [^{1}H]NMR δ = 3.88 (1H, m), 3.37 (2H, m), 1.50 (3H, d, J = 6.2 Hz), and 1.13 (3H, t, J = 7.0 Hz); [^{13}C]NMR δ = 60.7 (CH), 58.8 (CH), 52.0 (CH), 34.6 (CH_2), 32.4 (CH_2), 30.1 (CH_2) 25.8 (CH_2), 24.7 (CH_2), 20.4 (CH_3), 18.0 (CH_2), and 11.0 (CH_3).

IVd. [^{1}H]NMR δ = 4.05 (1H, m), 3.78 (1H, m), 3.46 (1H, m), 1.56 (3H, d, J = 6.8 Hz), and 1.13 (3H, t, J = 6.8 Hz); [^{13}C]NMR δ = 61.7(CH), 55.7 (CH), 49.2 (CH), 28.8 (CH_2), 28.3 (two CH_2) 26.9 (two CH_2), 20.0 (CH_3), 18.7 (CH_2), and 11.0 (CH_3).

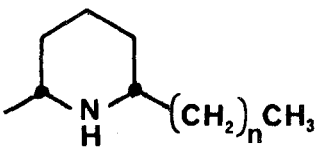
2-Methyl-6-(3-hydroxynonyl)pyridine (VIII). This hydroxy alkylpyridine was prepared in the same manner as V using 1-octene oxide. Distillation provided a pale yellow liquid, bp 115–120° C (0.1 mm Hg): IR 3400, 1590, and 1578 cm^{-1}; [^{1}H]NMR (60 MHz) δ = 7.5 (1H, d of d, J = 7.5, Hz), 6.98 (2H, br d, J = 7.5 Hz), 5.27 (1H, s), 3.68 (1H, m) 2.97 (2H, t, J = 7.0 Hz), 2.52 (3H, s),

2.8–1.6 (12H, m), and 0.86 (3H, br t); MS, *m/z* (rel. %) 235 (1, $M^{+}\cdot$), 234(2), 218(4), 160(3), 150(31), 133(2), 132(3), 121(14), 120(32), 108(8), 107(100), 93(5), 92(4), 79(3), 77(3), 66(2), 55(3), 43(4), and 41(4).

cis-*2-Methyl-6-(3-hydroxynonyl)piperidines* (*IX*). A solution containing 16.7 g of VIII and 0.6 g of PtO_2 in 130 ml of acetic acid wasy hydrogenated at 3.5 atm pressure of hydrogen overnight. The resulting mixture was worked-up as described for VI to give 16.3 g (95% yield) of a waxy solid, mp 41–44° C, which eluted as a single peak from a gas chromatograph on SP-1000. MS, *m/z* (rel %) 241(1), M^{+}), 240(1), 226(2), 156(5), 138(4), 99(6), 98(100), 96(3), 84(3), 82(2), 81(1), 70(3), 69(3), 57(3), 56(3), 55(5), 43(6), and 41(5).

(*5Z,9Z*)-*3-Hexyl-5-methylindolizidine* (*XI*). A solution containing 5 g of IX in 40 ml of acetic acid was oxidized as described above for VI to give 3.2 g (64% yield) of a pale yellow liquid, bp 95–100° C (0.07 mm Hg), IR 1710 and 1655 cm^{-1}. This material was treated with perchloric acid as described above, and the residue was hydrogenated overnight in methanol under 3.5 atm of hydrogen in the presence of 0.2 g of PtO_2. After the usual work-up, gas chromatographic analysis showed the presence of only one component which was isolated by preparative gas chromatography. IR 2835, 2790, 2720, 2590, 1455, 1380, 1320, 1305, 1263, 1205, 1170, 1135, 1110, and 1015 cm^{-1}; [^{1}H]NMR (360 MHz, CF_3COOH) δ = 3.65 (1H, m), 3.36 (1H, m), 3.22 (1H, m), 1.58 (3H, d, J = 6.8 Hz), 0.92 (3H, br t); [^{13}C]NMR 67.3 (CH), 63.0 (CH), 60.3 (CH), 39.7 (CH_2), 35.7 (CH_2) 31.9 (CH_2), 30.8 (CH_2), 30.2 (CH_2), 29.7 (two CH_2), 29.4 (CH_2), 27.0 (CH_2), 24.9 (CH_2), 22.7 ($CH_3$2), and 14.0 (CH_3); MS, *m/z* (rel % 223(1), $M^{+}\cdot$), 222(2), 208(3), 139(12), 138(100), 136(3), 95(3), 82(2), 70(2), 69(2), 68(2), 67(3), 55(5), and 41(6). Anal. Calcd for $C_{15}H_{29}N$: C, 80.65; H, 13.08; N, 6.27. Found: C, 80.49; H, 13.12; H, 6.26.

Analysis of Solenopsis conjurata *Alkaloids.* For analysis, solutions from the three collections of *S. conjurata* were reduced in volume to ca. 0.2 ml with a slow stream of nitrogen. The gas chromatograms of all three collections were quite similar (Figure 1, chromatograph I). The first component eluted at



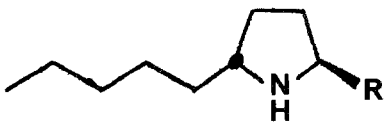
A, n=8

E, n=10

124° C and had a mass spectrum with important ions at m/z 167 ($M^{+\cdot}$), 152, and 138(100). The retention temperature and mass spectrum of this component were identical by coinjection and direct comparison to those of the first eluting isomer of 3-ethyl-5-methylindolozidine prepared by reductive amination of triketone III. (Figure 1, chromatogram II). The temperature program was held at 200° C after which components A–F eluted. The following structures could be assigned on the basis of mass spectra (MacConnell et al., 1971; Jones et al., 1982) and by direct comparison and coinjection with authentic samples under isothernal gas chromatographic conditions (160° C): A, *cis*-2-methyl-6-nonylpiperidine; B, 1,2-dimethyl-6-nonylpiperidine (probably *cis*); C, *trans*-2-methyl-6-nonlypiperidine; D, 1,2-dimethyl-6-nonylpiperidine (probably *trans*); E, *cis*-2-methyl-6-undecylpiperidine; F, hexadecanoic acid (Heller and Milne, 1982). None of the *trans* isomer of E was detected.

Analysis of Solenopsis (Diplorhoptrum) *Species AA Alkaloids.* The methylene chloride extract of 25 queens of *Solenopsis* (*Diplorhoptrum*) species A showed a single sharp peak (retention temperature = 175° C) upon gas chromatographic analysis. The mass spectrum of this component had important ions at m/z = 223 (M+), 208, and 138(100). This mass spectrum and retention temperature were identical with those of an authentic sample of indolizidine XI. No other alkaloids were detected in the extracts of this ant.

Analysis of Monomorium pharaonis *Alkaloids.* Gas chromatographic analysis of the methylene chloride extracts of *M. pharaonis* showed the expected (5*Z*,9*Z*)-3-butyl-5-methylindolizidine (monomorine I) and *trans*-5-(5-hexen-1-yl)-2-pentylpyrrolidine (XII) (monomorine III) as major components as well as 2-butyl-5-pentylpyrrolidine (XIII) (monomorine II) as a



XII R = $CH_2CH_2CH_2CH_2CH{=}CH_2$

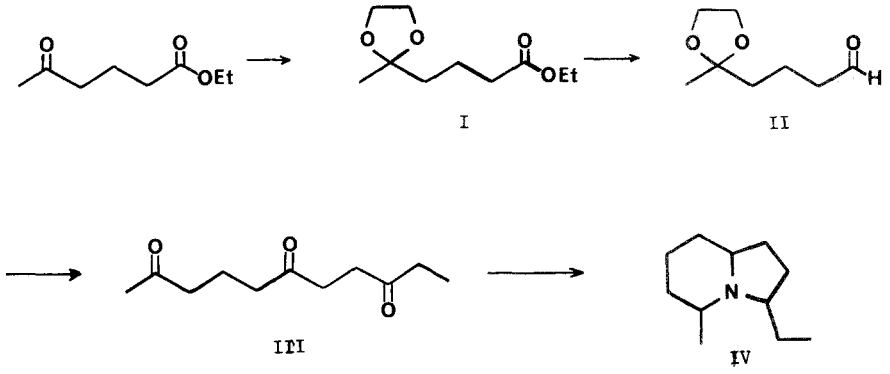
XIII R = *n*-Bu

minor component (Ritter et al., 1977a,b). This minor component was compared directly and by coinjection (isothermal, 120° C) with authentic samples of *cis*- and *trans*-pyrrolidine XIII (Jones et al., 1979); the 2-butyl-5-pentylpyrrolidine in *M. pharaonis* has a retention time identical with that of the later eluting *trans* isomer of XIII.

RESULTS

Gas chromatographic analysis of the alkaloidal venom components of *Solenopsis conjurata* (Figure 1) revealed a component eluting at 124° C whose mass spectrum corresponded to an isomer of 3-ethyl-5-methylindolizidine (IV). In addition, components A–F (Figure 1) were readily identified as *cis*-2-methyl-6-nonylpiperidine (A), 1,2-dimethyl-6-nonylpiperidine (B and D, probably *cis* and *trans*, respectively), *trans*-2-methyl-6-nonylpiperidine (C), *cis*-2-methyl-6-undecylpiperidine (E), and hexadecanoic acid (F), by their mass spectra and by direct comparison with samples of known stereochemistry.

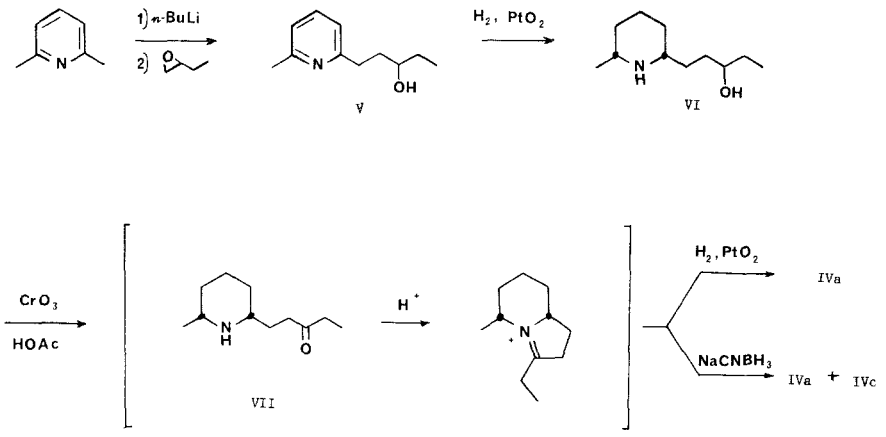
A mixture of the four possible isomers of indolizidine IV was prepared in the following manner (Scheme 1). 5-Oxohexanal ethylene ketal (II) was



SCHEME 1.

prepared from ethyl-5-oxohexanoate by standard methodology and condensed with ethyl vinyl ketone in the presence of a thiazolium salt catalyst to give the known 2,6,9-undecatrione (III) (Stetter and Mertens, 1981). The usual reductive amination conditions (Jones et al., 1980) were sufficient to cyclize triketone III and gave a mixture of all four possible stereoisomers of 3-ethyl-5-methylindolizidine (Figure 1, chromatograph II). The four isomers had identical mass spectra which matched that of the early eluting component from *S. conjurata.* Direct comparison and coinjection showed that the natural material was identical to the first eluting isomer, IVa (Figure 1).

In order to assign the stereochemistry of the isomers, and to provide a more convenient supply of the natural alkaloid, well-known indolizidine preparations from 2,6-lutidine were employed (Sonnet and Oliver, 1975). 2-Methyl-6-(3-hydroxypentyl)pyridine (V) was prepared from 2,6-lutidine anion (Scheme 2) and was hydrogenated to give the expected mixture of

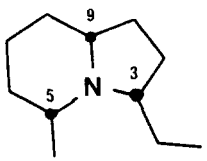


SCHEME 2.

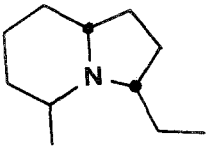
diastereoisomeric *cis*-2-methyl-6-(3-hydroxypentyl)piperidines (VI). Gas chromatographic analysis of VI under conditions that provide baseline separation of *cis* and *trans* piperidines (SP-1000) showed only one peak, suggesting that no *trans* isomer was present. In support of the assignment of *cis* stereochemistry to diastereoisomers VI, the *N*-benzyl derivatives of the mixture were prepared and their [^{1}H]NMR spectra showed a singlet at $\delta = 3.78$ or a pair of singlets at $\delta = 3.71$ and 3.78, one for each diastereomer depending on the solvent. There was no sign of the AB quartet that would be expected for the benzylic methylene group of the *trans* isomer (Hill and Chan, 1965). Furthermore, oxidation of the *N*-benzyl mixture with pyridinum chromate in pyridine provided only one ketone ([^{13}C]NMR and GC) as expected. Chromium trioxide oxidation of VI itself provided a mixture of the corresponding amino ketone and enamine (VII) which was converted to its iminium salt with perchloric acid. Catalytic hydrogenation of this salt provided only the indolizidine isomer IVa, while sodium cyanoborohydride reduction gave a 1 : 1 mixture of IVa and IVc (Scheme 2). These two isomers were also produced when VII was exposed to enamine-forming conditions (benzene–acid reflux) prior to cyanoborohydride reduction.

In light of previous studies of the isomers of monomorine I (Sonnet et al., 1979), these synthetic results, along with the [^{13}C] and [^{1}H]NMR spectra, reveal the stereochemistry of the isomers IVa–IVd (Figure 2).

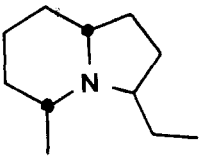
Strong indications of the 5*Z*,9*Z* configuration of IVa are that it is the sole product of a sequence of catalytic hydrogenations (Luning and Lundin, 1967; Sonnet and Oliver, 1975) and that it is the isomer first eluted in gas chromatographic analysis (Luning and Lundin, 1967; Spande et al., 1981).



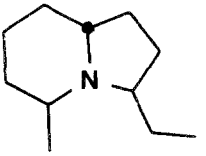
IVa



IVb



IVc



IVd

FIG. 2.

This assignment is confirmed by the NMR spectra of IVa. In the 360-MHz [^{1}H]NMR spectra of all the indolizidines, the alpha carbon methine signals appear as separated broad multiplets, and in IVa none of these signals appears further downfield than $\delta = 3.6$. This result correlates well with that obtained for (5*Z*,9*Z*)-monomorine I (Sonnet et al., 1979) as do the ^{13}C chemical shifts for the carbons and the C-5 methyl carbon (Table 1). The difference in the [^{13}C]NMR spectrum at C-3 arises from the difference in alkyl chain length. Thus, the first eluting isomer, IVa, as well as the natural alkaloid from *S. conjurata* is (5*Z*,9*Z*)-3-ethyl-5-methyl-indolizidine.

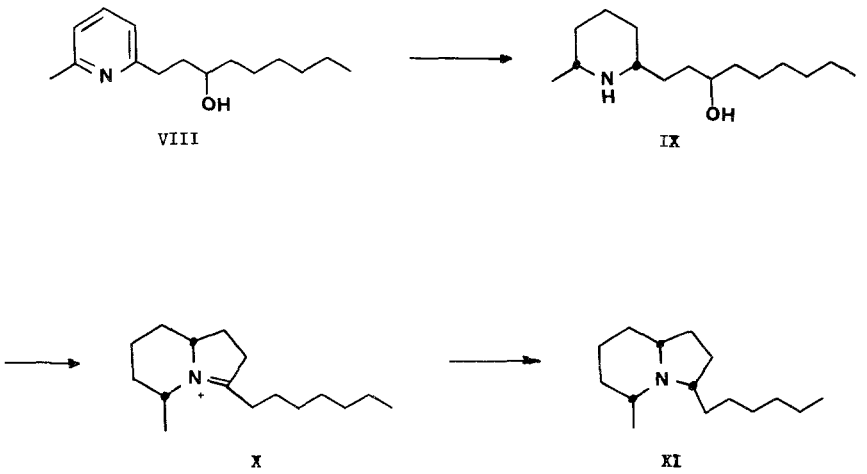
The second eluting (5*E*,9*Z*)-IVb is not formed from the *cis*-piperidine VI, and its ^{1}H and ^{13}C spectra can be closely correlated to those obtained previously for (5*E*,9*Z*)-monomorine I (Table 1) (Sonnet et al., 1979). Most striking is the C-5 methyl resonance for this isomer at 7.6 ppm, which is shifted at least 12.6 ppm upfield from the corresponding C-5 methyl signals in the other three isomers. This signal results from the C-5 methyl being axial and *trans* antiparallel to the nitrogen lone pair.

Of the last two isomers, only the third eluting (5*E*,9*E*)-IVc is formed along with (5*Z*,9*Z*)-IVa by sodium cyanoborohydride reduction of the iminium salt of VII. Since the precursor to VII is the *cis*-2,6-disubstituted piperidine VI, the piperidine rings of IVa and IVc are *cis* disubstituted, while the piperidine rings of IVb and IVd, which are not formed in this synthesis, are *trans* disubstituted. In addition, the NMR data for IVc and IVd also compare well to those reported for (5*E*,9*E*)-monomorine I and (5*Z*,9*E*)-monomorine I, respectively (Table 1).

TABLE 1. COMPARISON OF SELECTED NMR DATA FOR INDOLIZIDINE ISOMERS

	[^{13}C]NMR				[^{1}H]NMR	
Indolizidine isomer	5-CH_3	C-3	C-5	C-9	5-$C\underline{H}_3$	N-C<u>H</u>
(5*Z*,9*Z*)-Monomorine I	22.9	61.8	59.7	67.6	1.49	2.9–3.9 (3H)
(5*Z*,9*Z*)-IVa	22.7	64.4	60.1	67.3	1.58	3.23 (1H), 3.36 (1H), 3.56 (1H)
(5*Z*,9*Z*)-XI	22.7	64.4	60.1	67.3	1.58	3.22 (1H), 3.36 (1H), 3.65 (1H)
(5*E*,9*Z*)-Monomorine I	7.4	55.4	47.2	60.6	1.36	3.0–3.8 (2H), 3.8–4.3 (1H)
(5*E*,9*Z*)-IVb	7.6	55.6	47.4	58.7	1.45	3.40 (1H), 3.50 (1H), 4.02 (1H)
(5*E*,9*E*)-Monomorine I	20.9	56.3	51.8	60.6	1.40	3.0–3.7 (2H), 3.7–4.2 (1H)
(5*E*,9*E*)-IVc	20.3	58.8	52.0	58.9	1.50	3.38 (2H), 3.88 (1H)
(5*Z*,9*E*)-Monomorine I	20.6	55.0	48.6	61.2	1.46	3.1–3.6 (1H), 3.6–4.2 (2H)
(5*Z*,9*E*)-IVd	20.0	55.3	48.4	59.4	1.56	3.46 (1H), 3.78 (1H), 4.05 (1H)

The mass spectrum of a single volatile component in queens of *S.* (*Diplorhoptrum*) species AA indicated it to be one of the 3-hexyl-5-methylindolizidines [m/z = 223 ($M^{+\cdot}$, 3), 208(15), 138(100)]. (5*Z*,9*Z*)-3-Hexyl-5-methylindolizidine (XI) was prepared by the methodology described above (Scheme 3) and was identical in all respects of the natural alkaloid.



SCHEME 3.

Since the indolizidine alkaloid from *S. conjurata* is novel in having piperidine rather than pyrrolidine concommitants, and since the stereochemistry of these piperidines could be determined readily by direct comparison to authentic samples, it became necessary to examine the stereochemistry of the pyrrolidines that occur with monomorine I in *Monomorium pharaonis*.

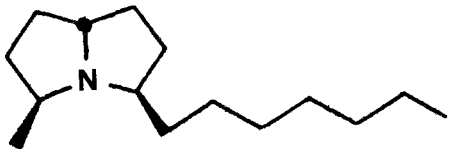
Gas chromatographic analysis of the extracts of a sample of this ant showed the presence of three alkaloidal components, exactly as had been described (Ritter, et al., 1977b). The stereochemistry of the major pyrrolidine component, *trans*-2-(5′-hexenyl)-5-pentyl pyrrolidine (XII) (monomorine III) had already been assigned, but the stereochemistry of the minor component, 2-butyl-5-pentylpyrrolidine (monomorine II), a monocyclic analog of monomorine I, had not (Ritter et al., 1977b). Direct comparison with an authentic sample (Jones et al., 1979) showed that the natural monomorine II (XIII) is also *a trans* disubstituted pyrrolidine.

DISCUSSION

Although 2,5-dialkylpyrrolidines have been found in a variety of *Monomorium* and *Solenopsis* species, and 2,6-dialkylpiperidines have been found widely distributed in *Solenopsis* species (Jones et al., 1982, and references therein), 3-alkyl-5-methylindolizidines had only been reported in *M. pharaonis* where they occur with at least four typical 2,5-dialkylpyrrolidines (Ritter et al., 1975b). Since the venom alkaloids of *S. conjurata* are a mixture of the 2,6-dialkylpiperidines, typical of the genus, along with (5*Z*,9*Z*)-3-ethyl-5-methylindolizidine (IVa), a comparison of the stereochemistry of the alkaloids in these ants may provide information on the biosynthetic pathways utilized for the production of these compounds by some species of *Monomorium* and *Solenopsis*.

Since the ants *M. pharaonis*, *S. conjurata*, and *S.* (*Diplorhoptrum*) species AA produce only the single 5*Z*,9*Z* stereoisomers of their respective 3-alkyl-5-methylindolizidines, the biosynthetic pathways for these compounds must be quite selective, and may be common to both genera. Indeed, the 5*Z*,9*Z* isomer of monomorine I has been reported to be the only isomer to have attractant and arrestant activities in feeding studies with *M. pharaonis* (Edwards and Pinniger, 1978).

Comparison to the results of laboratory synthesis is instructive here. There is no reason for the initial reaction steps of the reductive amination of a triketone with sodium cyanoborohydride forming a bicyclic amine to be stereoselective; the product ratio appears to depend largely on ring strain and steric hindrance in the possible product. This is the case in the formation of the previously described 3-heptyl-5-methylpyrrolizidine (XIV), where the 5*Z*,8*E* isomer was formed nearly ten times as readily as the other three possible isomers (Jones et al., 1980). Here the strain of a pair of *trans* fused five-membered rings, or the hindrance of an alkyl group forced into the cavity of a pair of *cis* fused five-membered rings, is undoubtedly responsible. In the preparation of 3-ethyl-5-methylindolizidine (IV) by this method however, the four possible isomers are formed in quantities of the same order of magnitude



XIV

(Figure 1, chromatograph I), indicative of the relatively smaller differences in strain and steric hindrance between the isomers.

Although the complete biosynthetic pathways for the *Monomorium* and *Solenopsis* alkaloids remain to be elucidated, with one exception (Jones et al., 1982) the alkaloids all possess an unbranched odd-numbered carbon skeleton cyclized with nitrogen. This feature is reminiscent of a number of plant-derived piperidines with structures thought to arise from the reductive amination of an α-polyketoacid (Leete, 1963), and of the coccinellid alkaloids for which the polyacetate pathway has been demonstrated (Tursch et al., 1975). Because the final stereochemistry of the *Monomorium* and *Solenopsis* indolizidines is probably determined during the closure of the last-formed ring, the stereochemistry of their monocyclic concomitants is of special interest. To date there have been no reports of a *cis*-2,5-dialkylpyrrolidine from either genus, even though the pyrrolidine ring of the indolizidines is *cis*-2,5 disubstituted. This is not the case for the previously described 3-heptyl-5-methylpyrrolizidine (XIV), in which both five-membered rings can be viewed as *trans* disubstituted (Jones et al., 1980). As a matter of fact, even 2-butyl-5-pentylpyrrolidine (XIII) (monomorine II), the monocyclic analog of monomorine I, is of the *trans* configuration. On the other hand, the occurrence of *cis*- and *trans*-2,6-disubstituted piperidines together is well known, although the *trans* isomer always predominates in the venoms of workers (MacConnell et al., 1976). In *S. conjurata*, however, 2-nonyl- and 2-undecyl-6-methylpiperidine are present mainly in the *cis* configuration (A and E) with a smaller amount of the *trans*-2-nonyl-6-methylpiperidine and none of the 2-undecyl-6-methylpiperidine detected. Of these compounds found in other *Solenopsis* (*Diplorhoptrum*) species (Jones et al., 1982), only the *trans* configuration occurs. Consideration of the stereochemistry of the alkaloids of *M. pharaonis* and *S. conjurata* together suggests that in the biogenetic cyclization forming the 5*Z*,9*Z* indolizidines, the six membered ring may be formed and saturated first, followed by the formation of the five-membered ring. This pathway contrasts with the reverse order of cyclization proposed earlier (Ritter et al., 1981; Talman et al., 1974) based, most reasonably, on the presence of the analogous pyrrolidine (XIII) in the gland.

The present proposal based on stereochemistry identifies the pyrrolidines and indolizidines as alternatives from a common intermediate.

The role of indolizidine alkaloids in ant behavior remains to be determined. Although in the case of *M. pharaonis*, a venom-derived indolizidine possesses trail-following activity (Ritter et al., 1977b), the true trail pheromone is a sesquiterpene aldehyde that is far more active than the alkaloid (Ritter et al., 1977a). On the other hand, indolizidines are demonstrably active as ant repellents (Edwards and Pinniger, 1978), and it would not be surprising if these compounds constitute key weapons in the defensive arsenals of selected species in the genera *Monomorium* and *Solenopsis*. Indeed, the defensive punch of venoms dominated by indolizidines and pyrrolidines or piperidines may be considerably augmented by the presence of mixtures of monocyclic and bicyclic alkaloids. If the mode of action of indolizidines differs from those of pyrrolidines and piperidines, it will further emphasize how some ant species in the genera *Solenopsis* and *Monomorium* have exploited sociality by challenging their competitors with a venom that is characterized by both alkaloidal diversity and idiosyncratic nitrogen heterocycles.

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ISOLATION AND IDENTIFICATION OF COTTON SYNOMONES[1] MEDIATING SEARCHING BEHAVIOR BY PARASITOID *Campoletis sonorensis*

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Abstract—In laboratory bioassays, the parasitoid *Campoletis sonorensis* was attracted to the following sesquiterpenes isolated from cotton essential oil: α-humulene, γ-bisabolene, β-caryophyllene oxide, spathulenol, β-bisabolol, and a new, naturally occurring bisabolene-related alcohol, (2-*p*-tolyl-6-methylhept-5-en-2-ol) which we name gossonorol. This is the first report of spathulenol in cotton. β-Caryophyllene, a major component of cotton, was not attractive to the parasitoids. The response of the parasitoids to these compounds and the possibility of augmenting parasitoid activity in the field by manipulating plant secondary metabolites is discussed.

Key Words—Parasitoid, Hymenoptera, Ichneumonidae, *Campoletis sonorensis,* synomone, sesquiterpene, α-humulene, γ-bisabolene, β-caryophyllene oxide, spathulenol, gossonorol, β-bisabolol.

INTRODUCTION

The parasitoid–host interaction is mediated to a great degree by chemical stimuli evolved by the host, its food, or associated organisms (Vinson, 1976). A parasitoid initially seeks a certain environment, and it does so regardless of the presence or absence of hosts (Doutt, 1964). The host, however, occurs only in specific locations within that environment. It would

[1]Synomone: A chemical produced or acquired by an organism that benefits both the emitting and receiving organisms (Nordlund and Lewis, 1976).

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be advantageous for a female parasitoid to be able to find those habitats most likely to yield hosts. Factors that retain a parasitoid on a food plant of its host may also be selected in the host habitat location sequence.

Vinson (1975) observed *Cardiochiles nigriceps* Viereck searching host-free, greenhouse-grown tobacco plants, which had been placed in an open field. The presence of volatile chemicals that orient the parasitoids to the plant was proposed. Camors and Payne (1972) found that the host tree terpene α-pinene and the host southern pine beetle sex pheromones attracted the parasitoid, *Heydenia unica* Cook and Davis, to the area of a southern pine beetle attack. We recently showed (Elzen et al., 1983) that *Campoletis sonorensis* (Cameron) orients to and searches host-free plants in the laboratory and that volatile and contact chemicals were involved. The growing terminals, buds, and flowers of sorghum, cotton, and tobacco were most attractive. The activity of these plants was extractable in ethyl ether. Thus, we concentrated our extraction and chemical isolation efforts on cotton terminals, flowers, and squares. In the present study we describe the isolation, identification, and bioassay results on those chemicals involved.

METHODS AND MATERIALS

Insects. C. sonorensis were reared from a laboratory culture of tobacco budworm [*Heliothis virescens* (F.)] larvae maintained on a diet described by Vanderzant et al. (1962). Adult parasitoids were caged in 600-ml plastic cartons covered with nylon screen and held at 26 $\pm$ 2° C in a 16:8 light–dark photochamber, and fed a 50% honey-distilled water solution on cotton pads. Mated, postovipositional, 3 to 10-day-old female *C. sonorensis,* chosen randomly from the culture, were used in all bioassays.

Bioassays. The response of *C. sonorensis* females to crude cotton extract and fractions of extracts was assessed by the Petri-dish bioassay and Petri-dish Y-tube bioassay described previously (Elzen et al., 1983). In the Petri-dish bioassay, five parasitoids were observed for 3 min with the number of antennal palpations lasting longer than 1 sec and the number of ovipositor probes being recorded. The Petri-dish Y-tube bioassay consisted of counting the number of parasitoids (total = 15) which entered either side of the Y-tube openings in a 3-min period. When extracts, fractions, or pure chemicals were tested, they were applied to 3 $\times$ 6-mm pieces of white felt placed in the bioassay chambers.

Chemical Extraction and Isolation. Freshly collected flowers, terminals, and buds (1 kg) of greenhouse-grown cotton, cultivar Stoneville 213, were surface washed for 3 hr in two 1-liter volumes of ethyl ether (pesticide analysis grade, Burdick and Jackson Laboratories). The washes were pooled

and vacuum evaporated at 27°C to near dryness. Procedures developed for purifying labile aldehydes in cotton, such as gossypol and the heliocides, were used. The residue was chromatographed over 20 g of Florisil, 60/100A, in a 2 × 45-cm column using EtOAc–hexane–HOAc(10:90:0.25) as the developing solvent (Stipanovic et al., 1978). The first 80 ml of yellow eluate from the column was vacuum evaporated at 27°C and redissolved in 10 ml of ethyl ether. This fraction was analyzed on a Tracor 550 gas chromatograph with flame ionization detector, modified with an all-glass variable split-ratio collection system. Separations were obtained on a 1.83-m × 4-mm-id glass column of 5% OV-101 on Chromosorb G, 80–100 mesh, nitrogen flow rate of 60 ml/min, run isothermally at 160°C. Individual peaks for bioassay or identification were collected in 40-cm × 1-mm-id glass capillary tubes under liquid nitrogen. Peaks were rechromatographed on a 1.83-m × 4-mm-id glass column of 5% OV-275 on Chromosorb G, 80–100 mesh at 120°C at previously noted conditions in the Tracor gas chromatograph.

Chemical Identification. Mass spectral data were obtained with a Hewlett-Packard 5995B gas chromatographic–mass spectrometer with a 1.83-m × 2-mm-id column of 3% OV-101 on gas chrom Q with a 3-min delay at 100°C, programed at 5°C/min to 270°C, and a helium flow rate of 20 ml/min. Some data were taken on a Finnigan 1020 GC-MS with a 1.83-m × 2-mm-id column of 5% OV-101 on Chromosorb 750, 100–120 mesh, helium flow rate 20 ml/min, with a 2-min delay at 80°C, programed at 20°C/min to 180°C.

Nuclear magnetic resonance data were obtained with a JEOL FX90Q FT NMR with 1.7-mm microprobe on individual peaks eluted from collection tubes in 25 μl $CDCL_3$. A Bruker 500 FT NMR was used to obtain the 500-MHz nuclear magnetic resonance spectrum.

Commercial standards for spectral confirmation and use in bioassays were purchased from the Givaudan Corporation (β-caryophyllene oxide), CA Aromatics Company (β-caryophyllene, γ-bisabolene), Fluka A.G. (humulene), obtained as gifts, or synthesized.

RESULTS

Bioassays of 10-μl aliquots of crude cotton ethyl ether extract and 10-μl aliquots of cotton Florisil column eluate from extracts resulted in positive searching behavior (Table 1) similar to behavior shown by *C. sonorensis* toward fresh cotton buds (Figure 1 and Elzen et al., 1983). Subsequent fractionation by gas chromatography revealed several peaks (Figure 2),

TABLE 1. RESULTS OF PETRI-DISH BIOASSAY OF *C. sonorensis* RESPONSE TO COTTON EXTRACTS AND FRACTIONS

Sample[a]	Antennal palpations[b]
Cotton extract	20.0 ± 4.1 (5)
Column eluate	21.6 ± 2.9 (5)
GC-0	0 (5)
GC-0′	11.1 ± 3.8 (8)
GC-1	10.1 ± 5.0 (8)
GC-2	6.3 ± 2.1 (8)
GC-2′	17.1 ± 2.2 (8)
GC-3	12.8 ± 4.7 (8)
GC-4	10.8 ± 2.3 (8)
Ethyl ether	0 (9)

[a]Mean ± standard deviation (*N*).
[b]10-μl samples applied to white felt.

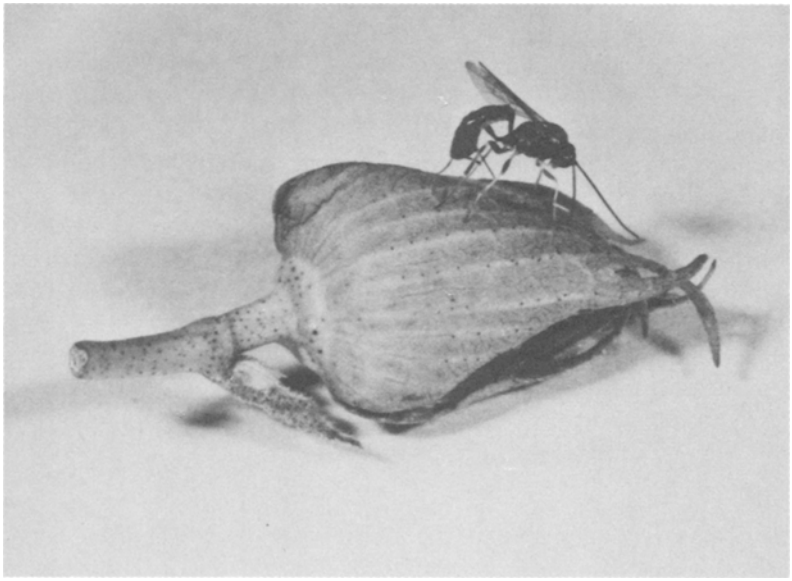

FIG. 1. Female *C. sonorensis* searching a host-free cotton bud.

some of which were active on bioassay (Table 1). The probing behavior previously reported (Elzen et al., 1983) was not stimulated by the fractions collected, further supporting the previous assumption that this response by the parasitoid was due to contact chemicals. Retention times for observed peaks on two different GC columns are shown in Table 2. The single

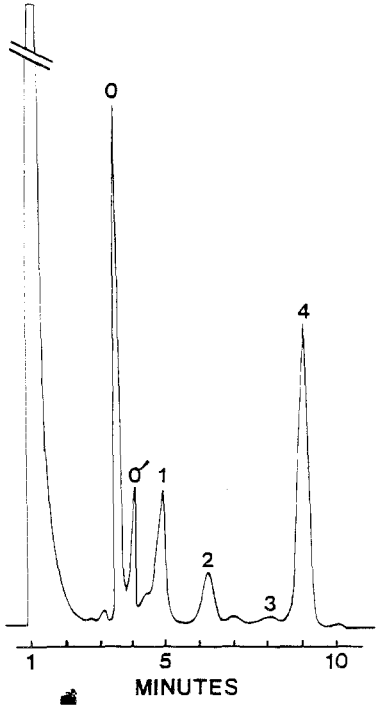

FIG. 2. GC trace of cotton extract.

TABLE 2. RETENTION TIMES OF COTTON EXTRACT PEAKS ON TWO GC COLUMNS

Peak	Retention Time (min.)	
	OV-101	OV-275
0	3.6	0.8
0′	4.1	1.2
1	4.9	1.4
2	6.5	4.0
2′		5.4
3	7.8	9.2
4	9.1	16.0

symmetrical peak 2 was resolved into two completely separated fractions on OV-275. Bioassay data for peak 2′ from OV-275 are included in Table 1.

Peak 0. The mass spectrum of peak 0 had a molecular ion at 204 and compared favorably with the spectrum shown in Heller and Milne (1978) for (C_{15} H_{24}) compound 87-44-5, β-caryophyllene. The [^{1}H]NMR spectrum was identical to that shown by Warnoff and Srinivasan (1973) and Bohlmann

and Zdero (1978) for β-caryophyllene. Spectral and chromatographic properties of β-caryophyllene purchased from C.A. Aromatics Co. were identical to those of peak 0.

Peak 0'. The mass spectrum of peak 0' was similar to α-humulene ($C_{15}H_{24}$, compound 6753-98-6), shown in Heller and Milne (1978), with a molecular ion at 204. [^{1}H]NMR and [^{13}C]NMR (Table 3) data were congruent with those of α-humulene (Figure 3) shown by Thompson et al. (1971) and Dev (1960) and those of an authentic sample purchased from Fluka A.G.

Peak 1. The mass spectrum of this peak showed a molecular ion at 204. The [^{1}H]NMR spectrum of this peak compared favorably with that shown by Minyard et al. (1966) for γ-bisabolene (Figure 3). The [^{13}C]NMR spectrum (Table 3) matched that of γ-bisabolene ($C_{15}H_{24}$) purchased from C.A. Aromatics Co.

Peak 2. There was good correlation between the mass spectrum (M^+220) of peak 2 and that of β-caryophyllene oxide (Figure 3) purchased from Givaudan Corp. A molecular formula of $C_{15}H_{24}O$ was indicated. The [^{13}C]NMR (Table 3) matched that presented by Bohlmann and Zdero (1978), and the [^{1}H]NMR matched that shown by Warnoff and Srinvasan (1973).

Peak 2'. The mass spectrum of peak 2' showed a parent peak at 220. An [^{1}H]NMR taken at 90 MHz was too complicated for easy interpretation. The [^{13}C]NMR spectrum (Table 3) indicated the possible presence of a

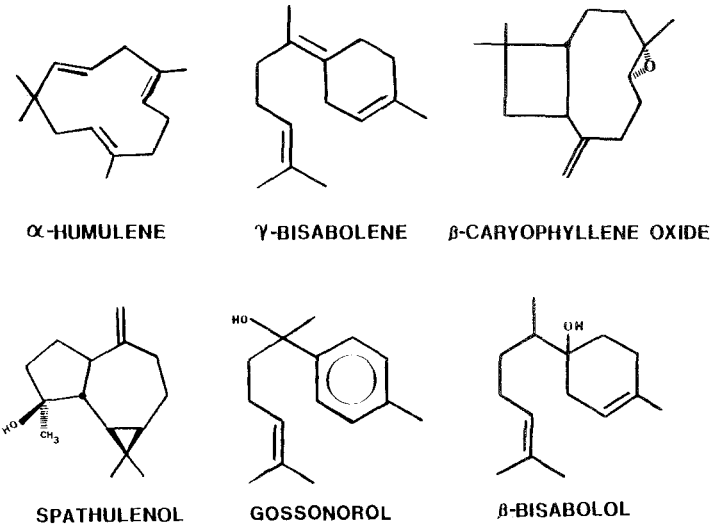

FIG. 3. Structures of active compounds isolated from cotton.

TABLE 3. [^{13}C]NMR DATA

Peak	Humulene (peak 0')	Bisabolene (peak 1)	Caryophyllene oxide (peak 2)	Spathulenol (peak 2')	Gossonorol	Bisabolol (peak 4)
1	140.9	134.0	151.2	153.3	144.8	133.8
2	138.9	131.2	112.1	106.1	135.7	131.1
3	133.0	128.4	62.7	80.8	131.6	124.7
4	127.6	125.6	58.7	54.3	128.6	118.4
5	125.9	124.5	50.6	53.3	124.5	72.0
6	124.9	120.9	48.2	41.7	124.2	41.9
7	42.0	34.4	39.4	38.8	74.5	34.3
8	40.4	31.5	38.8	29.9	43.6	30.9
9	39.7	29.3	33.4	28.6	30.2	30.9
10	37.2	26.9	29.7	27.5	25.4	27.0
11	27.0	26.7	29.6	26.6	22.8	26.5
12	23.3	25.5	29.4	25.9	20.7	25.5
13	17.8	23.2	26.8	24.7	17.4	23.1
14	15.0	17.7	21.2	20.2		17.5
15		17.5	16.5	16.2		13.5

tertiary alcohol. A 500-MHz [^{1}H]NMR was obtained (Figure 4) and the structure of spathulenol was deduced from it. A formula of $C_{15}H_{24}O$ was indicated. Since proton spectra are not well reported for this compound in the literature, [^{1}H]NMR assignments are shown in Figure 4. The mass spectrum was in good agreement with that presented by Motl et al. (1978)

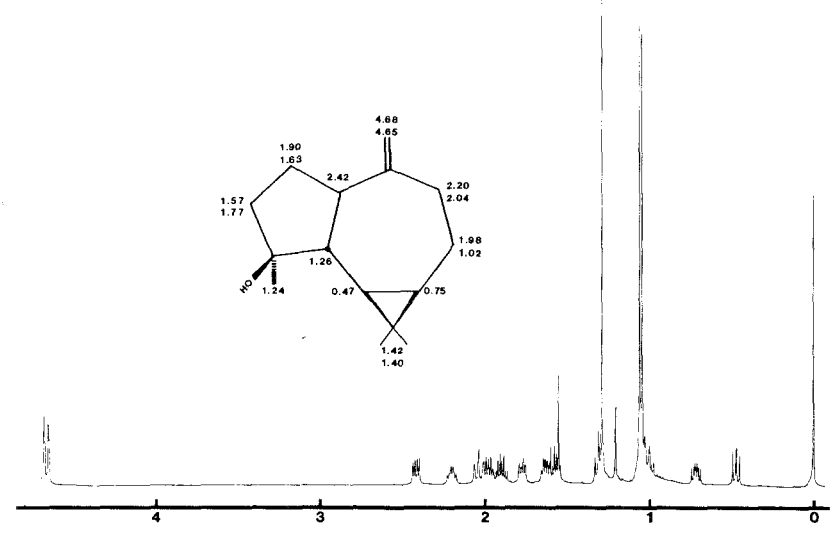

FIG. 4. 500-MHz [^{1}H]NMR of spathulenol with peak assignments.

and the ^{13}C spectrum compared well with that of Juell et al. (1976). An authentic sample of spathulenol provided by Dr. F. Bohlmann was identical in spectral and chromatographic properties with component 2'.

Peak 3. The mass and [^{1}H]NMR spectra (90 MHz) of compound 3 are shown in Figures 5 and 6. The mass spectrum showed a parent peak at 218. From the [^{1}H]NMR spectrum, an AB pattern at low field indicated a *para*-disubstitued benzene moiety. The broad one-proton multiplet at 5.11δ indicated that a triply substituted double bond was present. The methyl singlet at 2.34δ indicated a methyl attached to a benzene ring. The methyl singlet at 1.66δ indicated methyl attached to a tertiary alcohol carbon, and the six hydrogen singlet at 1.53δ indicated two methyls on a double bond. The broad NMR peak centered at 1.9δ could be attributed to two adjacent methylenes. The mass spectrum showed a base peak at 135, which indicates the substituted benzylic fragment $(CH_3C_6H_4C(CH_3)OH)^+$. The structure 2-*p*-tolyl-6-methylhept-5-en-2-ol (Figure 3) would fit all spectral data. A molecular formula of $C_{15}H_{22}O$ was indicated. This compound was prepared essentially as reported previously (Manjarrez and Guzman, 1966) by a Grignard reaction between *p*-bromotoluene and 6-methyl-5-heptene-2-one, except that the reaction was quenched by addition of one equivalent of tetrasodium EDTA in water. The ether extract separated by GC yielded >98% of a single component identical in all spectral and chromatographic qualities to peak 3.

Peak 4. The mass spectrum of peak 4 showed a molecular ion at 222, in

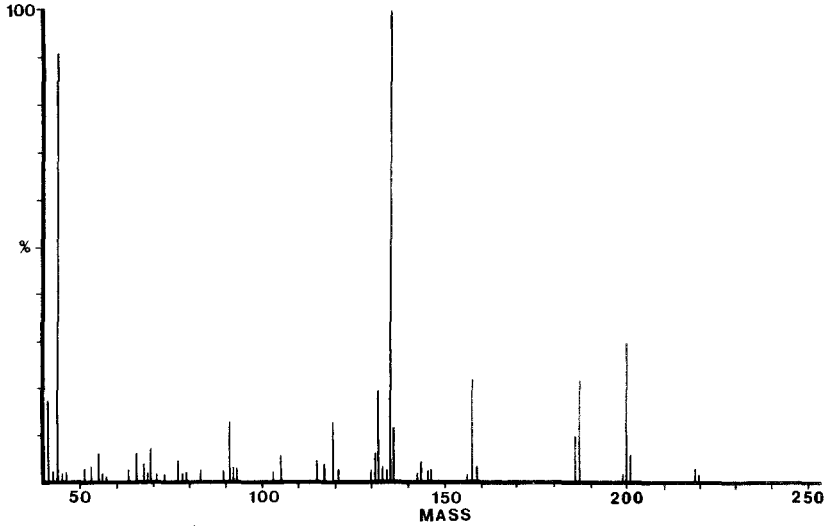

FIG. 5. Mass spectrum of gossonorol.

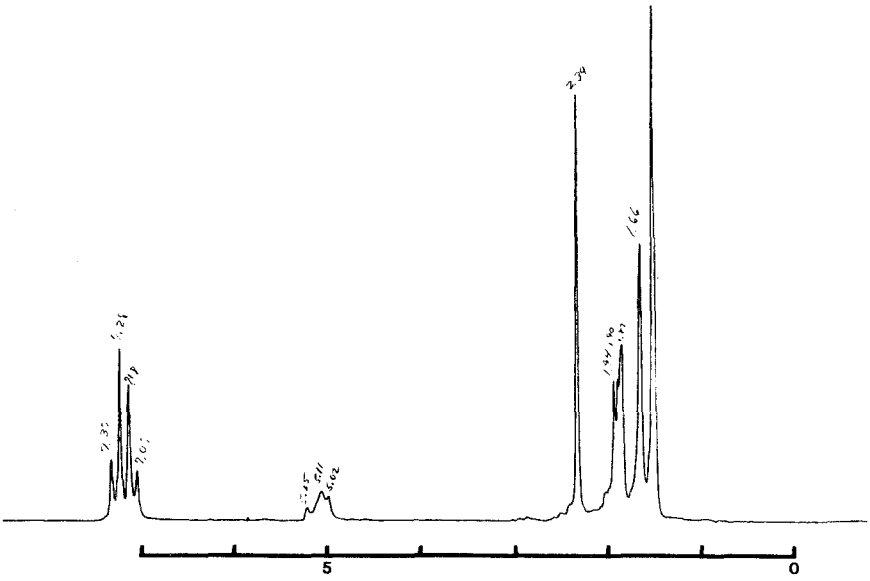

FIG. 6. 90-MHz [^{1}H]NMR of gossonorol.

good agreement to the mass spectrum in Heller and Milne (1978) for β-bisabolol (Figure 3), 1-(1,5-dimethyl-4-hexenyl)-4-methyl-3-cyclohexen-1-ol. The [^{1}H]NMR spectrum for this peak matches that given by Minyard et al. (1968) for β-bisabolol ($C_{15}H_{26}O$). The [^{13}C]NMR spectrum is given in Table 3.

Additional Bioassays. Futher characterization of the behavior by *C. sonorensis* to dilutions of standard commercial sesquiterpenes, spathulenol, synthetic peak 3, and naturally occurring β-bisabolol (purified as described by Minyard et al., 1968) produced the results shown in Table 4. These results confirm, to a reasonable degree, the inactivity of β-caryophyllene in eliciting parasitoid responses. Caryophyllene oxide was weakest among all compounds tested in eliciting close range antennal searching. The most active compound was spathulenol (Table 4), which is the most interesting structually (Figure 3), consisting of 7-, 5-, and 3-membered rings. Only mixtures of all compounds at observed optimum concentrations gave responses by *C. sonorensis* similar to those obtained to cotton extracts (Table 1).

Chemicals found in cotton buds in this experiment were tested for activity in the Petri-dish Y-tube olfactometer described previously (Elzen et al., 1983). Test results (Table 5) indicate that γ-bisabolene, β-caryophyllene oxide, and GC peak 3 attract *C. sonorensis* females from a short distance. The greatest number of parasitoids entering the test side of the Y-tube was

TABLE 4. RESULTS OF PETRI-DISH BIOASSAY OF DILUTIONS OF COMMERCIAL, SYNTHETIC, AND ONE NATURAL SESQUITERPENE HYDROCARBON

Sample[a]	Optimum conc. (μg)	Antennal palpations[b]
β-Caryophyllene		0
α-Humulene	5	12.8 ± 3.8 (8)
γ-Bisabolene	5	11.8 ± 3.0 (8)
β-Caryophyllene oxide	5	5.1 ± 2.2 (8)
Spathulenol	2	16.8 ± 2.2 (8)
Gossonorol	2	13.1 ± 2.0 (8)
GC-β-Bisabolol	1	11.7 ± 1.9 (8)
All terpenes except caryophyllene	As noted above	20.4 ± 2.8 (8)

[a]Mean ± SD (*N*).
[b]Source noted in text.

TABLE 5. RESULTS OF PETRI-DISH Y-TUBE BIOASSAY OF COTTON CHEMICALS

	No. entering tube[b]	
Sample[a]	Test side	Solvent blank side
Column eluate	8.8 ± 1.1 (8)	0
β-Caryophyllene	0 (5)	0
α-Humulene	0.7 ± 0.9 (7)	0.1 ± 0.4
γ-Bisabolene	7.9 ± 1.9 (7)	0
β-Caryophyllene oxide	3.7 ± 1.4 (7)	0
Spathulenol	0 (7)	0.1 ± 0.4
Gossonorol	5.1 ± 1.8 (7)	0
β-Bisabolol	0.6 ± 1.1 (7)	0.4 ± 0.5
All terpenes except caryophyllene	9.0 ± 1.8 (10)	0

[a]Mean ± SD (*N*).
[b]Source noted in text.

seen when all chemicals having apparent contact or short-range attractiveness were combined.

DISCUSSION

The terpenes reported herein, with the exception of gossonorol and spathulenol, have been previously reported in cotton essential oil. Humulene and γ-bisabolene were isolated from smoothleaf cotton (Deltapine) by Minyard et al. (1966), β-bisabolol from the same variety by Minyard et al.

(1968), and β-caryophyllene and β-caryophyllene oxide were isolated from that variety by Thompson et al. (1971). Hedin et al. (1972) stated that β-bisabolol was the major volatile alcohol in cotton; we found this to be the case in our isolates (Figure 2). These compounds were previously isolated as part of a series of experiments to identify cotton constituents attractive to the boll weevil, *Anthonomus grandis* Boheman (Gueldner et al., 1970). We have shown in this report that some of the sesquiterpenes present in cotton, and previously reported, are attractants or searching stimulants for the parasitoid *C. sonorensis* in the laboratory.

The compound isolated from peak 3 (gossonorol) and identified as 2-*p*-tolyl-6-methylhept-5-en-2-ol, has not, to our knowledge, been found previously in nature. The name gossonorol, derived from gossypium and sonorensis, is proposed for this structure. It was previously used in the synthetic scheme of γ-bisabolene (Manjarrez and Guzmán, 1966), α-curcumene (Hall et al., 1975), and curcumene ether (Mashraqui and Trivedi, 1978). This terpene has a heavy, slightly floral scent, somewhat reminiscent of commercial air-freshner.

Spathulenol has been reported in the essential oil of *Artemisia vulgaris* L. and *Artemisia dracunculus* L. (Juell et al., 1976) and in chamomile oil (Motl et al., 1978). Spathulenol has been synthesized (Surburg and Mondon, 1980) and its structure deduced (Bowyer and Jefferies, 1963). The recovery of spathulenol from cotton by our procedure is apparently the first report of the compound in *Gossypium* spp.

In field tests β-caryophyllene was an attractant for adult predatory green lacewings, *Chrysopa carnea* Stephens, whereas caryophyllene oxide was not. In contrast, the predator *Collops vittatus* Say was caught in traps containing caryophyllene oxide but was not caught in significant numbers in traps containing β-caryophyllene (Flint et al., 1979). *C. sonorensis* females also responded to β-caryophyllene oxide, although weakly in comparison to other compounds tested, and showed no response to β-caryophyllene. Hedin et al. (1973) noted that caryophyllene was a major component in the aroma of a cotton field. The atmosphere above cotton plants was also shown to contain 50–60% β-bisabolol and γ-bisabolene, and 30–40% other terpenes and β-caryophyllene oxide (Thompson et al., 1971). Since parasitoids responded to these latter chemicals in laboratory bioassays, we could speculate on their possible importance to certain parasitoids in a field situation. Supplemental releases of *C. sonorensis* in cotton have shown promise for controlling the tobacco budworm (Lingren, 1977). The possibility of increasing parasitization rates by manipulation of synomones (if not in this case, then perhaps others) is strongly suggested by these data and by previous studies (Altieri et al., 1981).

Reports of parasitoid attraction to known plant secondary metabolites

are infrequent in the literature. As previously noted, bark beetle parasitoids were attracted by the monoterpene, α-pinene, after a southern pine beetle attack, although some interaction with sex pheromones was involved (Camors and Payne, 1972). *Diaeretiella rapae* (Sulzer), a parasitoid of aphids on crucifers, was attracted to mustard oil (allyl isothiocyanate) present in collards. The parasitoid preferred collard over beet, which does not contain mustard oil (Read et al., 1970). While these studies are good examples of parasitoid attraction to plant-produced chemicals, the compounds tested were not arrived at through systematic bioassay, isolation, and identification of attractive factors; rather, they were commercial products reported to be major constituents of the plants on the basis of the chemical literature. Other chemicals could be important in attraction as well. We have identified several cotton chemicals which influence the behavior of *C. sonorensis* and ascertained that several chemicals are involved in the natural state. Other types of chemicals may be involved, particularly in the probing behavior shown previously, and possibly in the case of attraction to sorghum (Elzen et al., 1983). Preliminary GC results indicate that active sorghum extracts contain none of the compounds identified from cotton. Thus, another group of chemicals may elicit parasitoid searching as well. We plan to resume fractionation of attractive sorghum extracts in the near future.

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A *Caenorhabditis elegans* DAUER-INDUCING PHEROMONE AND AN ANTAGONISTIC COMPONENT OF THE FOOD SUPPLY

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Abstract—The free-living soil nematode *Caenorhabditis elegans* forms a nonfeeding dispersal stage at the second molt called the dauer larva when exposed to environmental cues indicating crowding and limited food. An improved bioassay, tenfold more sensitive than that used previously, has been used in the characterization of the two chemical cues which act competitively in controlling this developmental process. The pheromone concentration provides a measure of the population density; it enhances dauer larva formation, and inhibits recovery (exit) from the dauer stage. The pheromone is a family of related molecules which are nonvolatile, very stable, and possess physical and chromatographic properties similar to those of hydroxylated fatty acids and bile acids. A food signal, with effects on development opposite those of the pheromone, is produced by bacteria, and is also present in yeast extract. In contrast to the pheromone, the food signal is a labile substance which is neutral and hydrophilic.

Key Words—*Caenorhabditis elegans*, pheromone, nematode, dauer larva, development.

INTRODUCTION

Caenorhabditis elegans is a free-living, soil nematode used as a simple animal model for the study of a wide range of biological problems (for reviews, Riddle, 1978; Zuckerman, 1980). Under favorable growth conditions, feeding on bacteria, *C. elegans* passes through four larval stages (L1 through L4) after

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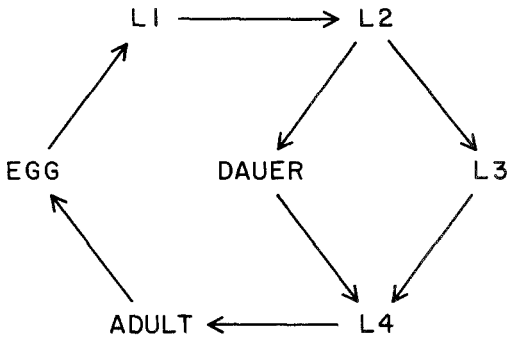

FIG. 1. Life cycle. Under favorable growth conditions, *C. elegans* passes through four larval stages, L1–L4, to reach the adult. High population density and limited food induce dauer larvae, which form at the second molt. When dauer larvae are placed in fresh medium, they recover, resume development, and molt into L4 larvae.

hatching from the egg, to reach a hermaphroditic adult in approximately three days (Byerly et al., 1976). Each self-fertilizing hermaphrodite produces 200–300 progeny. Conditions of high population density and a low food supply result in the appearance of a specialized larval form at the second molt called the dauer (German: enduring) larva (Figure 1). The dauer larva is a developmentally arrested, nonfeeding dispersal form adapted to prolonged survival in a harsh environment (Cassada and Russell, 1975; Klass and Hirsh, 1976). The environmental factors which influence development of the dauer larva include a pheromone, the food supply, and temperature (Golden and Riddle, 1982; Golden and Riddle, 1984a).

The concentration of the dauer-inducing pheromone apparently serves as a measure of the population density; higher concentrations enhance dauer larva formation and inhibit recovery (exit) from the dauer stage. At least two *Caenorhabditis* species (*C. elegans* and *C. briggsae*) produce a common pheromone activity, while other soil nematodes produce a pheromone not active on these *Caenorhabditis* species (Golden and Riddle, 1982). The *Caenorhabditis* pheromone is produced throughout the life cycle. Efficient induction of dauer larva formation requires continuous exposure to the pheromone after the middle of the first larval stage. Dauer-inducing conditions produce a prolongation of the L2 intermolt period, and result in a morphologically distinct L2 larva called the L2d (Golden and Riddle, 1984b).

Food-signal activity, produced by *E. coli* and found in yeast extract, has effects opposite that of the pheromone; it inhibits dauer larva formation and enhances recovery (Golden and Riddle, 1982). The worms' response is determined by the relative concentration of the two chemical cues, each of which is capable of exerting its influence in the absence of the other.

Sex-attractant pheromones have been described for a number of

nematode species (Anya, 1976; Ward and Bone, 1983), and pheromone-based biological control of *Nippostrongylus* and *Heterodera* has been proposed (Bone and Shorey, 1977; Rende et al., 1982). Pheromones affecting nematode larval development may offer additional potential for use in biological control, and *C. elegans* may be a useful laboratory model for the study of pheromone production and response. We report here the improvement of a rapid and convenient dauer larva recovery bioassay for the *Caenorhabditis* dauer-inducing pheromone and its use in the study of the chemical and physical properties of the pheromone and a competitive food signal.

METHODS AND MATERIALS

Nematode Growth. General growth conditions for *C. elegans* strain N2 have been described (Brenner, 1974). Pheromone was obtained from 200–1000 ml fully grown (4- to 7-day-old) liquid cultures of nematodes, grown in 1- or 2.8-liter Bellco baffled flasks on a rotary shaker at 20°C until the bacterial food supply was exhausted. The supernatant fraction from these cultures is called "depleted culture medium." The food source was a 5% (w/w) suspension of *E. coli* strain X1666 in S medium (Sulston and Brenner, 1974).

Pheromone Bioassay. The bioassay (Golden and Riddle, 1982) was modified to improve its sensitivity. The sample to be assayed was diluted in a fourfold series with M9 buffer (Brenner, 1974), and 30 μl of each dilution were placed into a well of a microtiter dish. Assays always included M9 buffer alone and a six-step dilution series (1/4 to 1/4096) of filter-sterilized, unfractionated depleted culture medium as controls.

N2 dauer larvae, for use in the bioassay of both pheromone and food signal, were obtained from 5-ml liquid cultures (as above) incubated in 18 × 150-mm test tubes on a rotary shaker at 20°C. Cultures were started daily with an inoculum of 20–100 worms obtained from an NG agar plate (Brenner, 1974). Dauer larvae were harvested from 12- to 14-day-old cultures and were prepared for the bioassay by layering 0.5 ml of the culture on top of 1 ml of 15% (w/w) Ficoll (in 0.1 M NaCl) in a 5-ml conical tube. Dauer larvae settled into the lower solution in 5 min, while nondauer, starved worms and debris remained at the interface. The upper layer and interface were removed by aspiration, and the dauer larvae in the lower layer were washed three times with water by low-speed centrifugation. Approximately 20 purified dauer larvae were dispensed into each assay well, suspended in 30 μl of an autoclaved solution of 0.05% Bacto yeast extract in M9 buffer. The microtiter dish was incubated at room temperature, 23°C, for 4 hr and then each assay well was visually scored for inhibition of dauer larva recovery with the use of a Wild M5 stereomicroscope.

Food-Signal Bioassay. Thirty microliters of a fourfold dilution series of

sample in M9 buffer was placed into microtiter wells. M9 buffer alone, and a dilution series of autoclaved 1% yeast extract served as controls. Dauer larvae from 9- to 10 day-old cultures were prepared as for the pheromone bioassay, but added to the assay wells in M9 buffer containing 0.1% filter-sterilized depleted culture medium (as used in the pheromone bioassay), without added yeast extract. The assay was incubated and scored as in the pheromone bioassay except that a sample was scored for stimulation of recovery.

Pheromone Extraction. A crude preparation of pheromone was obtained from depleted liquid culture medium by organic extraction. One to five liters of medium was reduced in volume by drying under a stream of air at 100° C, and then centrifuged at 10,000*g* for 10 min. The supernatant was completely dried in a vacuum oven at 60° C. This residue was extracted 4–6 times with 95% ethanol until the extract was only slightly colored. The extracts were combined and dried under a stream of air at 60° C. Unless otherwise stated, this oily residue served as the starting material for further analysis. Pheromone was extracted into water for experiments on stability and physical properties or extracted into the appropriate solvent for application to chromatography columns or thin-layer plates.

Analytical Procedures. In experiments testing the stability of the pheromone and food signal to the various physical and chemical treatments described in Results, the samples were monitored with the appropriate bioassay along with untreated controls. Solubility of the pheromone and food signal in various solvents was determined by partitioning between the aqueous and organic phases. The two phases were separated, dried, and resuspended into M9 buffer before bioassay.

Fractions were collected from column chromatography of the food signal, and directly tested for activity in a bioassay. Column chromatography of the pheromone using organic solvents required that the solvent first be evaporated from each fraction under a stream of air at 60° C. The residue was resuspended with M9 buffer and tested in the pheromone bioassay.

For thin-layer chromatography (TLC) of both the pheromone and the food signal, the developed thin-layer plate was dried in a vacuum oven at 80° C for 1 hr to remove traces of solvent, then sections (usually 1 cm) of adsorbent were scraped from the plate, placed into 13 × 100-mm test tubes, and M9 buffer was added to just cover the adsorbent (typically 0.2–0.5 ml). This suspension was vortexed a number of times over a period of 10–20 min and the adsorbent was pelleted by centrifugation at 2000*g* for 5 min. Samples from the supernatant were assayed for activity in the appropriate bioassay. The Eastman plastic-backed chromagram sheets (Table 3) were cut into strips, which were placed into test tubes and eluted similarly to the adsorbent scraped from glass plates. The R_f ranges given in Tables 4 and 5 represent the sections of the TLC plate positive for activity.

Paper electrophoresis was used to confirm the pheromone's negative

charge. A sample was applied to the center of a 1.5 × 35-cm strip of Whatman 3-mm paper saturated with buffer (80 mM Tris base, 10 mM sodium acetate, 3 mM Na_2EDTA, adjusted to pH 8.05 with acetic acid), and subjected to 250 V for 50 min. Bromophenol blue was used as a marker. Glycine was included as a control for endoosmosis and visualized with ninhydrin. Fractions were tested for pheromone activity by eluting sections of the strips into M9 buffer, then testing aliquots in the pheromone bioassay.

Food-Signal Purification. Food signal was partially purified from yeast extract on a relatively small scale to obtain material for analysis by TLC. The starting material was 1 g of Bacto yeast extract dissolved in 10 ml of water. Lipids were extracted with three sequential organic extractions, one volume of each: chloroform, diethyl ether, and ethyl acetate. The aqueous phase was deionized by passing through a 10-g Dowex 1 × 8 column followed by a 30-ml water wash. The eluate was then passed over a 10-g Dowex 50W × 4 column followed by a 30-ml water wash. The combined eluate was then passed through a 2-g DEAE cellulose column followed by 30 ml of water. The final column eluate, about 100 ml, was freeze-dried and resuspended with 5 ml water for use in subsequent chromatography experiments.

Food signal also was partially purified from bacterial cultures. Two liters of supernatant obtained from an overnight culture of *E. coli* X1666 was lyophilized and resuspended in 400 ml water. This was autoclaved, cooled on ice, and centrifuged at 10,000*g* for 10 min. The supernatant was extracted three times with chloroform–methanol (2:1), and the aqueous phase was lyophilized. The residue was resuspended in 100 ml water, centrifuged at 10,000*g* for 10 min, and the supernatant was collected and stored at 2° C until used as sample for column chromatography.

Materials. Thin-layer plates were Analtech silica gel H, 250 μm layer; microcrystalline cellulose, 250 μm layer; and Kodak, Eastman chromagram sheets, 13179 silica gel. Column chromatography materials included Whatman DE-52 microgranular cellulose, Dowex 1 × 8-400 (Sigma), Dowex 50W × 8-200 (Bio-Rad), EM Reagents silica gel 70–325 mesh, Sephadex G10-120 and G25-150 (Sigma), Bio-Gel P2 (Bio-Rad), and Sigma boric acid gel. Chloroform and methanol were Mallinckrodt nanograde, and other organic solvents and all chemicals were reagent grade. Enzymes were from Sigma. Difco Bacto yeast extract was used as the source of food-signal activity.

RESULTS

Dauer-Inducing Pheromone

Improved Bioassay. The dauer-inducing pheromone is assayed by its inhibition of dauer larva recovery in the presence of a standardized amount of food signal (Golden and Riddle, 1982). Pheromone activity is measured by

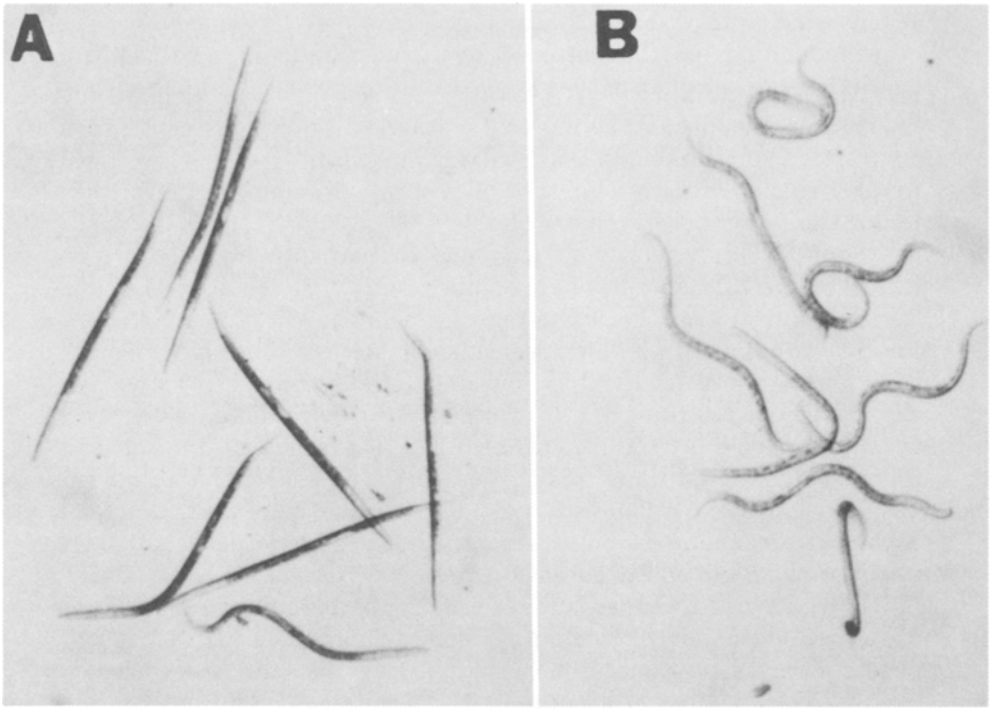

FIG. 2. Dauer larva recovery bioassay. (A) *C. elegans* dauer larvae incubated in the presence of 0.05% yeast extract and the dauer-inducing pheromone are inhibited from recovering, retain their thin, dark appearance, and usually remain rigid and motionless. (B) Dauer larvae incubated in the absence of added pheromone recover to resume development within 4 hr, increase movement, and begin feeding. (Worms are approximately 400 μm in length.)

testing a dilution series of a pheromone sample for the greatest dilution which inhibits recovery (Figure 2). The bioassay is semiquantitative; fourfold differences in pheromone concentration were reliably detected. The sample dilution series typically produced assay wells scored as positive or negative, separated by a single assay well scored as a "borderline" response, in which some dauer larvae were inhibited from recovery and some were not. The number of units of pheromone activity in a sample was calculated as the reciprocal of the final dilution that produced the borderline response (Golden and Riddle, 1982). The ratio of pheromone to food signal, and not the absolute amounts of either stimulus, influences dauer larva recovery. The bioassay as described here is tenfold more sensitive than the original assay and allows detection of pheromone activity at a 1/2000 dilution of exhausted liquid culture medium.

The sensitivity of the bioassay has been improved by changes in the preparation and age of the dauer larvae, and the food-signal source. For

sensitive and reproducible assays, both the age of the dauer larvae and their growth conditions must be controlled. The liquid cultures were started with a relatively small inoculum of 20–100 worms and incubated at 20° C for 12–14 days to provide dauer larvae which worked well in the pheromone bioassay. Dauer larvae become progressively more predisposed to recovery during the 1- to 2-week period after they are formed, so that the apparent sensitivity to pheromone decreases over 1000-fold during this period (Golden and Riddle, 1984b). Sensitivity decreases fivefold between days 10 and 14. Growth on *E. coli* strain X1666 was found to provide dauer larvae better suited to the bioassay than growth on the uracil auxotroph, OP50, which is used as food on NG agar plates. It was important to avoid contamination of the cultures from which dauer larvae were obtained; mold or bacterial contamination adversely affected the suitability of the dauer larvae for the recovery bioassay. An autoclaved solution of 0.05% yeast extract in M9 buffer (one tenth the concentration previously used) was used as the source of food signal in the bioassay and was found to retain activity for at least one month when stored at 4° C.

Physical Properties. The bioassay was used to measure pheromone activity present in samples during the determination of the pheromone's physical properties and its partial purification. Filter-sterilized depleted culture medium (see Methods and Materials) was assayed for pheromone activity before and after the various treatments. The semiquantitative nature of the pheromone bioassay allows the detection of a fourfold loss in activity. Pheromone activity is not volatile; it is stable to heat, treatment with acid or base, and evaporation to dryness under a stream of air at 100° C (Golden and Riddle, 1982). It remains stable in filter-sterilized, depleted culture medium stored at room temperature for over one year. The pheromone is not degraded by incubation in cultures of *E. coli* or in month-old *C. elegans* cultures. The following enzymes do not detectably affect pheromone activity when added to a concentration of 1% and incubated for 2 hr at 37° C: proteinase K, RNase, A, DNase I, phosphodiesterase, alkaline phosphatase, and carboxypeptidase A. The pheromone is also stable to treatment with 1% periodic acid. These results suggest that the pheromone may be very stable in the natural soil environment.

The pheromone was found to be soluble in water and some organic solvents. Pheromone could be obtained from dry residue at intermediate steps during purification by extraction into organic solvents including 95% ethanol, acetone, chloroform, and chloroform–methanol (2:1). Chloroform extracted the pheromone least efficiently. At neutral pH, the majority of the pheromone activity partitioned to the aqueous phase in biphasic solutions of water and diethyl ether, chloroform, heptane, cyclohexane, isopropyl ether, or petroleum ether. It was partially extracted into organic phases of ethyl acetate and chloroform–methanol (2:1). If biphasic water and diethyl ether, or

chloroform–methanol (2:1) solutions were adjusted to a pH less than 2, the majority of the pheromone partitioned to the organic phase, presumably because of the neutralization of a negative charge. The pheromone's negative charge was confirmed by its electrophoretic mobility in comparison with an uncharged control. Pheromone activity moved toward the anode when subjected to paper electrophoresis at pH 8, but migrated behind a bromophenol blue marker.

Partial Purification. Diethylaminoethyl (DEAE) cellulose anion exchange columns (Kates and Morris, 1975) were used for partial purification of the pheromone. The pheromone sample was an organic extract from depleted culture media or was partially purified by silicic acid column chromatography (see below) before application to the DEAE column. The sample was suspended in chloroform and applied to the column followed by a chloroform wash of five column volumes. Pheromone activity was eluted from the column with ten column volumes of chloroform–methanol (1:1) or with two or three column volumes of chloroform–acetic acid (4:1). DEAE had a relatively low capacity for the pheromone and was easily overloaded, especially when the starting material was a crude 95% ethanol extract. Dowex 1 (anion) and Dowex 50W (cation) ion-exchange resins produced anomalous results; the pheromone was retained on both columns, probably because of the hydrophobic nature of the column matrix.

Table 1 shows that increasing proportions of methanol in chloroform eluted pheromone from a silicic acid column (Kates and Morris, 1975), and that a ratio of 1:1 was needed to elute the major quantity of pheromone from the column. A step gradient was used for routine partial purification of pheromone activity (Table 2). The pheromone sample was applied to the column in chloroform, and the column was washed with additional chloro-

TABLE 1. SILICIC ACID COLUMN CHROMATOGRAPHY OF DAUER-INDUCING PHEROMONE[a]

Fraction[b]	Solvent	Pheromone
1	chloroform	−
2	chloroform–methanol (99:1)	−
3	chloroform–methanol (98:2)	−
4	chloroform–methanol (96:4)	+
5	chloroform–methanol (85:15)	+
6	chloroform–methanol (50:50)	+++
7	methanol	+

[a]Column volume was 1.8 ml in a Bio-Rad 2-ml econocolumn with reservoir. Pheromone sample reextracted into chloroform.
[b]Each fraction was 5 ml of solvent.

TABLE 2. PREPARATIVE SILICIC ACID COLUMN CHROMATOGRAPHY OF PHEROMONE[a]

Fraction[b]	Solvent	Pheromone[c]
1	chloroform	20
2	chloroform	60
3	chloroform–methanol (2:1)	6000
4	chloroform–methanol (2:1)	100
5	chloroform–methanol (2:1)	0
6	methanol	100

[a]Column volume was 1.8 ml in a Bio-Rad 2-ml econocolumn with reservoir. Pheromone sample was from a 95% ethanol extract of depleted liquid medium, reextracted into chloroform.
[b]Each fraction was 10 ml of solvent.
[c]Pheromone units per milliliter, as measured by the original pheromone bioassay procedure.

form. Almost all of the pheromone activity was eluted with the first chloroform–methanol (2:1) fraction. This procedure (Table 2) has been successfully scaled-up approximately 20-fold for the purification of greater quantities of pheromone.

Pheromone partially purified by silicic acid and DEAE cellulose column chromatography was concentrated and used as the sample for thin-layer chromatography (TLC) on silica gel adsorbent. Table 3 shows the R_f values for a number of solvent systems on two types of silica gel. The R_f values are based on the fractions which contained pheromone activity when eluted from the silica gel and tested in the bioassay. The mobility of the pheromone in these solvent systems was similar to that of hydroxylated fatty acids, prostaglandins, or bile acids (Bailey et al., 1977; Eneroth, 1976; Waldi, 1965). The

TABLE 3. THIN-LAYER CHROMATOGRAPHY OF PHEROMONE ON SILICA GEL

Solvent	R_f[a]	R_f[b]
Water		0.64–0.79
Chloroform–methanol–acetic acid–water (90:8:1:0.8)[c]	0.41–0.58	0.13–0.35
Chloroform–acetic acid (90:3)[c]	0.28–0.55	0.01–0.08
Chloroform–methanol–acetic acid (90:5:1)[c]	0.56–0.80	
Chloroform–methanol–acetic acid (90:5:5)[c]	0.56–0.69	0.07–0.22
Chloroform–methanol–water (80:20:1)		0.40–0.66
Chloroform–methanol–28% ammonium hydroxide (80:20:1)		0.01–0.13
Diethyl ether–methanol–acetic acid (90:1:2)[c]	0.56–0.88	0.26–0.43

[a]Eastman chromagram sheet, silica gel with polyacrylic acid binder, 100 μm layer, 2 × 9-cm strips.
[b]Analtech silica gel H, 250 μm layer, 2.5 × 20 cm.
[c]Solvent from Bailey et al. (1977).

pheromone was always found to "streak" and cover a large portion of the plate, when compared to the discrete spots of contaminant substances visualized by illumination with short-wave ultraviolet light. Attempts to visualize the pheromone with various staining techniques included the use of iodine vapor, phosphomolybdic acid, 2',7'-dichlorofluorescein, and chromic–sulfuric acid (Mangold, 1965). In no case were we able to correlate pheromone activity with a visible spot or streak.

The pheromone is a set of related compounds. The results of an experiment designed to test the homogeneity of the pheromone are presented in Table 4. A column-purified pheromone sample was chromatographed on silica gel plates, and fractions were assayed for pheromone activity. The three fractions containing the majority of the pheromone activity were reapplied to three separate lanes on a second TLC plate and chromatographed under the same conditions used for the first TLC. Each fraction retained a distinct R_f corresponding to its original R_f in the first chromatogram, with only a slight spreading of activity (Table 4). This indicates that the pheromone is a heterogeneous mixture of molecular species and that streaking of activity is due to separation of the component activities. There was no noticeable loss of total pheromone in the fractions representing the streak of pheromone activity in this solvent system or any of the other solvent systems used for TLC of the pheromone. Therefore, there is no apparent synergism between the individual species of pheromone, and their activities seem to be simply additive.

Food Signal

Food-Signal Bioassay. The bioassay is based on the stimulation of dauer larva recovery in the absence of added pheromone. Dauer larvae, purified as in the pheromone bioassay, were placed into a solution of M9 buffer con-

TABLE 4. PHEROMONE HETEROGENEITY[a]

Fraction number	R_f
First chromatogram	
4	0.20–0.30
5	0.30–0.47
6	0.47–0.59
Second chromatogram	
4	0.15–0.35
5	0.27–0.51
6	0.45–0.66

[a]Thin-layer chromatography on Analtech silica gel H, solvent was chloroform–methanol (4:1). See text for details.

taining 0.1% filter-sterilized, depleted nematode culture medium and added to the sample to be tested for food-signal activity. Stimulation of recovery was scored as positive for food signal. The dauer larvae used in the food-signal bioassay differed from those used in the pheromone bioassay only by their age. Younger dauer larvae, from cultures 9–10 days old (dauer larvae form on days 3 and 4), were used because they did not recover when placed into control wells. Dauer larvae from cultures older than 11 days were induced to recover simply by their removal from depleted culture medium (Cassada, 1975) even if exogenous food signal was not added. As in the pheromone bioassay, a dilution series of each sample was assayed to provide quantitation of the food-signal concentration in the samples. A dilution series of autoclaved 1% yeast extract and medium from stationary-phase bacterial cultures served as positive controls. Food-signal activity was not stable in aqueous solutions at room temperature, but solutions retained their activity for at least one month when stored at 4°C.

Preliminary Characterization. Food signal activity was detected in a number of commercial bacteriological media including: yeast extract, beef-blood serum, corn meal agar, bean pod agar, and a small amount was detected in peptone. Food signal activity was absent from other media such as: casamino acids, soy peptone, tryptone, potato dextrose agar, gelatin, and powdered skim milk. It is not known whether all food signals are the same compound, or if there are a number of different compounds which produce the same effect. Yeast extract was initially used as the source of food signal for the experiments presented here, although food signal of highest activity was found in medium from bacterial cultures. Food signal found in bacterial cultures also was analyzed, and it has similar physical properties to the food signal found in yeast extract.

The food signal present in yeast extract is unstable in aqueous solution and appears to be easily oxidized. Aqueous solutions stored at room temperature lose 90% of their activity after 1–2 months; this loss is diminished at 4°C. Solutions dried at room temperature or at 100°C lose 90–100% of their activity, respectively. The food-signal activity is stable to treatment with 1 N hydrochloric acid or 1 N potassium hydroxide at 70°C for 1 hr. It is stable to boiling and autoclaving and is not volatile. Treatment with 1% periodic acid at room temperature decreases activity by 90%. The food signal's sensitivity to periodic acid suggests that it may contain a *cis*-diol group, and therefore could potentially bind to a boronate resin affinity column, but activity was not retained on a boric acid gel column.

The food-signal activity is water soluble and is not extracted into organic solvents. It apparently is not strongly charged and is not bound to either anion or cation exchange resins including Dowex 1, DEAE cellulose, Dowex 50W, or Bio-Rex 70. It passes through dialysis tubing and an Amicon UM-2 ultrafiltration filter, indicating that it consists of low-molecular-weight mate-

rial (less than 1000). Gel exclusion chromatography was used to provide a better indication of the food signal's molecular size and as a step in its purification. A sample partially purified by organic extractions and deionization was applied to a Sephadex G10 column with water as the mobile phase. The food-signal activity was found in the void volume along with a blue dextran marker, although a significant portion of the activity was present in fractions immediately following the void volume. This indicates a molecular weight of less than 700. A second sample was applied to a Sephadex G25 column. The food signal was not excluded from G25 and was eluted following the excluded fractions marked with blue dextran. The G25 fractions positive for food signal were pooled and concentrated for analysis by TLC.

Table 5 contains the R_f values for food-signal activity in several different solvent systems on both silica gel and microcrystaline cellulose layers. The food signal's behavior in the various TLC systems was similar to that of pyrimidine nucleosides (Cohn, 1961; Pataki, 1968; Mangold, 1965). In contrast to the pheromone, food-signal activity does not streak on TLC plates.

Food-signal activity obtained from bacterial culture medium has properties very similar to those of the activity present in yeast extract. The bacterial food signal was partially purified by Bio-Gel P2 (exclusion limit 1800) column chromatography using 1 mM ascorbic acid (used as an antioxidant), pH 7.0, as eluant. Fractions containing food-signal activity were pooled, lyophilized, resuspended in 7 ml 10 mM ascorbic acid, and stored at 2° C. Five milliliters of this sample was then applied to a 200-ml silicic acid column and eluted with 1 mM ascorbic acid. Fractions containing food signal were collected as for the Bio-Gel P2 column except the sample was resuspended in 3 ml of water and stored frozen as 1-ml aliquots for TLC. The sample was chromatographed on silica gel H plates with water, and activity was present in fractions with an R_f of 0.56–0.70, while a thymidine standard migrated at an R_f of 0.76. The slight difference in the R_f of the food-signal activity from bacterial cultures in

TABLE 5. THIN-LAYER CHROMATOGRAPHY OF FOOD SIGNAL

Absorbant	Solvent	R_f
Silica gel[a]	water	0.66–0.82
Avicel[b]	ethyl acetate–ethanol–water (12:2:1)	0.00–0.05
Avicel	water	0.90–1.0
Avicel	*n*-butanol–acetic acid–water (6:2:2)	0.33–0.52
Avicel	ethyl acetate–formic acid–water (7:2:1)	0.56–0.65
Avicel	isopropyl alcohol–hydrochloric acid–water (39:10:11)	0.66–0.82

[a]Analtech silica gel H, 250 μm layer, 2.5 × 20 cm.
[b]Analtech microcrystalline cellulose, 250 μm layer, 2.5 × 20 cm.

comparison with that present in yeast extract chromatographed under the same conditions (Table 5) may not reflect a difference in food-signal compounds but might result from differences in the samples due to the different methods of preparation.

The physical properties of the food signal suggested that it may be a nucleoside or a modified nucleoside such as those found in tRNA. However, the four standard nucleosides, adenosine, guanosine, cytodine, and uridine lacked food-signal activity. Both yeast and *E. coli* tRNA were hydrolyzed with alkali and digested with phosphatase to produce free nucleosides, but no food-signal activity was found. Identical treatment of a control 1% yeast extract solution did not alter the food-signal activity.

Based on these preliminary data, no conclusion can be reached as yet regarding the identity of the food-signal substance or substances. However, the food signal's physical properties and wide distribution are in marked contrast to that of the pheromone.

DISCUSSION

Environmental cues regulate *C. elegans* larval development. The combined influence of these cues determines what proportion of a population will form dauer larvae and whether previously formed dauer larvae will recover and resume development. The adaptive significance of dauer larva formation may arise from a "feast or famine" lifestyle. Localized concentrations of microbial growth on decaying animal or vegetable matter may support rapid proliferation of a soil nematode population, which then begins to form large numbers of dauer larvae when the food supply nears exhaustion. These dispersal forms survive for months in the absence of food, then resume development under conditions of low population density. Adults may subsequently reproduce by self-fertilization.

We have used a dauer larva recovery bioassay in the study of two chemical cues which have opposite influences on dauer larva development (Golden and Riddle, 1982). A pheromone induces dauer larva formation and inhibits recovery, and a component of the food supply inhibits formation and enhances recovery of dauer larvae. The bioassay for these two cues was improved to provide greater sensitivity and reproducibility by closely regulating the growth conditions and age of the dauer larvae used in the bioassay.

The pheromone produced by *C. elegans* is a heterogeneous mixture of related compounds. The pheromone's physical and chromatographic properties are similar to those of hydroxylated fatty acids or bile acids. In the case of vertebrate bile acids, a large amount of heterogeneity can be imposed by different patterns of hydroxylation on the steroid ring. Nematodes require sterols in their diet (Van Fleteren, 1974), but it is not known if these sterols are

metabolized to compounds with the properties we have described for the pheromone. Nevertheless, it may be possible to adapt methods developed for the purification of bile acids to the purification and eventual identification of the dauer-inducing pheromone.

Thus far, the only method of detection of the pheromone is measurement of its biological activity. The amount of active substance in our preparations is unknown and may be very small. Thus, purification of sufficient pheromone for structural analysis may require a large scale-up of our present procedures. A chemical means of detecting pheromone on TLC plates would facilitate purification.

The food signal found in yeast extract and bacterial culture medium has physical and chromatographic properties similar to those of nucleosides or carbohydrates. Its sensitivity to periodic acid suggests the presence of a *cis*-diol group. It is possible that food signal is a common metabolite produced by many organisms. Further characterization of its physical properties and comparison of its chromatographic mobilities with known standards may lead to its identification.

Genetic analysis of dauer larva formation has included the characterization of mutant strains (Riddle et al., 1981) which have abnormal responses to environmental cues (Albert et al., 1981; Golden and Riddle, 1984a). Also, a mutant defective in pheromone production has been isolated, and it is unable to form dauer larvae normally, unless exogenous pheromone is supplied. Analysis of mutants affected in pheromone production and response will provide an additional tool in the study of environmental influences on dauer larva development.

An understanding of the basic mechanisms involved in the control of *C. elegans* dauer larva development may provide insight into systems of chemical communication in other nematode species. Other Rhabditidae exhibit a dauer stage (Bovien, 1937). Environmental cues may regulate larval development of some parasitic nematodes, such as *Anguina agrostis* (Gupta and Swarup, 1972) and *Ditylenchus dipsaci* (Dropkin, 1980) which produce dauer larvae, or other species such as *Bursaphelenchus xylophilus* which produce dauer-like dispersal stages (Ishibashi and Kondo, 1977). Thus, it is possible that a detailed understanding of how defined environmental cues affect *C. elegans* may provide insight into control of parasitic species.

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Journal of Chemical Ecology, Vol. 10, No. 8, 1984

Letter to the Editor

EXPERIMENTAL DESIGN AND ECOLOGICAL REALISM

In a recent letter, Rose (1982) raised certain questions concerning a paper on crayfish communication by Itagaki and Thorp (1981) which concluded that individuals of *Procambarus clarkii* do not recognize the sex of conspecifics by detection of pheromones. Rose's (1982) comments and Thorp and Itagaki's reply (1982) were particularly interesting, since Itagaki and Thorp's (1981) conclusions were in contrast to earlier results demonstrating pheromone detection of conspecific sex by males in this species (Ameyaw-Akumfi and Hazlett, 1975).

I tend to agree with many of the points in Rose's letter. However, I wish to question one of his assumptions—that the experimental design of Itagaki and Thorp was appropriate. In testing crayfish for behavioral changes following exposure to "conditioned water," we (Ameyaw-Akumfi and Hazlett, 1975) utilized a nonflow (static) system, whereas Itagaki and Thorp (1981) used a flow-through system. Itagaki and Thorp argued that the flow-through system avoided possible contamination from the build-up of waste products. Their concern was at least partially based upon earlier results which indicated the production of stress pheromones in a related species, *P. acutus* (Thorp and Ammerman, 1978). Itagaki and Thorp (1981) agree that the specific differences in our respective results on *P. clarkii* may be methodological, but I wish to raise a general question of appropriateness of experimental procedures.

Thorp and Itagaki (1982) are correct in pointing out that as scientists we must do our best to falsify hypotheses and avoid playing advocacy science. But the ecology of the organisms we study can render some experimental designs questionable. For example, *Procambarus clarkii*, the red swamp crayfish, occurs in lentic waters and is almost never found in streams (see Spohrer et al., 1975). It is strongly associated with burrows. Given an organism that lives in "static systems," is it meaningful to test it in a flowing system? Surely if an aerial system of testing were judged to be methodologically cleaner, we would not consider it a realistic means of hypothesis falsification for aquatic animals. Crayfish such as *Orconectes virilis* live in burrows in both lotic and lentic environments and evidence of sex recognition by chemicals has been obtained by both static (Ameyaw-Akumfi, 1976) and flow-through (Tierney and Dunham, 1982) methods.

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Dunham (1978) and others have outlined methodological points which should be taken into account by those testing for pheromonal communication, a number of them clearly improvements on the methods we have used. However, I would stress that ecological realism must also be considered in our experimental design, if our attempts to falsify hypotheses are to increase our understanding of biology.

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Letter to the Editor

THEORY AND PRACTICE IN CRAYFISH COMMUNICATION STUDIES

In an earlier paper (Itagaki and Thorp, 1981), we concluded that " . . . chemical communication between adult crayfish does not occur or is not efficient at distances greater than the effective range for visual communication," and that " . . . there is no definitive evidence in crayfish for interadult pheromones which act over short distances or which are related to nonsexual responses (e.g., alarm, stress, and/or agonistic behaviors)." Readers of this journal are accustomed to reports of the discovery or identification of sex pheromones in insects and other species. It is not surprising, therefore, that heretical reports to the contrary (e.g., Itagaki and Thorp, 1981; Thorp and Itagaki, 1982) generate more than their share of controversy (e.g., Rose, 1982; Hazlett, 1984). It is a credit to the editorial staff that unorthodox conclusions are occasionally published. In the present paper, I elaborate on the ecological conditions which would promote chemical communication in crayfish (specifically sex identification) and the experimental mechanisms necessary for demonstrating this phenomenon (hence, the title: theory and practice . . .). The raison d'être for writing this paper is, in part, as a reply to Hazlett's (1984) comments on sex pheromones in the crayfish *Procambarus clarkii* (Girard).

Theory. Should we expect crayfish to possess long-distance sex pheromones just because such chemicals are active in many insects and some crustaceans? Decidedly not, in my opinion. Natural selection should promote development of sex pheromones for species in which (1) mate location is difficult or haphazard, (2) females are receptive for relatively short periods (Itagaki and Thorp, 1981), or (3) other types of premating reproductive isolating mechanisms are ineffective. The first two conditions are not met for most crayfish including *P. clarkii.* This species copulates during a lengthy period, primarily from July through October in Louisiana, and males maintain mature spermatozoa during all but approximately six weeks of the year (Penn, 1943). High densities of mixed-sex populations during much of the year and the ability of females to store mature spermatozoa in the annuli for many months (Penn, 1956) nullifies any potential problem in obtaining

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mates. Furthermore, females are not limited to a critically short postmolt mating period as is the case for some marine crustaceans (e.g., Dunham, 1978). These ecological and reproductive characteristics suggest that sex pheromones would not be necessary in the reproductive process of *P. clarkii*.

Use of pheromones as reproductive isolating mechanisms has been examined only once in crayfish (i.e., Tierney and Dunham, 1982). In that study, the average time of conspecific pursuit and copulation among pairs of *Orconectes propinquus* and pairs of *O. virilis* was 29.8% of the experimental period. In contrast, congeneric copulations were frequent, but the average time spent pursuing and copulating was only 8.3% of the period. The authors concluded that chemical cues might be an important reproductive isolating mechanism in these crayfish since morphologically and behaviorally similar species of crayfish often occur sympatrically in nature without apparent hybridization. Their experimental observations, however, imply that chemical cues, if present, are either not totally effective or operate only over short distances. Since I have not tested this particular question, I do not wish to take issue with their conclusions except to point out that the hard exoskeleton of crayfish (and intricate copulatory structures) should provide a relatively effective "lock-and-key" mechanical, reproductive isolating mechanism.

Tierney and Dunham also noted that congeneric amplexus in mixed laboratory populations was usually brief and often terminated when the female escaped prior to receiving the male's spermatozoa. This experiment suggests that behavioral and mechanical isolating mechanisms, and perhaps postmating mechanisms, may alone be adequate explanations for the infrequent discovery in nature of hybrid species of crayfish, assuming they could be recognized if present. I remain convinced, therefore, that one should not expect to find "long-distance" sex pheromones in crayfish (and, in particular, in *P. clarkii*) based solely on knowledge of their reproductive and ecological habits. This conclusion does not obviate the need for more studies, however, since it is based on theory and evidence which supports the null hypothesis of no chemical communication (rather than the more acceptable negation of a null hypothesis).

Practice. The proper procedures for conducting studies of sex pheromones in crustaceans need not be reviewed here since they have been adequately dealt with elsewhere (Dunham, 1978). Suffice it to say that no experiment purporting to show sex pheromones in crayfish has met these demanding, but necessary, criteria. Hence, in the remainder of this section, I will address the particular comments by Hazlett (1984) regarding experimental design and ecological realism.

I agree with Hazlett's cautionary comments that ecological characteristics of a species must be considered when designing experiments. I do not agree, however, that only static systems are appropriate for behavioral testing of *P. clarkii*. He has assumed that *P. clarkii* is a totally lentic crayfish and is

". . . almost never found in streams . . . ," a statement which he erroneously attributes to Spohrer et al. (1975). This is simply not the case. *P. clarkii* is primarily a lentic crayfish but is also found in streams. Hobbs (1974) describes the habitat of this species as "lentic and lotic situations and burrows." In 8% of the habitats sampled by Penn (1956), *P. clarkii* occurred in slow-moving streams. Brown (1959) compared the habitats of this species in Illinois and Louisiana and found 40% of the *P. clarkii* sampled in running water, a figure which he believed somewhat elevated by the occurrence of frequent rains during his collection period.

Part of the misconceptions concerning the distribution of *P. clarkii* may be caused by the dominance of reports on this species from people commercially harvesting them in the easily accessible ponds and rice paddies of the South. Also, their occurrence in floodplain swamps, e.g., those of the Atchafalaya River in Louisiana (Penn, 1956), may lead some people unfamiliar with these environments to assume erroneously that such crayfish are in completely static habitats. Hazlett's (1984) characterization of *P. clarkii* as "strongly associated with burrows" is not incorrect, but might mislead readers. This species, a secondary burrower, lives in burrows primarily in the winter (males and females) and sometimes in the early summer (females) rather than in the late summer and early fall when copulation occurs in shallow warm water (Penn, 1943). I conclude, therefore, that an investigator would be justified in testing *P. clarkii* for sex pheromones in either a static apparatus or a slowly flowing system, as was done in Itagaki and Thorp (1981), if all else were equal.

The problem with the static apparatus used in previous studies purporting to have demonstrated sex pheromones in crayfish (i.e., Ameyaw-Akumfi and Hazlett, 1975) is not the lack of flow, per se, but the accumulation of toxic wastes, with or without pheromones, which could result from holding stimulus crayfish for 1–2 days in head tanks. If sex pheromones are important in initiating and maintaining amplexus in crayfish or in simple sex identification, why is it necessary to require 1–2 days of conditioning before this water will elicit some response? Is it likely that any aquatic invertebrate would have to be exposed in nature to a member of the opposite sex for that length of time before sex recognition was possible?

The evidence for chemical recognition of sex by lentic or lotic crayfish species is not convincing. I have tested for chemical sex recognition in static systems with the crayfish *Procambarus acutus* (Thorp and Ammerman, 1978; "stress pheromones" speculated upon but not identified), *P. troglodytes*, and *Cambarus latimanus* (the latter two are unpublished data), and in flowing systems with *P. clarkii* (Itagaki and Thorp, 1981) and *Orconectes inermis* (unpublished data). In all cases, chemical sex recognition outside their visual range was not evident. Tierney and Dunham's (1982) study of chemical communication in two species of *Orconectes* did not involve experiments on

sex recognition (as incorrectly stated by Hazlett, 1984) since they did not compare the responses of a crayfish to water conditioned by males with reactions to water conditioned by females. Only the PhD dissertation of Ameyaw-Akumfi (1976; published earlier in a journal as Ameyaw-Akumfi and Hazlett, 1975) has reported chemical sex recognition in crayfish, and this study suffers from procedural problems as discussed by me previously and by Dunham (1978) in a generic sense.

In conclusion, I believe that both theory and laboratory evidence indicate that crayfish can chemically detect another organism but do not communicate chemically through sex pheromones at distances outside their range of visual communication. While Dr. Hazlett would probably still not agree with this conclusion, I am sure that we are in agreement with the need to undertake more laboratory experiments and field observations before we can make more than tentative conclusions about the behavior and ecology of crayfish.

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Letter to the Editor

CHEMICAL COMMUNICATION IN CRAYFISH: Physiological Ecology, Realism and Experimental Design[1]

In writing my response (Rose, 1982) to Itagaki and Thorp (1981) concerning sex pheromones and *Procambarus clarkii*, I compiled a list of potential criticisms and reinterpretations of their methods, data, interpretations, and conclusions. I then compiled the evidence for and against each item. Some items subsequently were included while others were not. Hazlett (1984) has recently resurrected one item (not included) and examined this item in the broader context of the role of realism in ecological experimental design. The present contribution addresses both the specific and general questions raised by Hazlett.

The specific question brought forth by Hazlett is whether or not lentic animals should be tested behaviorally under lotic conditions. He suggests they should not; I believe experimental design depends on the specific question being examined and that there are no hard and fast rules. There are two issues relevant to Itagaki and Thorp's experiments: (1) the effectiveness of the stimuli tested, and (2) the appropriateness of the overall design.

1. In the crustacean pheromone literature, one finds cause to doubt the effectiveness of Itagaki and Thorp's (1981) stimuli. In brief, Itagaki and Thorp drew stimulus waters from "head" tanks that contained animals for a 90-min acclimation period. Test duration was 20 min. Therefore the total time that the "stimulus" animals were in the head tanks was 110 min. Ryan (1966) reports that there was "no regularity of response" when "stimulus" animals were in "head tanks" for less than 2 hr in his tests on sex pheromones in portunid crabs (similar data for crayfish does not exist to my knowledge). Furthermore, Christofferson (1978) has shown restraint to inhibit release of pheromone in this same crab for up to several hours. It is not inconceivable that animals respond similarly (at least with respect to micturition) to situations of "restraint" or handling and introduction into a new environment. (There is substantial evidence that some crustacean pheromones are borne in the urine produced by the antennal glands.) It is therefore possible that Itagaki and Thorp's apparatus was frequently either (1) devoid of sex pheromone or (2) subjected to extremely low levels (but see Rose, 1982) especially if urine is the vehicle for putative sex pheromone in *P. clarkii*.

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2. *Procambarus clarkii* is the red swamp crayfish. Current flow rates of swamps vary tremendously depending on microlocation. In some areas water is virtually stagnant, while in others there is appreciable flow. It is therefore difficult to evaluate the flow rate in Itagaki and Thorp's apparatus (1.5 cm/sec) with respect to *P. clarkii* and chemoreception. Commercially raised crayfish from Louisiana are raised under sprinkler systems (Waubun Laboratories, Schriever, La., personal communication; these animals are commercially available through Carolina Biological Supply). Itagaki and Thorp used commercially available animals. One may question whether or not a signal perceived by such animals will be appropriately acknowledged behaviorally under such experimental conditions as described (see also below). Furthermore, Gleeson (1980) has found some behaviors attenuated or partially lacking in the blue crab when observed under laboratory conditions.

Hazlett also raises a more general question concerning the role of ecological realism in experimental design. This role varies. In some instances one is interested in the "normal situation" (e.g., some crayfish communication studies), and one would like to mimic, as nearly as possible, the natural situation. In such studies one changes only those parameters necessary for standardization of results and quantification of effect, if any effect exists. Other studies, however, require the examination of some aspect of an organism at or near its physiological limits, e.g., Schneider's (1957) studies of pheromone detection by moths. Thus, in order to assure the best experimental design, one must use an integrative approach and draw upon the findings of all physical and biological sciences.

Frequently there are physiological tests for the demonstration of reception of specific stimuli (e.g., electroretinograms, single unit electrophysiological recordings, etc.) or the components necessary for reception (e.g., microspectrophotometry of visual pigments), although there may be difficulty in extracting signal from noise. There are also anatomical criteria for the demonstration of certain receptor types, e.g., cones in the cat retina (see Blough and Yager, 1972). Anatomy and physiology should be studied in conjunction with ecology (see Ameyaw-Akumfi and Hazlett, 1975). Positive results from anatomical and physiological tests may suggest perception (see below), but behavioral results are necessary for its demonstration. Clear statement of appropriate hypotheses eases selection of experimental design and interpretation of results.

Factors such as motivation of subject animals may further complicate the choice of the most appropriate experimental design. Three examples are drawn from the literature on vision in which motivation and testing conditions have altered results, and it is likely that as the pheromone literature increases, examples from this field will be available also: (1) Rose and Menzel (1981) have behavioral evidence suggesting that foraging honeybees exhibit differences in their ability to discriminate colored disks depending on location

(hive entrance vs. food source) and independent of illumination of those disks. It seems doubtful that the receptive cells or their physiology are different under the two sets of circumstances. (2) Food-deprived cats (80% free-feeding weight, 22 hr hungry) can discriminate colors while satiated cats will not Mello and Peterson, 1963; Daw and Pearlman, 1970). (3) Weiskrantz et al. (1974), studying "blindsight" in man have shown that striate cortex lesion results in functional blindness with respect to verbal tests for sightedness, but "sightedness" with respect to nonverbal responses to visual stimuli. These examples serve to illustrate that animals are often aware of stimuli even though they may not be responsive. Furthermore, they may be aware of, and responsive to, additional cues or motivational states, as in (1) above, which are unforseen by the experimenters yet are important to the test subjects.

Thus one must be extremely cautious in the design and choice of behavioral experiments. In many instances one measures performance, not motivation nor perception. One must bear this in mind always, especially in the interpretation of negative results. It is possible to infer perception (and therefore the existence of something perceived) from differential performance in different test situations, but one cannot conclude nonperception (and nonexistence of perceivable stimuli) from nondifferential performance. Realism may be manifest in terms of experimental habitat, stimulus frequencies and intensities, and overall experimental design. Presumably one is more likely to obtain accurate results when the experimental situation closely approximates the natural one (see Hazlett, 1984). Efforts at ecological realism, however, should not encroach upon the interpretation of differential results. Furthermore, behaviors observed under laboratory conditions may not be indicative of those occurring in the field; behaviors may be lacking, attenuated, inappropriate, or different. Therefore it is frequently beneficial for an investigator to consider (and where possible incorporate) all that is known about the animal, or system, under study in terms of habitat, behavior, physiology, biochemistry, evolution, and genetics when designing realistic ecological experiments aimed at answering particular questions.

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Errata

ON THE NATURE OF CHEMICAL COMMUNICATION BY CRAYFISH IN A LABORATORY CONTROLLED FLOW-THROUGH SYSTEM

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p. 1065, l. 11: should read ". . . . The stimulus water was from . . ."
p. 1067, l. 5: should read ". . . between members of the same species, and . . ."

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MARKING URINE AND PREPUTIAL GLAND SECRETION OF MALE BANK VOLES (*Clethrionomys glareolus* L.) Chemical Analyses and Behavioral Tests

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Abstract—Urine and preputial gland secretion of male bank voles were analyzed by gas chromatography (GC) and mass spectrometry (MS) (selected ion monitoring, SIM). GC-MS analyses showed the presence of hexadecyl acetate in preputial gland secretion and in marking urine but not in metabolic urine. Female bank voles responded more strongly to marking urine of males than to metabolic urine, and they responded more to preputial gland secretion (pure or added to urine) than to metabolic urine. Dominant males spent more time and marked more frequently in response to hexadecyl acetate-enriched urine than to urine alone. The opposite reaction was shown by subordinate males. The results suggest that the acetate functions in the bank voles' dominance interactions.

Key Words—Bank vole, *Clethrionomys glareolus*, preputial gland, urine, chemical analysis, behavior.

INTRODUCTION

Urine marking behavior in bank voles and the development of their preputial glands are correlated with sex and sexual maturation (Johnson, 1975; Christiansen et al., 1978; Christiansen, 1980). Male *Clethrionomys* voles form stable dominance hierarchies observed both in the field (in *Clethrionomys rufocanus*; Viitala, 1977) and in the laboratory (in *Clethrionomys glareolus*, Sörensen, 1981). In mice (*Mus musculus*) males demonstrate their dominance through higher urinary marking frequencies (Desjardins et al., 1973) and through qualitative differences in their urine (Jones and Nowell, 1974).

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In the bank vole, dominant males have larger preputial glands than subordinates (Gustafsson et al., 1980). This suggests the possibility of a correlation between social dominance and production of preputial gland secretion. Also dominant male bank voles mark significantly more frequently than subordinates when confronted with different types of intruders (Hoffmeyer, 1983). Female bank voles are able to distinguish urine odors of dominant and subordinate males and are more attracted by those of the dominants (Hoffmeyer, 1982). Christiansen (1976, 1980) suggested that a sex attractant of male bank voles is produced by the preputial glands and mixed with the urine, as in mice (Bronson, 1966, 1976).

The aim of this paper was to relate the behavioral reactions of male and female bank voles to the chemical composition of urine and preputial secretion of males.

METHODS AND MATERIALS

Animals. The bank voles (*Clethrionomys glareolus*) were all descendants of a wild stock kept in the laboratory (Gustafsson et al., 1980). They were kept in plastic mouse cages (40 × 20 × 15 cm) on a photoperiod of 18 hr light and 6 hr dark and at a temperature of 22 ± 5° C. They were sexed at the age of 10 days and weaned at 18–21 days, when they were caged in groups of five, males and females separate. All individuals were housed in the same room, which presumably stimulated their sexual maturation. The male urine donors as well as the test animals were selected from all-male groups of 3–5 individuals with stable dominance hierarchies. To determine social dominance in groups of bank voles, we used the intruder method modified after Sörensen (1981). Dominance is positively correlated to specific types of behavior (e.g., flank-scratching with hindleg, displacement digging, attack). Subordination is correlated to escape and to submissive upright posture (Sörensen, personal communication). According to weekly testing, the males had held the same social status for at least one month prior to the chemical testing. This was to assure long-term physiological correlates of social status, which may affect male odors (review by Brown, 1979).

Collecting Urine and Preputial Gland Secretion. Marking urine for behavioral tests was collected on 265 × 130-mm filter paper, prewashed in methylene chloride. Urinations from 10 min of marking by individual dominant males (average total trace length 100 cm), delineated under UV light at 254 nm, were cut and the paper pieces extracted with methylene chloride (5 ml). The extracts were stored at −20° C. In collection of markings for chemical analyses, the test animal was allowed to mark for 10 min on a glass plate (200 × 200 mm). The deposited material was extracted with a total amount of 0.5 ml methylene chloride. The solution was concentrated to 10 μl and stored at −20° C until examination.

Metabolic urine was collected in cages described by Jones et al. (1973), but modified for voles to have separate sleeping, feeding, and urination sections. The males were kept individually in the cages overnight. A male donor was used for urine collection at intervals of at least four days. The urine was extracted with twice its volume of methylene chloride. The organic fraction was stored at −20° C. Metabolic urine that was to be compared with marking urine was dropped on filter paper and then extracted similarly. For the behavioral tests, 5 μl were used as odor sample. For chemical analyses, organic fractions from 8 ml urine were concentrated almost to dryness.

Preputial gland secretion from killed specimens was squeezed from the large efferent ducts and transferred to glass capillaries together with a small amount (40 μl) of methylene chloride per specimen. For behavioral tests, 5 μl from one specimen was used after evaporation of the solvent. For chemical analyses, pooled samples from four specimens were used. The solution was concentrated to 10 μl of which 1-μl samples were used to obtain mass spectra.

Gas Chromatography. (GC) was preformed on a Perkin-Elmer model 900 equipped with a flame ionization detector (FID). A 25-ml fused silica capillary column (ID 0.20 mm) dynamically coated with OV-101 was used. The injector temperature was 230° C. The split valve was opened 60 sec after injection. After another 120 sec, the column temperature was linearly increased by 4° C/min from ambient to 220° C.

Mass Spectrometry. (MS) A Ribermag R10-10c quadrupole GC-MS data acquisition system equipped with a Carlo Erba model 4160 GC was used. Separation was performed on a 25-m fused silica column, ID 0.20 mm, dynamically coated with SE-54 as stationary phase. One-microliter samples were injected splitless at a column temperature of 100° C. The split valve was opened 30 sec after the injection and the temperature increased linearly by 10°/min after 2 min to 220° C, where it was held constant. The injector temperature was 230° C. The electron energy used was 70 eV in both electron impact ionization (EI) and in chemical ionization (CI). The temperatures in the ion source were 125° C and 100° C, respectively. Ammonia at 1 torr in the ion source served as reactant gas.

Testing Female Bank Vole Responses. Females were tested between 2 and 3 months of age. Fourteen days before testing, they were transferred to larger cages (80 × 35 × 20 cm) and were fur-clipped for individual identification. They were weighed and treated with estradiol benzoate (OVEX B, 20 μg/kg body wt), once per day, for 3 days before the test. Microtines are reflex ovulators. They do not, like mice or rats, (spontaneous ovulators), come into estrous regularly. The estrogen treatment was given to facilitate estrous, which enhances female selectivity to male stimuli. Two hours before testing, an adult male was placed in the female's cage in a small wire net box. It was left there for 1 hr while frequent observations were made to assess the female's estrous state. This and the previous presence of father and brothers

constituted the females' only experience of males. Estrous female voles are recognizable because they always seek and stay close to the male in a way clearly different from that shown during agonistic interaction. Vaginal smears from female bank voles do not provide easily identifiable criteria as they do in the laboratory rat (Gustafsson and Westlin, personal communication).

For experiment 1, a special device for bioassay of response to olfactory stimuli in rodents was used (described by Christiansen et al., 1977). It consists of a 4-chamber choice box of Plexiglas/aluminum approx. 20 × 10 × 5 cm. Four Plexiglas tubes, with an inner diameter of 20 mm, open through holes in the long side. Each is provided with infrared photocells. Smaller tubes with an outer diameter of 19.5 mm can be inserted into the fixed tubes. The odor to be tested is applied on a filter paper strip mounted on a cork which is placed in the smaller tube. The device is simple to clean and operates automatically. It was placed in the females' home cage. In each test, three samples were presented (two odor samples and one blank) in randomized positions. The females were tested groupwise (with only familiar group mates) for 2 hr. They could approach the apparatus freely and sniff the odor samples. Only the cumulative scores per stimulus per test period were calculated (Table 1).

Testing Male Responses. The males were between 3 and 6 months of age. A choice situation was used, where the animals' urine marking responses could be recorded.

For experiment 2, the males were presented with two samples per test.

TABLE 1. EXPERIMENT 1: RESPONSES OF FEMALE BANK VOLES TO MARKING AND METABOLIC URINE OF CONSPECIFIC DOMINANT MALES.[a]

N^b	Approach frequency (mean ± SE)		P (two-tailed)
	Marking urine	Metabolic urine	
12	21.3 ± 3.0 (63%)	12.4 ± 2.3 (37%)	< 0.01 ($T = 0$)
	Urine mixed with preputial secretion	Metabolic urine	
12	12.9 ± 1.9 61%	8.4 ± 1.8 39%	0.01 ($T = 3$)
	Preputial secretion	Metabolic urine	
12	12.7 ± 2.3 (60%)	8.6 ± 2.2 (40%)	< 0.05 ($T = 12.5$)

[a]The females were tested by an olfactometer (Christiansen et al., 1977, p. 11) in groups of five.
[b]N = number of female groups tested. T = critical value in the Wilcoxon matched-pairs signed-ranks test (Siegel, 1956).

These were presented in separate compartments (Plexiglas 20 × 9 × 12 cm) lined with filter paper on the floor. These compartments were connected to the test male's home cage by short (6 cm long) removable Plexiglas tubes (diameter 4 cm). The males were tested individually. They were isolated in their home cage for 1 hr before testing. Immediately before the start of a test, each odor sample was placed at the far end of one compartment, on the floor, and the compartments were connected to the male's cage. A test started when the test animal first entered a compartment and was continued for 10 min. The urine marking frequency of each male was determined by counting the number of marks present on each sheet of filter paper. The difference in number of marks deposited in the two compartments during the test period was calculated for each male (Table 2).

In the last experiment (experiment 3, see Table 3), testing male and female responses to hexadecyl acetate, the same set-up was used, but only the cumulative times spent in each compartment for 5 min active time were compared.

In all experiments each animal was presented with only one stimulus combination per day. On the next day a different combination was given, using samples from a new donor male. The animals were not tested with more than two combinations, which were given in random order. After the presentation of a combination to an animal, the scores per stimulus were compared and the difference calculated. At the end of a test series, a Wilcoxon matched-pairs signed-ranks test was applied. The females tested with the olfactometer (experiment 1) could freely approach the apparatus and choose between the stimuli. Testing in groups proved to be necessary to overcome the timidity of the females. The drawback was that the statistical unit then became

TABLE 2. EXPERIMENT 2: URINARY MARKING FREQUENCY OF DOMINANT MALE BANK VOLES EXPOSED TO 5 μl METHYLENE CHLORIDE EXTRACTS OF URINE MIXED WITH PREPUTIAL SECRETION OR METABOLIC URINE OF CONSPECIFIC MALES.[a]

	Urinary marking frequency (mean ± SE)		
N^b	Urine mixed with preputial secretion	Metabolic urine	P (two-tailed)
12	39.4 ± 5.7 (60%)	26.6 ± 3.7 (40%)	< 0.01 ($T = 5$)

[a] The samples were provided in separate compartments connected to the home cage. Testing of individuals.

[b] N = number of individuals tested. T = critical value in the Wilcoxon matched-pairs signed-ranks test (Siegel, 1956).

TABLE 3. EXPERIMENT 3: BANK VOLE RESPONSES TO HEXADECYL ACETATE (1 ng).[a]

	N^b	Number of individuals spending most time in compartment with		P^c	Cumulated time (sec) spent in compartment with		P^d
		Urine with acetate	Urine without acetate		Urine with acetate (mean ±SE)	Urine without acetate (mean ±SE)	
Dominant males	12	11	1	< 0.001	87.4 (±16.2)	58.5 (±11.7)	0.05
Subordinate males	10	0	10		57.1 (±11.8)	67.9 (±13.5)	<0.01
Females	12	3	9		68.4 (±13.8)	82.8 (±14.1)	NS

[a] Acetate and control samples were provided in separate compartments connected to the home cage of the test animal. Test period 5 min active time. The animals were tested individually.
[b] N = number of individuals tested. Statistical methods; dominant males ≠ subordinate males.
[c] P: χ^2 test.
[d] P: Wilcoxon matched-pairs signed ranks test (two-tailed); based on difference in cumulated times recorded for each individual.

the group. In the other test situation, using two different stimulus compartments (experiments 2 and 3), the test animal always had its home cage as a third possibility, so that choices could proceed independently.

RESULTS

Chemical Analyses. Gas chromatography analyses of preputial gland secretions indicated only small amounts of material of high volatility (Figure 1a and b). GC retention times of peaks 1 and 2 coincided with those of synthetic hexadecyl and octadecyl acetate, respectively. Mass spectrometric examination of the volatile material corresponding to peaks 1 and 2 revealed that the fractions contained only minute amounts of acetate (less than 1 pg per sample). Figure 2 shows the mass spectrum of a compound isolated from pooled extracts of preputial gland secretion of four dominant males. The fragmentation pattern was identical with that of authentic hexadecyl acetate. The peaks at m/z 61 and m/z 116 are characteristic for saturated acetates (Budzikiewicz et al., 1967). No molecular ions can be seen and the ions of highest mass (at m/z 224) are formed by the loss of acetic acid (M-60) from the molecule. Although we looked intensively by chemical ionization for octadecyl and octadecenyl acetates, we could not find any traces. Figure 3 shows a mass fragmentogram of preputial gland secretion from a dominant male, in the region of the GC retention time for hexadecyl acetate, with the mass spectrometer arranged for selected ion monitoring (SIM). Chromatogram a represents the integrated signals of ions of m/z 61.0 and chromatogram b signals the signals of ions of m/z 224.2 = M-60, strongly suggesting the presence of hexadecyl acetate in the preputial gland secretion. Also a corresponding fragmentogram of extracts of marking urine indicates the presence of this acetate in the urine (Figure 4). The same mass fragmentographic analysis was performed on an extract of metabolic urine from a dominant male. Although a very large volume (8 ml) of urine was extracted, no hexadecyl acetate was detected (Figure 5, top). Figure 5, bottom, shows the same extract with 600 pg of authentic acetate added. Interestingly, the fragmentogram (Figure 5 top) indicated the presence of ions of m/z 224.2 formed by cleavage of compound with a somewhat shorter GC retention time than that of hexadecyl acetate. Whether these ions reflect the presence of a hexadecyl acetate isomer in the metabolic urine or originate from an entirely different compound is not known.

Reactions of Bank Voles. Female bank voles were more attracted to marking urine than to metabolic urine (Table 1). The females also reacted significantly more frequently to urine supplemented with preputial gland secretion and to pure preputial gland secretion than to metabolic urine. Additional tests were made with dominant males (Table 2). They marked

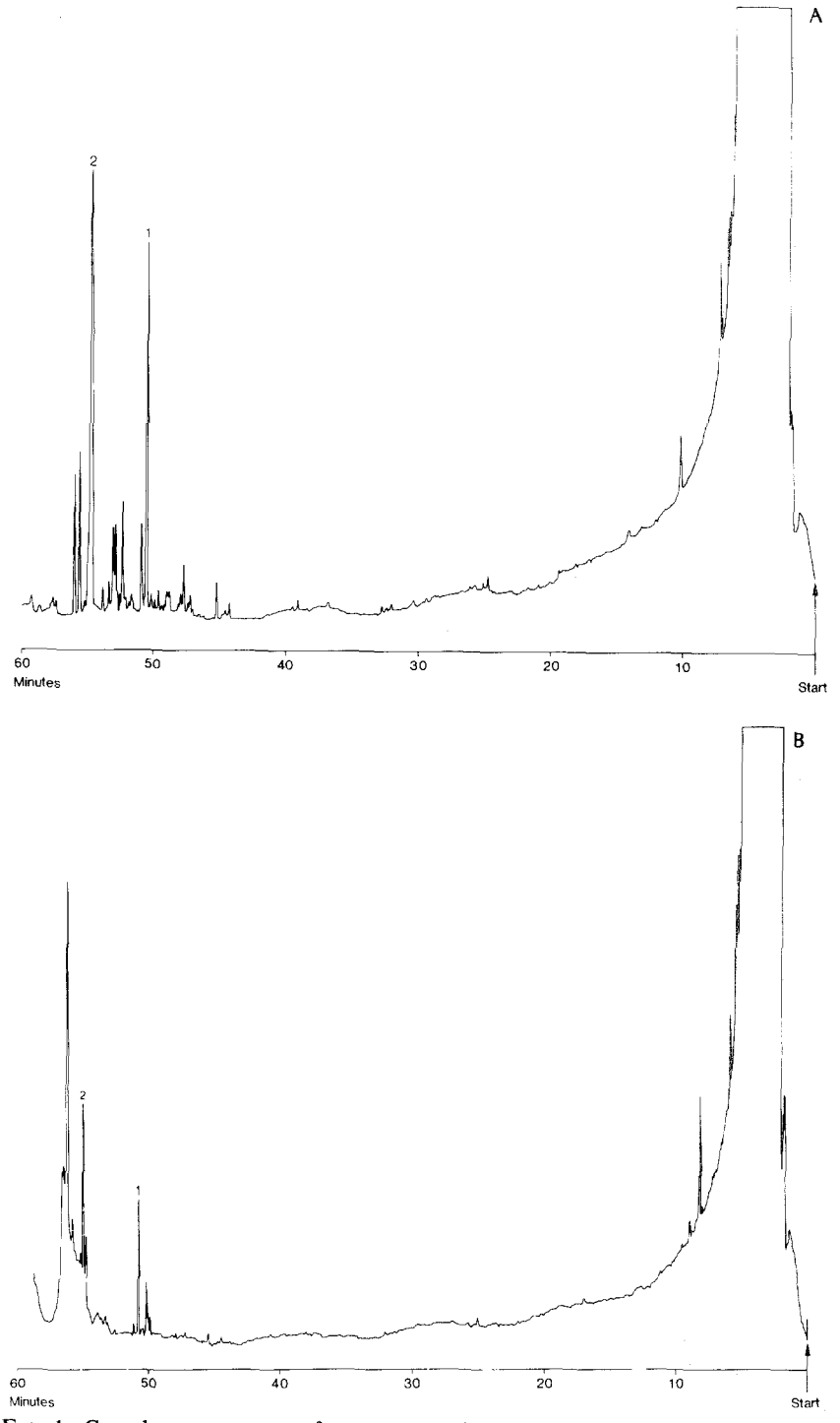

FIG. 1. Gas chromatograms of components in the preputial gland secretion from (A) a dominant male bank vole and (B) a subordinate male bank vole.

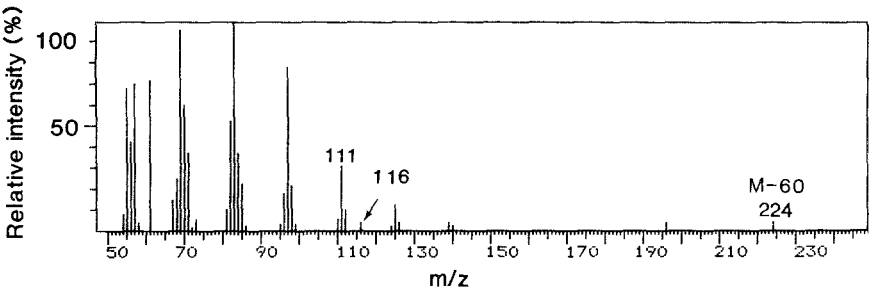

FIG. 2. Mass spectrum of a component isolated from a methylene chloride extract of a preputial gland secretion of a dominant male bank vole.

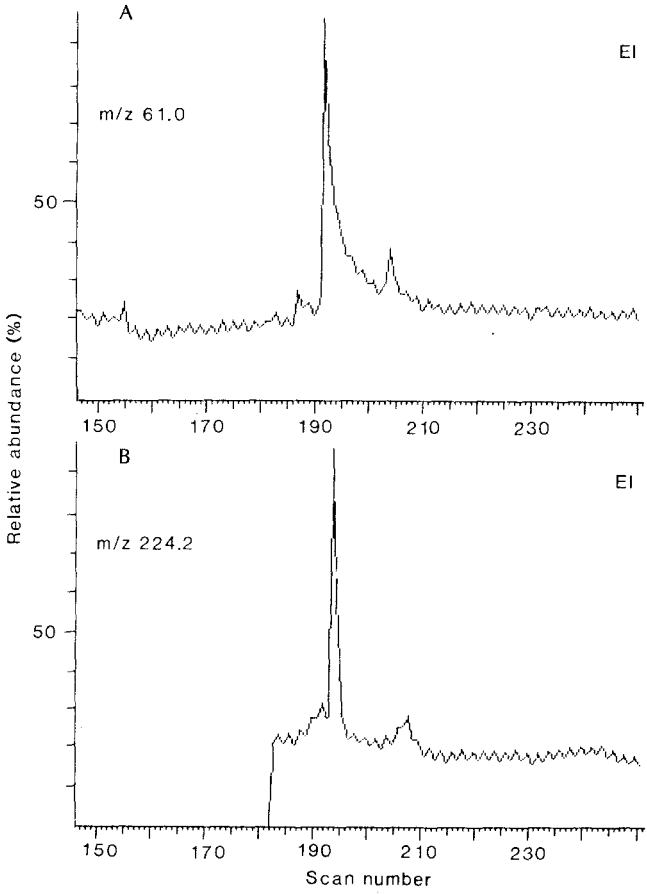

FIG. 3. Mass fragmentogram (SIM) of an extract of preputial gland secretion of a dominant male bank vole, monitoring of m/z 61.0 (A) and m/z 224.2 (B).

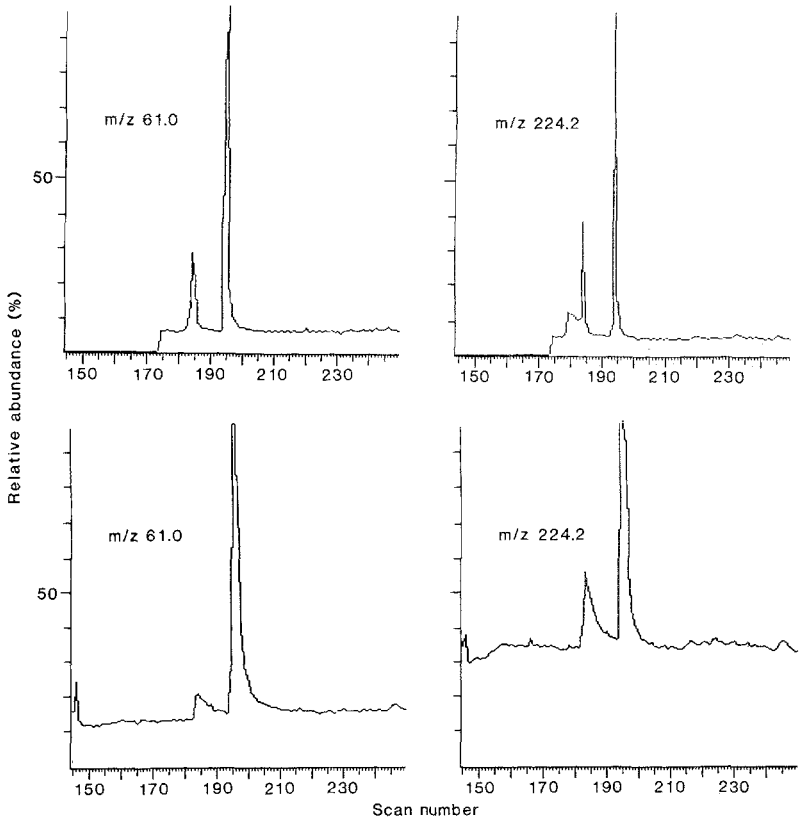

FIG. 4. Mass fragmentograms of a lipid extract of marking urine from one dominant male bank vole (top) and the extract with 600 pg hexadecyl acetate added (bottom). Monitoring as in Figure 3.

more frequently as a reaction to a mixture of urine and preputial gland secretion than to metabolic urine.

Dominant males in general spent more time with acetate-enriched urine than with controls (Table 3). They also performed urine marking more frequently in areas impregnated with acetate-enriched urine. Conversely the subordinate males spent less time with acetate-enriched urine (Table 3). In females the behavior was less consistent. The rank of the females was not known and female bank voles generally do not form as distinct dominance hierarchies as males do. The three females reacting positively behaved aggressively within their group, in contrast to the other females.

DISCUSSION

Whether preputial gland secretion is released in the urine or not has been a matter of debate. It has been uncertain whether the secretion is released only

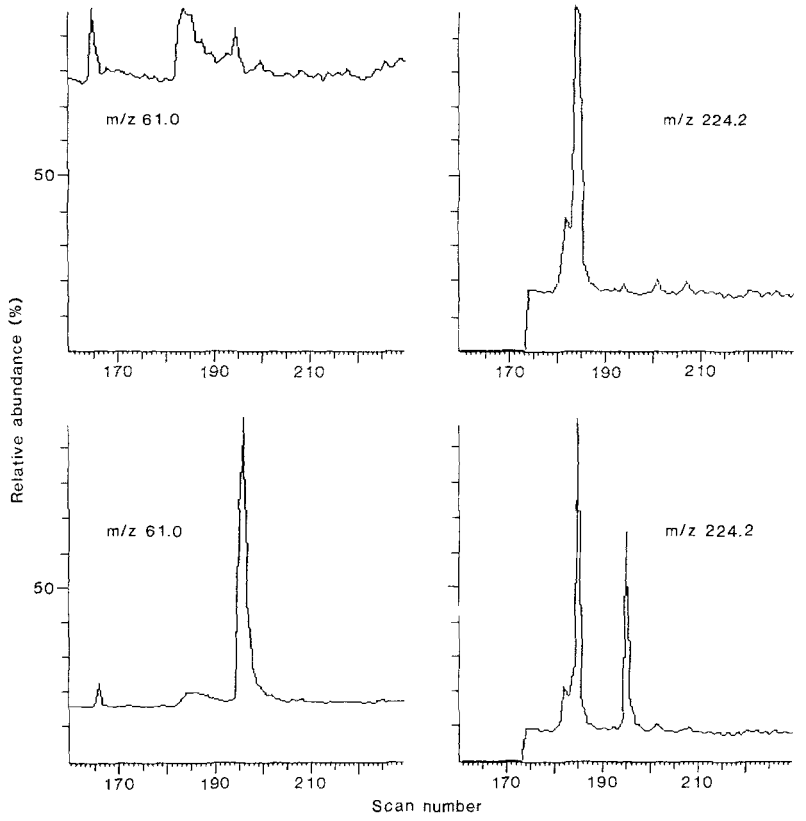

FIG. 5. Mass fragmentograms monitoring as in Figure 3 of a lipid extract of metabolic urine from one dominant male (above) and same extract with 600 pg hexadecyl acetate added (below).

in connection with urinary marking or in all eliminated urine. According to Bronson and Caroom (1971), a lipophilic factor, strongly attractive to sexually experienced female mice was found in a preputial gland homogenate and externally voided urine, but not in bladder urine from "preputial-ectomized" males. Bronson (1976) concluded that preputial secretions probably are mixed with urine during marking. Similar suggestions were made about bank voles by Christiansen (1980).

The results of this study showed that preputial secretion is associated with urinary marking. First, hexadecyl acetate was present in the preputial gland secretion and was found in marking urine, but not in metabolic urine. Second, both males and females reacted more strongly to urine mixed with preputial gland secretion when this mixture was presented as an alternative to metabolic urine.

Only dominant individuals reacted positively to the hexadecyl acetate.

This suggests that the acetate has a function in dominance interactions. Furthermore, dominant males mark more frequently than subordinates (Hoffmeyer, 1983) and have larger preputial glands (Gustafsson et al., 1980). Male bank voles generally reacted clearly to the hexadecyl acetate, while this was not the case with the females. This indicates that the acetate is more important in male–male competition than in intersexual interactions. The lack of a clear tendency in the results with the females was unexpected, because the females were attracted by marking urine and by preputial secretion added to urine. However, females may need the addition of more specific cues.

Testosterone is necessary for urinary marking in male mice (Maruniak et al, 1977; Bronson, 1979). The same appeared to be the case in bank voles (Christiansen, 1980). The synthesis of the marking compound hexadecyl acetate was found to be androgen dependent; testosterone treatment of subordinate male bank voles caused a change in the histology of their preputial glands and an increase of the amount of hexadecyl acetate (Brinck, 1983).

Social dominance relations may also affect the external release. In mice, urinary marking was inhibited in subordinate males, even when their testosterone levels were artificially elevated (Maruniak et al., 1977).

In the bank vole, high marking frequency was correlated with high social status and dominant male bank voles reacted to hexadecyl acetate (Table 3 and Hoffmeyer, 1983). Inhibited release of this compound in subordinate male bank voles could be one way of signaling subordination. In nature such low status signaling may be advantageous if subordinate males stay within the range of dominants (review by Rohwer and Ewald, 1981).

Acknowledgments—We thank G. Westerdahl for assisting in the mass spectometry, S. Douwes for preparing the line drawings, A. Odham for typing the manuscript, S. Erlinge and G. Odham for valuable comments on the manuscript. Grants were obtained from the Swedish and Danish Natural Science Research Councils.

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IDENTIFICATION OF NEW SEX PHEROMONE COMPONENTS IN *Trichoplusia ni*, PREDICTED FROM BIOSYNTHETIC PRECURSORS

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Abstract—In addition to the previously identified components (*Z*)-7-dodecenyl acetate and dodecyl acetate, sex pheromone glands of *Trichoplusia ni* release (*Z*)-5-dodecenyl acetate, 11-dodecenyl acetate, (*Z*)-7-tetradecenyl acetate, and (*Z*)-9-tetradecenyl acetate. Bioassays in a flight tunnel showed that a synthetic blend of these six compounds elicited complete flights to the source from 95% of the males tested and elicited hairpenciling responses at the end of the flights from 88% of the males tested. This blend was not significantly different from intact pheromone glands, which elicited complete flights to the source from 98% of the males tested and hairpenciling responses from 91% of the males tested. In contrast, the previously identified two-component blend elicited significantly fewer complete flights to the source (33%) and did not elicit hairpenciling responses from any of the males tested. The search for additional sex pheromone components was prompted by our previous identification of unusual fatty acyl moieties in the gland that seemed to be possible biosynthetic intermediates.

Key Words—Lepidoptera, Noctuidae, *Trichoplusia ni*, pheromone, (*Z*)-7-dodecenyl acetate, (*Z*)-5-dodecenyl acetate, 11-dodecenyl acetate, (*Z*)-7-tetradecenyl acetate, (*Z*)-9-tetradecenyl acetate, biosynthesis.

INTRODUCTION

The main sex pheromone component of the cabbage looper moth, *Trichoplusia ni* (Noctuidae), was identified as (*Z*)-7-dodecenyl acetate (Berger,

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1966). Dodecyl acetate was later identified as a second component, important mainly in close-range courtship behavior (Bjostad et al., 1980a). An extensive series of flight tunnel tests by Linn and Gaston (1981a,b) provided a detailed comparison of the behavioral effects of a large number of blends of these two components.

We recently demonstrated that the main pheromone component, (*Z*)-7-dodecenyl acetate, arises by chain shortening of the fatty acyl moiety (*Z*)-11-hexadecenoate to (*Z*)-9-tetradecenoate and then to (*Z*)-7-dodecenoate, which is reduced and acetylated (Bjostad and Roelofs, 1983). Appreciable amounts of (*Z*)-11-octadecenoate and (*Z*)-9-hexadecenoate were also observed in the gland, as were smaller amounts of (*Z*)-7-tetradecenoate and (*Z*)-5-dodecenoate. It was apparent that (*Z*)-7-tetradecenoate could arise from (*Z*)-11-octadecenoate by chain shortening and that (*Z*)-5-dodecenoate could be produced by further chain shortening. Small peaks with the retention times of methyl (*Z*)-7-tetradecenoate and methyl (*Z*)-5-dodecenoate were in fact observed in capillary gas–liquid chromatograph (GLC) analyses of methanolyzed pheromone gland extracts, and small peaks with the retention times of (*Z*)-7-dodecenyl acetate and (*Z*)-5-dodecenyl acetate were also observed.

Because the (*Z*)-11-hexadecenoate and (*Z*)-11-octadecenoate moieties in the gland are apparently produced by a (*Z*)-11 desaturase acting on the saturated moieties hexadecanoate and octadecanoate, respectively, we suspected that tiny amounts of (*Z*)-11-tetradecenoate and 11-dodecenoate might also be produced by the desaturase. Small peaks with the correct retention times were observed in gas chromatograph traces, as were small peaks with the retention times of (*Z*)-11-tetradecenyl acetate and 11-dodecenyl acetate. A summary of the proposed biosynthetic routes is shown in Figure 1. We now report the chemical identifications of these compounds, and a behavioral evaluation of their status as pheromone components.

METHODS AND MATERIALS

Extracts. The insects were reared on a semisynthetic medium (Shorey and Hale, 1965), and females were separated from males as pupae. Adults were maintained on a light cycle (14:10 light–dark). Pheromone glands were dissected from the ovipositors of 3 to 4-day-old females with fine forceps and allowed to stand overnight in 1 ml glass-distilled dichloromethane in a 4-ml vial with a Teflon-lined screw cap.

Volatile extracts were made from individual females essentially as described by Baker et al. (1981) and Pope et al. (1982) by trapping volatiles from the gland on glass wool, but we found that larger quantities of pheromone components were obtained by introducing a stream of nitrogen through a capillary tube whose outlet was about 1 mm from the gland. After a

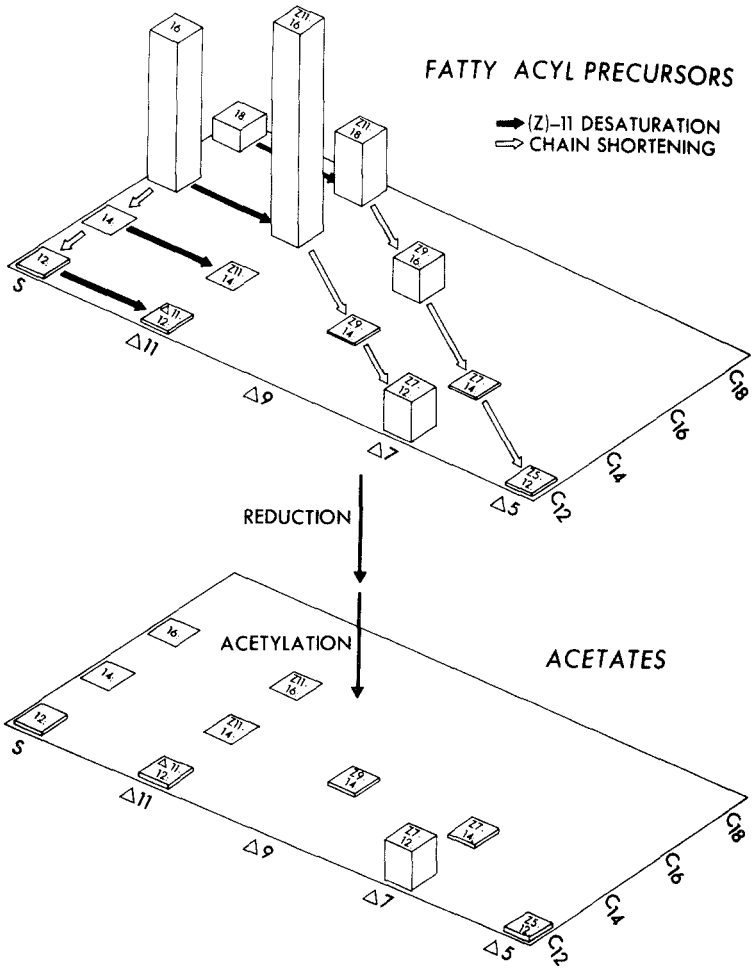

FIG. 1. Proposed biosynthetic pathways leading to compounds observed in the sex pheromone gland of *Trichoplusia ni*. Height of each block is proportional to amount of compound in gland. Names of compounds are abbreviated. Z7–12: fatty acyl precursor = (Z)-7-dodecenoate, Z7–12: acetate = (Z)-7-dodecenyl acetate, etc.

volatile extract had been made from an individual gland for 10 min, the gland was excised from the ovipositor and extracted with 10 μl of dichloromethane. Both the volatile extract and the gland extract from the same female were then analyzed by capillary GLC.

Chemical Identifications. Capillary GLC was conducted with a 45-m Carbowax 20 M column and with a 25-m cross-linked methyl silicone column, used with splitless injection and programed from 80°C to 200°C at 10°/min

after an inital delay of 2 min. Quantification of relative abundances of compounds was performed by electronic integration of peak areas (flame ionization detection). Packed GLC columns were 3% OV-101 (methyl silicone) on 100- to 120-mesh Gas-Chrom Q in a 2-m glass column (inside diameter, 4 mm) at 180°, and 10% XF-1150 (50% cyanoethyl, methyl silicone) on 100- to 120-mesh Chromosorb W-AW-DMCS in a 2-m glass column (inside diameter, 2 mm) at 140°. The resolution of the XF-1150 column deteriorated rapidly if used at temperatures higher than 140° C. Fractions were collected from packed GLC columns with 30-cm glass capillary tubes.

Crude extracts of pheromone glands in dichloromethane were injected onto an OV-101 packed column, and the fractions containing 12-carbon acetates and 14-carbon acetates were recovered by rinsing through each collection tube with 0.5 ml dichloromethane. Positional isomers were resolved by collection from the XF-1150 packed column. Each isomer was identified on the basis of ozonolysis, base methanolysis, and acetylation.

For determination of double-bond positions by ozonolysis (Beroza and Bierl, 1967), a solution of ozone was prepared in 20 μl of CS_2 in a small test tube (5 mm diameter) in a bath of acetone and dry ice (−78° C). A carbon disulfide solution of a given isomer to be ozonized was injected into this solution with a syringe, a stream of nitrogen was applied to dispel excess ozone, and a carbon disulfide solution containing 1 μg triphenylphosphine was injected to decompose the ozonide. The solution was analyzed by capillary GLC, and the retention time of the acetoxy aldehyde was compared with the retention times of acetoxy aldehydes of synthetic positional isomers.

Base methanolysis was performed to verify the conversion of acetates to the corresponding alcohols. A dichloromethane solution of a given isomer in a 4-ml vial with a Teflon-lined screw cap was evaporated to apparent dryness with a stream of nitrogen, and 0.5 ml of a solution of KOH in methanol (0.5 M) was added. After 1 hr at 25° C, 1 ml water and 1 ml hexane were added to the vial, and the mixture was shaken. The hexane portion was reduced in volume by evaporation with a nitrogen stream, and the retention time of the methanolysis product was determined by capillary GLC.

Acetylation was performed to verify the conversion of alcohols to the corresponding acetates. Acetyl chloride (0.1 ml) was added to a given isomer in a 4-ml vial, was allowed to stand at 25° C for 1 hr, and was then evaporated to apparent dryness with a stream of nitrogen. Hexane (0.5 ml) was added to recover the acetylation product, and after concentration with a stream of nitrogen, the solution was analyzed by capillary GLC.

Behavioral Tests. Males 2–3 days old were used for behavioral tests, using procedures described by Linn and Gaston (1981a,b) and Linn and Roelofs (1981). The design and application of the flight tunnel have been described by Miller and Roelofs (1978). Males were tested individually in the fight tunnel, at the midpoint of the scotophase at a light intensity of 0.3 lux,

with a windspeed of 50–55 cm/sec at 21° C. As emphasized in an earlier report (Linn and Gaston, 1981a), care in handling the male moths was critical in achieving high proportions of successful male flights. Males were allowed to acclimate quietly at 0.3 lux in the room housing the flight tunnel for 1 hr before testing, and each male was moved slowly to the tunnel for testing.

Synthetic compounds for behavioral testing were prepared in our laboratory, and isomeric purity greater than 99.9% was verified by capillary GLC analysis. Polyethylene caps were prepared by adding dichloromethane solutions of synthetic compounds to the insides of the caps with Pasteur pipets. After 1 hr the caps were closed and left in the hood for 36 hr. Caps were held in glass vials at −10° C when not in use. Sex pheromone glands were bioassayed by excising the glands from five females and placing them on the head of an insect pin mounted on a cork. In addition, individual pheromone glands were bioassayed using the same gland extrusion tube (described in Baker et al., 1981) used in the volatile extraction apparatus.

RESULTS

Chemical Identifications. Analysis of crude gland extracts by capillary GLC indicated the presence of a number of compounds in small amounts in addition to the main pheromone component (*Z*)-7-dodecenyl acetate. Volatile extracts from the glands of individual females contained only a few of the compounds observed in gland extracts (Figure 2). The compounds in the gland extracts were separated by GLC (Tables 1 and 2). Most of these compounds were acetates. The acetate functional group was verified in each case by methanolysis to form the corresponding alcohol (and comparison of the retention time with that of the same synthetic compound) and by reacetylation of the alcohol to assure that the retention times of the parent compound and of its reacetylation product were identical. The chain length of the parent acetate was verified by comparison of its retention time with that of synthetic acetates. Double-bond positions were determined by comparison of the retention times of the acetoxy aldehydes from ozonolysis with the retention times of the acetoxy aldehydes from ozonolysis with the retention times of acetoxy aldehydes from ozonolysis of synthetic unsaturated acetates.

All retention times were determined with the Carbowax 20M GLC capillary column. This column was able to resolve most positional isomers, as indicated by tests with synthetic compounds. Synthetic (*Z*)-5, (*Z*)-7, and (*Z*)-9-dodecenyl acetates were separated, as were synthetic (*Z*)-5, (*Z*)-7, (*Z*)-9, and (*Z*)-11-tetradecenyl acetates. The retention times of synthetic (*Z*)-9-dodecenyl acetate and 11-dodecenyl acetate were identical on the Carbowax 20 M column (within 0.01 min, according to cochromatography), but these compounds could be separated on the methyl silicone column.

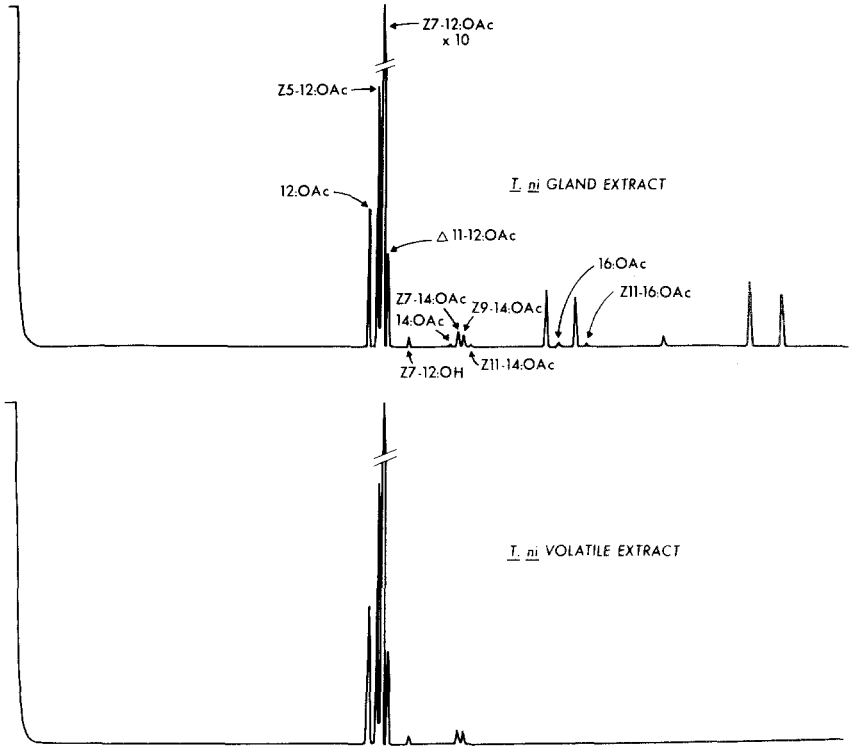

FIG. 2. Capillary GLC traces of gland extract and volatile extract of *Trichoplusia ni*. Names of compounds are abbreviated. Z7-12:OAc = (Z)-7-dodecenyl acetate, Z7-12:OH = (Z)-7-dodecenol, etc. The Z7-12:OH peak was added for completeness in the gland extract trace, but three of four extracts lacked this compound (see text).

Ozonolysis indicated that (Z)-5, (Z)-7, and 11-dodecenyl acetates all occurred in the gland, but (Z)-9-dodecenyl acetate was apparently completely absent (acetoxy aldehyde as little as 1% the amount that was observed from ozonolysis of 11-dodecenyl acetate would have been detected). Moreover, (Z)-7, (Z)-9, and (Z)-11-tetradecenyl acetates all occurred in the gland, but (Z)-5-tetradecenyl acetate was apparently completely absent [less than 0.01% the amount of (Z)-7-dodecenyl acetate].

An anomaly became apparent with respect to the occurrence of dodecenyl alcohols in the gland extracts and volatile extracts. Capillary GLC analyses of volatile extracts from individual glands indicated the presence of a small peak with the retention time of (Z)-7-dodecenol, and this peak was also apparent in analyses of extracts of the individual excised glands (Table 2). Four extracts of 50 glands each were also prepared, and the dodecenyl alcohol peak was apparently absent from three of the extracts (less than 0.05% of the

TABLE 1. RELATIVE PROPORTIONS OF COMPOUNDS IDENTIFIED FROM SEX PHEROMONE GLAND EXTRACTS OF *Trichoplusia ni* (50 GLANDS EACH)[a]

Compound[b]	Extract 1	2	3	4	$\bar{X}$	SD
1. 12:OAc	4.55	5.43	4.93	4.17	4.77	0.54
2. Z5-12:OAc	7.83	4.85	8.38	7.72	7.20	1.59
3. Z7-12:OAc	100.00	100.00	100.00	100.00	100.00	
4. 11-12:OAc	2.79	3.14	2.98	2.75	2.92	0.18
5. 14:OAc[c]					0.04	
6. Z7-14:OAc	0.45	0.31	0.29	0.40	0.36	0.075
7. Z9-14:OAc	0.26	0.26	0.28	0.31	0.28	0.024
8. Z11-14:OAc[c]					0.03	
9. 16:OAc	0.17	0.11	0.12	0.08	0.12	0.039
10. Z11-16:OAc	0.17	0.10	0.11	0.06	0.11	0.046
11. 12:OH[d]				0.03		
12. Z5-12:OH[d]				0.09		
13. Z7-12:OH[d]				0.81		
14. 11-12:OH[d]				0.01		

[a]Relative proportions were calculated with respect to the main component Z7-12:OAc.
[b]Names of compounds are abbreviated. Z7-12:OAc = (Z)-7-dodecenyl acetate, Z7-12:OH = (Z)-7-dodecenol, etc.
[c]Detected by capillary Carbowax 20 M analysis of C_{14} acetate fraction from OV-101 packed column; direct analysis of gland extract would have required overloading capillary column with (Z)-7-dodecenyl acetate.
[d]Only present in one extract of four extracts prepared; relative proportions determined by analysis of acetate derivatives.

amount of (Z)-7-dodecenyl acetate), but was present in the fourth [0.8% the amount of (Z)-7-dodecenyl acetate]. The fourth extract was fractionated by GLC on OV-101, and the dodecenyl alcohol fraction was acetylated with acetyl chloride and analyzed by GLC on the Carbowax 20 M capillary column. Peaks with the retention times of the acetates of dodecanol, (Z)-5-dodecenol, (Z)-7-dodecenol, and 11-dodecenol were observed in essentially the same proportions as the native acetates in the gland (Table 1).

Males Responses to Pheromone Glands. Males were tested individually in a flight tunnel to make a comparison of pheromone glands with synthetic blends (Table 3). Single pheromone glands were displayed from the same extrusion tube used to make volatile extracts, assuring that the handling procedures for bioassays and for volatile extractions were the same. In addition, five excised pheromone glands were placed on the head of an insect pin mounted on a cork, and male responses to this treatment were compared with their responses to polyethylene caps containing synthetic blends. The estimated release rate of (Z)-7-dodecenyl acetate from the five glands was

TABLE 2. RELATIVE PROPORTIONS OF COMPOUNDS FROM VOLATILE EXTRACTS AND GLAND EXTRACTS OF INDIVIDUAL SEX PHEROMONE GLANDS OF *T. ni*[a]

Compound[b]	Female 1	2	3	4	5	6	Mean	SD
1. 12:OAc								
Volatiles	8.19	6.83	6.49	7.54	5.71	8.38	7.19	1.03
Gland	10.80	7.61	6.49	8.32	7.71	7.94	8.15	1.43
2. Z5-12:OAc								
Volatiles	10.83	10.68	8.26	4.32	10.69	11.13	9.35	2.59
Gland	10.80	10.15	8.27	4.46	10.82	10.05	9.09	2.45
3. Z7-12:OAc								
Volatiles	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
Gland	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
4. 11-12:OAc								
Volatiles	2.51	2.36	2.13	3.48	2.80	3.38	2.78	0.55
Gland	2.90	2.28	2.13	3.40	3.23	3.10	2.84	0.52
5. Z7-14:OAc								
Volatiles	1.89	1.74	0.71	0.23	0.49	1.25	1.05	0.68
Gland	1.98	3.17	0.71	0.47	1.62	1.12	1.51	0.99
6. Z9-14:OAc								
Volatiles	0.76	0.62	0.24	0.12	0.12	0.88	0.46	0.34
Gland	0.79	2.92	0.24	0.35	0.50	0.62	0.90	1.01
7. Z7-12:OH								
Volatiles	1.51	2.11	0.59	0.35	1.70	1.13	1.23	0.67
Gland	1.58	0.89	0.59	0.35	0.62	1.12	0.86	0.44

[a]Relative proportions were calculated with respected to the main component Z7-12:OAc.
[b]Names of compounds are abbreviated. Z7-12:OAc = (Z)-7-dodecenyl acetate, Z7-12:OH = (Z)-7-dodecenol, etc.

100ng/min (Bjostad et al., 1980b), comparable to the release rate of dodecyl acetate from polyethylene caps (6.1 μg/hr) measured by Kuhr et al. (1972). Single glands elicited complete flights to the source from 98% of the males tested, and the treatment with five excised glands elicited complete flights from 95% of the males tested.

Male Responses to Synthetic Blends. Only the compounds that appeared in the volatile extracts were tested in synthetic blends in the flight tunnel bioassays (Table 3). Because it was unclear whether or not the (Z)-7-dodecenol in the volatile extracts was an artifact, a blend lacking it and a blend including it were both tested. The blend lacking (Z)-7-dodecenol (blend A) elicited complete flights from 95% of the males tested and was not significantly different from intact pheromone glands, which elicited complete flights from 98% of the males tested. The blend including (Z)-7-dodecenol (blend B) elicited complete flights form only 33% of the males tested. The

TABLE 3. RESPONSES OF MALE *Trichoplusia ni* IN FLIGHT TUNNEL TO COMPOUNDS IDENTIFIED FROM FEMALE SEX PHEROMONE GLAND EXTRACTS[a,b]

Treatment	Number tested	Taking flight	Plume orientation	Upwind flight	Source contact	Hairpencil display
Five excised glands	40	39[a]	38[a]	38[a]	38[a]	38[a]
Single gland (intact female in holder used for volatile extractions)	40	40[a]	39[a]	39[a]	39[a]	37[a]
Blend A: 12:OAc (0.16 mg) Z5-12:OAc (0.17 mg) Z7-12:OAc (3.00 mg) 11-12:OAc (0.10 mg) Z7-14:OAc (0.0093 mg) Z9-14:OAc (0.0078 mg)	40	40[a]	39[a]	38[a]	38[a]	35[a]
Blend B: blend A Z7-12:OH (0.024 mg)	40	40[a]	31[b]	26[b]	13[b]	2[b]
Blend C: 12:OAc (0.16 mg) Z7-12:OAc (3.00 mg)	40	36[a]	20[c]	14[c]	13[b]	0[b]

[a]Names of compounds are abbreviated. Z7-12:OAc = (*Z*)-7-dodecenyl acetate, etc. Values in each column with different letters are significantly different ($P \leq 0.05$) according to method of adjusted significance levels for proportions (Ryan, 1960).

two-component blend identified previously (Bjostad et al., 1980a) elicited complete flights from 33% of the males tested (blend C), but none of these males exhibited the characteristic hairpenciling behavior that usually occurs after completed flights to females (Gothilf and Shorey, 1975). The six-component synthetic blend reported here (blend A) elicited hairpenciling from 88% of the males tested. The blend including (*Z*)-7-dodecenol (blend B) elicited hairpenciling from 5% of the males tested.

The low number of male responses to the synthetic blend containing (*Z*)-7-dodecenol (blend B) is inconsistent with our observation that this compound occurs in volatile extracts. The intact pheromone glands used for bioassay were displayed from the same glass holder used in the apparatus for volatile extraction, and this seems to rule out the possiblity of a difference in handling procedures. In order to check the possibility of hydrolysis during the volatile extraction, a small piece of filter paper bearing 10 μg of (*Z*)-7-dodecenyl acetate was tested in the volatile extraction apparatus, but no (*Z*)-7-dodecenol was found in the volatile extract [less than 0.05% the amount of (*Z*)-7-dodecenyl acetate recovered]. Because gland extracts typically lacked (*Z*)-7-diodecenol, and because males responded poorly to a blend containing it, the weight of evidence seems to suggest that its presence in volatile extracts was an artifact, but we have not yet been able to identify the source.

DISCUSSION

After the identification of (*Z*)-7-dodecenyl acetate from the gland extracts of *T. ni* by Berger (1966), a number of studies by Shorey and coworkers demonstrated the importance of this single compound in attracting males for mating. These included trapping experiments with synthetic (*Z*)-7-dodecenyl acetate (Saario et al., 1970; Gaston et al., 1971; Kaae and Shorey, 1972; Kaae et al., 1973a,b) and experiments demonstrating disruption of communication by the release of synthetic (*Z*)-7-dodecenyl acetate from many point sources (Shorey et al., 1967, 1972; Gaston et al., 1967; Kaae et al., 1974; Farkas et al., 1974).

A second sex pheromone component, dodecyl acetate, was later isolated from *T. ni* pheromone gland extracts and volatile extracts (Bjostad et al., 1980a), and its behavioral role was rigorously investigated with flight tunnel studies (Linn and Gaston, 1981a,b). This compound was initially detected using packed columns for quantitative analyses of (*Z*)-7-dodecenyl acetate in individual *T. ni* sex pheromone glands (Bjostad et al., 1980b). The columns used were not able to resolve positional isomers, however, and compounds present in small amounts were difficult to detect because the peaks from packed GLC columns were relatively broad. A capillary GLC became available for subsequent work on the pathway of (*Z*)-7-dodecenyl acetate biosynthesis in *T. ni* (Bjostad and Roelofs, 1983), and the much greater resolving power and sensitivity of capillary GLC allowed the detection of a number of additional compounds in gland extracts of *T. ni*.

A variety of empirical studies have been conducted to determine the behavioral effects of synthetic compounds that are structurally related to (*Z*)-7-dodecenyl acetate. The geometric isomer (*E*)-7-dodecenyl acetate was behaviorally neutral in trapping experiments (McLaughlin et al., 1975), but

was marginally effective in disruption experiments (Kaae et al., 1974). The functional group analog (*Z*)-7-dodecenol was tested in trapping experiments in which it was dispensed along with (*Z*)-7-dodecenyl acetate, and dramatic reductions in trap catch were observed (Tumlinson et al., 1972). In flight tunnel experiments in which (*Z*)-7-dodecenyl acetate and (*Z*)-7-dodecenol were dispensed simultaneously, males made copulatory attempts at a point considerably downwind from the source, although (*Z*)-7-dodecenyl acetate dispensed alone elicited copulatory attempts at the source (McLaughlin et al., 1974). Attempted disruption with (*Z*)-7-dodecenol was somewhat successful (Kaae et al., 1974). Steck et al. (1982a,b) tested 30 synthetic compounds for trace coattractant activity in *T. ni* and other noctuid moths with a two-stage screening procedure. In the first stage, (*Z*)-7-dodecenyl acetate was mixed with 10% of each of the 30 compounds and tested by trapping in the field. Compounds that greatly reduced trap catches relative to those obtained with (*Z*)-7-dodecenyl acetate alone were retested at 1% and 0.1% levels, with the rationale that these compounds might be trace coattractants causing inhibition only at superoptimal levels. A significant increase in trap catches of *T. ni* was observed when 0.5% (*Z*)-7-tetradecenyl acetate was added to (*Z*)-7-dodecenyl acetate. This complements our observation that (*Z*)-7-tetradecenyl acetate is released from sex pheromone glands of *T. ni*, and is present at 0.5–1% the amount of (*Z*)-7-dodecenyl acetate.

The biosynthesis of the main pheromone component (*Z*)-7-dodecenyl acetate involves chain shortening of (*Z*)-11-hexadecenoate to (*Z*)-9-tetradecenoate and then to the immediate fatty acyl precursor (*Z*)-7-dodecenoate (Bjostad and Roelofs, 1983). We suspected that the corresponding acetates (*Z*)-11-hexadecenoate and (*Z*)-9-tetradecenoate might be produced in addition to (*Z*)-7-dodecenoate. This proved to be true. The gland also contains (*Z*)-11-octadecenoate in abundance, and we reasoned that chain shortening of this compound would produce (*Z*)-9-hexadecenoate, (*Z*)-7-tetradecenoate, and (*Z*)-5-dodecenoate and that the corresponding acetates might also be expected. (*Z*)-7-Tetradecenyl acetate and (*Z*)-5-dodecenyl acetate were observed, but (*Z*)-9-hexadecenyl acetate was not; this was not surprising, because (*Z*)-11-hexadecenoate is much more abundant in the gland than (*Z*)-9-hexadecenoate, and (*Z*)-11-hexadecenyl acetate was only slightly above the limit of detection. Finally, because the (*Z*)-11-hexadecenoate and (*Z*)-11-octadecenoate in the gland were apparently produced by a (*Z*)-11 desaturase, it seemed likely that (*Z*)-11-tetradecenoate and 11-dodecenoate might also occur in small amounts and that the corresponding acetates might be produced as well. This also proved to be true.

Although biosynthetic considerations led us to propose the presence of a number of additional compounds that were in fact observed in the gland, we were equally interested in isomers that were not observed in the gland. For example, (*Z*)-5-dodecenyl acetate, (*Z*)-7-dodecenyl acetate, and 11-dodecenyl

acetate were observed, but (*Z*)-9-dodecenyl acetate was not. In the biosynthesis of (*Z*)-7-dodecenyl acetate, a relatively small proportion of the large (*Z*)-11-hexadecenoate pool in the gland is chain shortened. The biosynthesis of (*Z*)-5-dodecenyl acetate similarly appears to involve chain shortening of a relatively small proportion of the large (*Z*)-11-octadecenoate pool in the gland. The biosynthesis of (*Z*)-9-dodecenyl acetate would therefore be expected to involve chain shortening of only a small proportion of the (*Z*)-11-tetradecenoate in the gland, but so little of this compound is present that chain shortening apparently produces a negligible amount of the precursor (*Z*)-9-dodecenoate. (In the biosynthesis of 11-dodecenyl acetate, the precursor 11-dodecenoate is apparently produced directly by the desaturase and does not involve chain shortening). As another example, (*Z*)-7-tetradecenyl acetate, (*Z*)-9-tetradecenyl acetate, and (*Z*)-11-tetradecenyl acetate were present in the gland, but (*Z*)-5-tetradecenyl acetate was not. Presumably this is because the precursor (*Z*)-5-tetradecenoate would arise from chain shortening of (*Z*)-11-eicosenoate, which was not detected in these pheromone glands.

In light of these interpretations, we were particularly interested in a recent paper by Klun et al. (1983) reporting that the sex pheromone of the noctuid moth *Loxagrotis albicosta* comprises the components dodecyl acetate, (*Z*)-5-dodecenyl acetate, (*Z*)-7-dodecenyl acetate, and 11-dodecenyl acetate, in the respective proportions 5:5:1:5. These are the same C_{12} acetates that we found in *Trichoplusia ni* (albeit in different proportions), and the isomer (*Z*)-9-dodecenyl acetate is evidently absent from pheromone gland extracts of *L. albicosta* just as it is absent from gland extracts of *T. ni*. We suggest that the biosynthetic features discussed above with reference to *T. ni* may explain the isomeric array of *L. albicosta*, including a (*Z*)-11 desaturase that produces large amounts of (*Z*)-11-hexadecenoate and (*Z*)-11-octadecenoate (and produces much smaller amounts of (*Z*)-11-tetradecenoate and 11-dodecenoate), and also including a chain-shortening system involved in making (*Z*)-5-dodecenyl acetate from (*Z*)-11-octadecenoate, in making (*Z*)-7-dodecenyl acetate from (*Z*)-11-hexadecenoate, and in making dodecyl acetate from hexadecanoate.

Volatile extracts of *T. ni* contained fewer compounds than gland extracts and in much smaller amounts. Some compounds that were observed in very tiny amounts in gland extracts, such as (*Z*)-11-hexadecenoate and (*Z*)-11-tetradecenoate, were below the limit of detection in volatile extracts. Only the six compounds dodecyl acetate, (*Z*)-5-dodecenyl acetate, (*Z*)-7-dodecenyl acetate, 11-dodecenyl acetate, (*Z*)-7-tetradecenyl acetate, and (*Z*)-9-tetradecenyl acetate were observed in all gland extracts and in all volatile extracts. The male response to this blend (95% completed flights) was not significantly different from the response to intact pheromone glands (98% completed flights) in flight tunnel bioassays. It is conceivable that additional components

involved in sex pheromone communication in *T. ni* may be released in trace amounts, as observed in other moths (Steck et al., 1982a,b, and references therein), but male responses to the synthetic blend of six compounds reported here were so high that it would have been impossible to detect any further contribution of other compunds.

Volatile extracts also contained a small amount of (*Z*)-7-dodecenol (Table 2), but of four gland extracts analyzed, three lacked (*Z*)-7-dodecenol altogether, and synthetic blends containing (*Z*)-7-dodecenol greatly reduced male responses (Table 3), an effect observed by other workers (Tumlinson et al., 1972; McLaughlin et al., 1974). We have not ruled out the possibility that the (*Z*)-7-dodecenol in the volatile extracts may be an artifact.

We conclude from these experiments that the complement of six acetates observed in volatile extracts of *T. ni* pheromone glands elicits the complete courtship sequence from significantly more males than does the previously identified blend, (*Z*)-7-dodecenyl acetate plus dodecyl acetate. Although all six acetates are released by females, it may be that the significant increase in male response is only due to some of them. A comprehensive set of tests is underway to determine if each of the six compounds has a significant effect on male behavior.

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RESPONSE OF COLORADO POTATO BEETLES, *Leptinotarsa decemlineata* (Say), TO VOLATILE COMPONENTS OF TANSY, *Tanacetum vulgare*

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Abstract—The responses of Colorado potato beetle, *Leptinotarsa decemlineata* (Say), to volatile components of tansy, *Tanacetum vulgare* L., were investigated in order to establish a chemical basis for observed reduction in beetle populations when potatoes, *Solanum tuberosum* L., were interplanted with tansy. Colorado potato beetles exhibited avoidance behavior to tansy oil, volatiles from intact tansy plants, a "hydrocarbon fraction" of tansy oil, obtained by fractionation on alumina, and five of the 13 known components of tansy oil that were tested. One constituent of tansy oil, α-pinene, attracted beetles.

Key Words—Colorado potato beetle, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae, tansy, *Tanacetum vulgare*, potato, *Solanum tuberosum*, interplanting, volatile compounds, insect attractance, insect avoidance.

INTRODUCTION

Popular gardening literature is full of advice on how to prevent insect damage to horticultural plants, but much of this "gardening lore" is not supported by objective investigations. Interplanting of crop plants with other plants that are said to have insect-repelling properties is an often-recommended procedure. With the increased interest in integrated pest management, experiments in interplanting, as a means of insect control, have been reported recently in the literature (Latheef and Irwin, 1980; Theunissen and Denouden, 1980).

In a review of host-plant resistance to pests, Hedin et al. (1977) pointed out the paucity of work on naturally occurring insect repellents by finding only 12 such compounds reported in the literature. More recently, a number

of insect-repelling or -attracting substances have been isolated from many plants, such as: bay leaves (Verma and Melvan, 1981), Javanese vetivar (Subhash et al., 1982), *Lasiantheae fructosa* (Mikami et al., 1981), alfalfa (Buttery and Kamm, 1980), and others. On searching the popular literature for plants that are alleged to have insect-repelling properties, one finds that most of them are herbs or other aromatic plants.

For three successive seasons, Rodale Organic Gardening and Farming Research Center (ROGFRC) Emmaus, Pennsylvania, with our cooperation, conducted interplanting experiments in the field. Various interplanting schemes were tried including: peppers (*Capsicum annuum* cv. Calif. Wonder), potatoes (*Solanum tuberosum*, cv. Katahdin), cucumbers (*Cucumis sativus* cv. Straight Eight), cabbages (*Brassica oleracea* cv. Danish Ballhead), with catnip (*Nepeta cataria*), coriander (*Coriandrum sativum*), eucalyptus (*Eucalyptus globulus*), marigold (*Tagetes patula*), nasturtium (*Tropaeolum majus*), onion (*Allium cepa*), wormwood (*Artemisia absinthium*), sage (*Salvia officinalis*), and tansy (*Tanacetum vulgare*).

Statistically significant reductions of insect populations were achieved at ROGFRC in some interplanting systems (Matthews et al., 1983). For example, Colorado potato beetle population on potato plants was reduced 60–100% when interplanted with tansy and 58–83% when interplanted with catnip, while green peach aphid (*Myzus persicae*) populations on peppers were reduced 82% when interplanted with catnip and 59% when interplanted with tansy as compared to monocultural plantings.

With the knowledge that statistically significant reductions of insect populations occur in crops interplanted with tansy, the purpose of the present study was to test the hypothesis that volatile compounds produced by tansy repel Colorado potato beetles in the vicinity of the plant. Von Rudloff (1963) separated 26 terpenes from the volatile oil of tansy and positively identified 22 of them. In a second investigation, Von Rudloff and Underhill (1965) studied seasonal variations in the volatile oil of tansy. Hethelyi et al. (1981) studied five genotypes of tansy and identified six more compounds in the oil. Thus, many of the volatile compounds that might be expected to occur in the vapor phase around the tansy plant are known. The Colorado potato beetle (*Leptinotarsa decemlineata*) was selected, since it has been widely studied for its response to volatile compounds produced by the potato plant, and it is known to be sensitive and specific in its responses to olfactory stimuli (Wei-Chun Ma and Visser, 1978).

METHODS AND MATERIALS

Tansy (*Tanacetum vulgare*) plants were harvested at ROGFRC, and the essential oils of the plants were isolated by steam distillation in our laboratory.

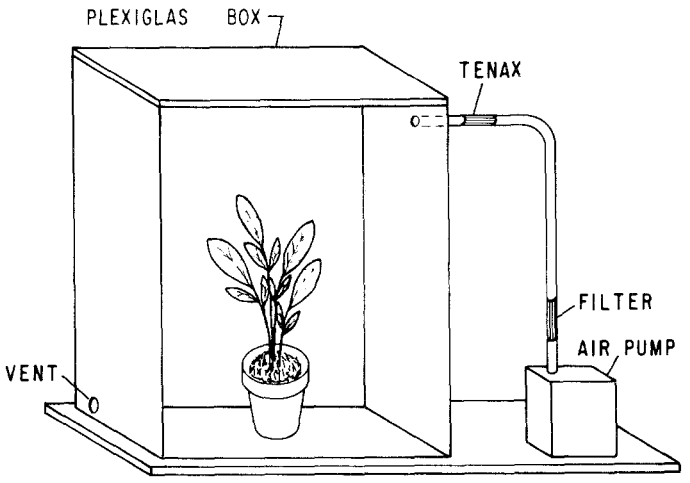

FIG. 1. Apparatus for collection of volatiles produced by intact tansy plants.

Commercial tansy oil from La Pine Scientific Company[1] of Norwood, New Jersey, was used for comparison with oil isolated in our laboratory. Individual authentic chemicals known to be present in tansy oil (Von Rudloff, 1963) were obtained from chemical supply houses.

Distillation. Tansy (620 g of stems, leaves, and some flowers) was steam distilled, and the oil was extracted from the aqueous distillate with methylene chloride. The extract was concentrated on a rotary evaporator to yield 9.3 g of tansy oil.

Headspace Vapor Collection. A method for collecting headspace volatiles from intact plants was developed to determine the volatile compounds given off by the plants. A box with a volume of 46.5 liters was constructed of clear Plexiglas (Figure 1). An air sampling pump was used to draw air through the box at a pumping rate of 1 liter/min. Intact tansy plants were placed in the box, and air circulated through the box was drawn by the pump into a glass tube filled with Tenax-GC (60/80 mesh). Tenax-GC was selected as the absorbent for volatiles of tansy, since once the volatiles are absorbed, they are stable on this polymer for up to 5 days at 0° C, and multiple collections can be made (Buckholz et al., 1980). Tenax-GC has a lower adsorption capacity than other polymers, but since volatiles given off by intact plants are produced in small quantities, this fact did not present a problem. After 24 hr of collection, material on the Tenax tube was eluted with acetone and analyzed by gas chromatography. Analysis of tansy oil obtained by steam distillation and the "headspace" vapor given off by intact tansy plants yielded similar chromato-

[1]Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

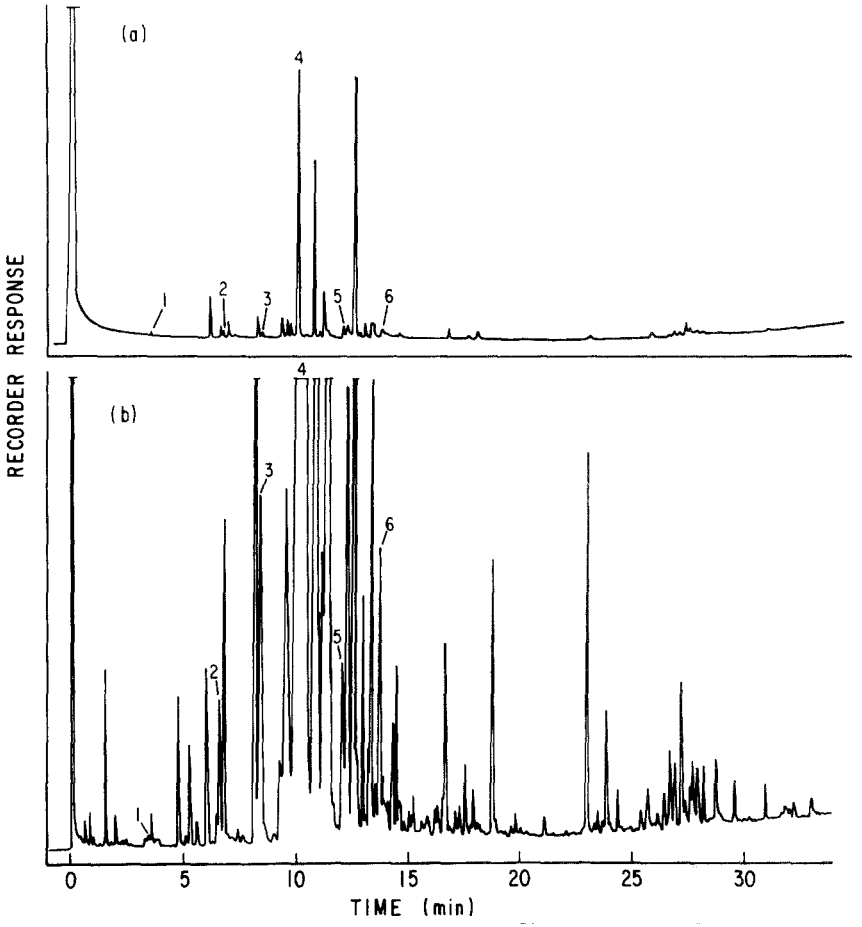

FIG. 2. Gas chromatograms of volatiles of tansy. (A) Volatiles collected from intact tansy plants. (B) Volatiles obtained by steam-distillation of tansy. Peaks: (1) α-pinene, (2) α-terpinene, (3) γ-terpinene, (4) thujone, (5) dihydrocarvone, (6) carvone.

grams (Figure 2), which indicated that the main components of the distilled oil also were given off in the vapor phase by intact plants.

Gas Chromatographic Method. A Varian gas chromatograph, model 3700 equipped with a 12-m, 0.25-mm-ID fused silica OV-101 capillary column was programed as follows: 5 min at 50° C then at 4° C/ min to 170° C, and then held at 170° C for 10 min. The column inlet pressure was 15 psi helium.

Repellency Tests. Colorado potato beetle colonies were maintained on potato plants (grown in our laboratory) under long day conditions provided by timed, artificial light. Before each experiment, the beetles were starved for 24 hr (Visser and Ave, 1978). Five beetles were used per test.

The following preliminary experiments were conducted. In one case, a

drop of full-strength tansy oil was placed on the rim of a large crystallizing dish. When a beetle that was placed on the rim approached the drop, it would back away, try to turn around, or fall from the rim; the latter is a typical reaction of the Colorado potato beetle when disturbed. A drop of water placed on the rim was used as a control. In another experiment, 20 mg of tansy oil was dissolved in 10 ml of acetone, and a strip of filter paper moistened with a 0.1-μl drop of this solution was introduced into the vicinity of beetles, and their reaction was observed. The beetles were repelled by strips of filter paper treated with tansy oil, but showed no reaction to acetone-treated strips. These experiments indicated that the beetles actually were repelled by tansy oil. In a subsequent experiment, two sprigs from a potato plant were placed in bottles filled with water and positioned at opposite ends of a cage containing 10 Colorado potato beetles. One sprig was moistened with tansy oil dispersed in water and the other with plain water. For the first 8 hr the beetles completely avoided the treated sprig. After 24 hr, six Colorado potato beetles had infested the untreated sprig, and it was almost completely eaten. The treated sprig was nearly intact except for one beetle that was feeding inside the top of the sprig where tansy oil solution had not reached.

Three additional repellency assays were conducted. First, to simulate the atmosphere produced by volatiles emanating from intact tansy plants, a cotton plug was saturated with tansy oil, or a component of tansy oil, and loosely inserted in a pipet. A stream of N_2 at a flow rate of 80 ml/min was directed through the pipet and exhausted in one side of a box. A second pipet was plugged with cotton, and the N_2 flow through this pipet was directed toward the other side of the box. Nitrogen was used instead of air to prevent possible oxidation of the substance tested. An overhead lamp was used to create even lighting. A freshly cut potato leaf was placed under each pipet, and five Colorado potato beetles were released in the middle of the box, and their activities were observed for 1 hr (Figure 3).

The second test involved comparison of feeding activity on two potato leaves, one plain and the other treated with the substance to be tested. One millimole of each individual compound to be tested was dissolved in 50 ml of acetone. For whole tansy oil, the concentration was 150 mg in 50 ml of acetone. Aliquots of acetone solutions were applied to potato leaves. A 100-μl aliquot was used in the 1-hr test and a 500-μl aliquot in the 2-hr test. As before, five beetles were placed in the box and observed for 1 hr. Substances that repelled or attracted beetles were repeated in a 2-hr test.

A third set of experiments involved placing beetles in a cheesecloth-covered dish (17 cm in diameter) containing one potato leaf moistened with a 500-μl solution of the substance to be tested and one untreated leaf for 24 hr. At the end of the test period both leaves were examined, and the repellency or the attractancy of the tested substance was determined by comparison of the extent of feeding on each leaf.

The three tests described above were conducted on the following sub-

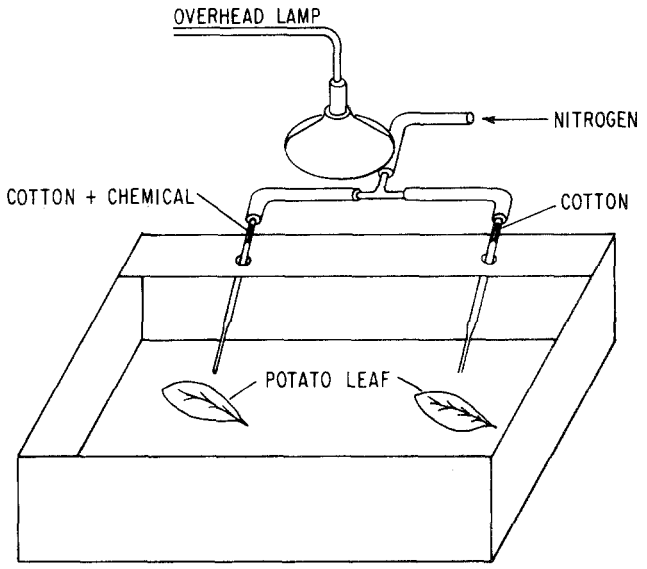

FIG. 3. Assembly for testing the response of Colorado potato beetles to volatiles produced by tansy.

stances: (1) tansy oil isolated in our laboratory; (2) tansy oil obtained from a commercial source; (3) tansy "headspace volatiles" collected for 24 hr, as already described, from intact plants, absorbed on a Tenax-filled tube, eluted with acetone, and concentrated under nitrogen (Table 1); (4) three fractions of distilled tansy oil: a "hydrocarbon fraction," an "aldehyde-ester fraction," and an "alcohol fraction" (Table 2), which were obtained by fractionating the oil on neutral Woelm's alumina with hexane, diethyl ether, and ether–methanol (90:10) eluents, respectively (Buttery and Kamm, 1980); and (5) thirteen individual compounds which are known to be present in tansy oil (Von Rudloff, 1963) in substantial amounts (Table 3).

RESULTS AND DISCUSSION

The phenomenon of repellency is complicated. These studies involved only one insect and one plant in very basic experiments. Even so, complications were encountered, because the composition of tansy oil varies considerably with season (Von Rudloff and Underhill, 1965) as well as from plant to plant, and because the compounds that vary are largely those that produced responses of Colorado potato beetles. Although all tansy plants were from the same source (ROGFRC), one batch of laboratory-distilled oil did not contain thujone (perhaps due to the fact that the seed company supplied seeds from

TABLE 1. COLORADO POTATO BEETLE RESPONSE TO VOLATILE OILS

Oil	Potato leaf under atmosphere of tansy volatiles	Potato leaf treated with tansy oil or volatiles collected from tansy plants		
	1 hr exposure	1 hr exposure	2 hr exposure	24 hr exposure
Tansy oil (commercial)	avoided[a]	avoided[b]	avoided[b]	avoided[b]
Tansy oil (laboratory preparation)	avoided[a]	avoided[b]	avoided[b]	sl. avoided[c]
Tansy headspace volatiles collected on Tenax	avoided[a]	avoided[b]	[d]	sl. avoided[c]

[a]Avoided leaf under atmosphere of tansy volatiles but fed upon leaf under nitrogen atmosphere.
[b]Avoided oil-treated leaf but fed upon untreated leaf.
[c]Slightly avoided—after 24 hr one beetle was found on the leaf but did not feed on it.
[d]Not observed at 2 hr.

different sources). The six components of the volatile oil of tansy which produced responses in the Colorado potato beetle were tentatively identified by GLC retention time. Presence of thujone was confirmed by mass spectral data, since this compound may be absent in some tansy plants.

The results of the experiments strongly suggest that there is a chemical basis for the repellent properties of tansy. First, tansy oil (both commercial and freshly distilled) produced avoidance behavior in Colorado potato beetles in all experiments (Table 1). Second, "headspace volatiles" from intact tansy plants had the same effect, demonstrating that the volatile components, in the

TABLE 2. COLORADO POTATO BEETLE RESPONSE TO FRACTIONS OF TANSY OIL

Oil fraction	Potato leaf under atmosphere of oil fraction	Potato leaf treated with fraction of oil		
	1 hr exposure	1 hr exposure	2 hr exposure	24 hr exposure
Hydrocarbon	avoided[a]	avoided[a]	avoided[a]	avoided[a]
Ester–aldehyde	accepted[b]	avoided[a]	sl. avoided[c]	no difference[d]
Alcohol	accepted[b]	avoided[a]	sl. avoided[c]	no difference[d]

[a]Avoided treated leaf, but fed upon untreated leaf.
[b]Fed upon treated leaf to some extent.
[c]Did not avoid the treated leaf completely, but did not feed upon it.
[d]Both leaves were fed upon.

TABLE 3. COLORADO BEETLE RESPONSE[a] TO INDIVIDUAL COMPOUNDS COMPRISING TANSY OIL

Compound	Potato leaf under atmosphere of compound	Potato leaf treated with compound		
	1 hr exposure	1 hr exposure	2 hr exposure	24 hr exposure
α-Pinene	attracted	attracted	attracted	attracted
dl-α-Pinene	no effect	no effect	[b]	no effect
α-Limonene	accepted	accepted	[b]	no effect
α-Terpinene	avoided	avoided	avoided	avoided
γ-Terpinene	sl. accepted	avoided	avoided	avoided
Cineole	no effect	avoided	avoided	no effect
ρ-cymene	sl. avoided	sl. avoided	accepted	avoided
α,β-thujone	avoided	avoided	avoided	avoided
L-Camphor	sl. accepted	avoided	sl. avoided	accepted
Terpinen-4-ol	no effect	sl. avoided	[b]	sl. avoided
Dihydrocarvone	avoided	avoided	avoided	avoided
Carvone	avoided	avoided	avoided	avoided
Borneol	accepted	avoided	sl. accepted	no effect

[a]Attracted = preferred treated leaf to untreated leaf; slightly avoided = did not avoid the leaf completely, but when on it, did not feed; slightly accepted = took a few bites before moving away; no effect = both treated and untreated leaf was fed upon; accepted = fed upon treated leaf to some extent; avoided = avoided treated leaf, but fed upon untreated leaf.
[b]Not observed at 2 hr.

aggregate, produce an avoidance response in Colorado potato beetle. As shown in Table 2, the strongest avoidance effect was produced by the "hydrocarbon fraction" obtained by fractionating tansy oil on alumina. Four components of tansy oil: α-terpinene, thujone, dihydrocarvone, and carvone, produced definite avoidance behavior in Colorado potato beetles (Table 3). To a slightly lesser degree, γ-terpinene had the same effect. One compound, α-pinene, attracted beetles.

While this study was limited in scope, the results confirm the validity of the concept of interplantings with tansy for the control of the Colorado potato beetle and identify at least some of the chemical substances responsible for avoidance behavior of Colorado potato beetles. Additional work under more rigid conditions is needed. Larger colonies of insects in more controlled environments should be used in experiments, quantitative composition of oils should be established, and different concentrations of compounds and their combinations should be examined in order to exploit more fully the use of tansy in interplantings, or the use of tansy oil or its constituents as insect repellents.

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ALKANES FROM SURFACE LIPIDS OF SUNFLOWER STEM WEEVIL, *Cylindrocopturus adspersus* (LECONTE)

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Abstract—The stem weevil, *Cylindrocopturus adspersus* (LeConte) (Coleoptera: Curculionidae) yields 3% of its body weight as extractable lipids (40 μg/weevil). The alkane fraction was composed of *n*-alkanes (38%) and branched alkanes (62%). The compounds were characterized by gas chromatography–mass spectrometry (GC-MS). The chromatogram contained several single-component peaks (9 of 25). Only seven dimethylalkanes were isolated (17.8%): 9,19- and 9,21-dimethylheptacosane; 9,19- and 9,21-dimethylnonacosane; 9,21- and 11,21-dimethylhentriacontane; and 11,21-dimethyltritriacontane. Important methylalkanes were: 2-methyltetra- and hexacosanes and 10-methylhexa- and octacosanes. Late-eluting gas chromatography peaks were composed of simple alkane mixtures or a single component.

Key Words—Insect hydrocarbons; sunflower stem weevil, *Cylindrocopturus adspersus;* Coleoptera; Curculionidae; 9,19-dimethylalkanes; 9,21-dimethylalkanes.

INTRODUCTION

The life history of a sunflower stem weevil, *Cylindrocopturus adspersus* (LeConte) (Coleoptera: Curculionidae) in North Dakota has been described by Casals (1976) and by Schulz (1978), but little is known of its biology, physiology, and behavior. We are reporting here the results of the mass spectral analysis of the complex alkane fraction of the cuticular waxes from the adult stem weevil. The surface lipids of insects serve to prevent both

desiccation and invasion of pathogens (Nelson, 1978; Blomquist, 1979). The results of the composition analysis of the hydrocarbon fraction of the surface lipids reported in this paper suggested rare "primer" molecules used for the biosynthesis of some of the methylalkanes isolated from the stem weevil.

METHODS AND MATERIALS

Larvae of a stem weevil, *C. adspersus,* were dug from dry sunflower stems that were field collected around Fargo, North Dakota, after harvest and stored at 3–6° C. Mature larvae obtained in this fashion were broken of diapause and allowed to continue development to adults at 25°C, 50% relative humidity. The procedure for sexing these insects and maintaining them through the adult stage was that of Reinecke (1981). Starting one week after eclosion, the adults were fed on sprouted sunflower seeds. Adults were sacrificed 0, 4, 8, and 14–21 days posteclosion and held at −10 to −15°C until used.

For gas chromatography–mass spectrometry (GC-MS) analysis, 140 adult weevils of mixed age and sex were extracted with 10 ml toluene for 24 hr at room temperature. The toluene was removed with a stream of nitrogen (N_2) at 45° and the residue dissolved in hexane. The resulting solution was percolated through a 20% $AgNO_3$-impregnated silica gel column (6 × 100 mm) to remove unsaturated compounds. Alkanes were eluted with hexane, and the solvent from the alkane fraction was removed under a N_2 stream. The residue was dissolved in isooctane and the solution stored over freshly activated 5 Å molecular sieve for 24 hr to remove the *n*-alkanes.

Gas chromatographic (GC) analyses were made before and after molecular sieving, using a Varian 3700 instrument equipped with a 3.05-m × 0.318-cm stainless-steel column packed with 3% OV-101 on Gas-Chrom Q (100–120 mesh). Helium (20 ml/min) was the carrier gas, and the instrument was programed at various rates, depending on the analyses, from 125 to 310° with a 2-min initial isothermal hold and an 8-min final isothermal hold. Hydrocarbons *n*-$C_{20}H_{42}$, *n*-$C_{24}H_{50}$, *n*-$C_{28}H_{58}$, *n*-$C_{32}H_{66}$, *n*-$C_{36}H_{74}$, *n*-$C_{40}H_{82}$, and *n*-$C_{44}H_{90}$ were used as GC standards to determine detector response and retention indices. Two internal standards were added to the crude extracts, viz., 3-methylnonadecane and 3-methylheneicosane at the rate of 1 μg/μl and are labeled peaks A and B, respectively, in Figure 1.

(GC-MS) was performed using a modified Varian 3700 gas chromatograph coupled to a Varian-MAT 112S mass spectrometer in the electron impact mode and fitted with a Carter Cook jet seperator. The GC glass column was 3.05_m × 0.635 cm OD and was packed with 3.5% OV-101 on

Gas-Chrom Q, 100–120 mesh. Helium was the carrier gas at a flow rate of 25 ml/min. Separations were performed by programing the instrument from 150 to 324° at 4°/min, and a MS scan was made every 4.8 sec. Data acquisition and storage were made with a PDP 11/34 RSX-11M 3.2 version computer dedicated to the mass spectrometer.

RESULTS AND DISCUSSION

The adult stem weevil had an average weight of 1.4 mg and yielded about 3% of its body weight in extractable surface lipids (40 μg/weevil). Approximately 6% of the lipids were alkanes and 0.3% alkenes. The alkanes were both normal and branched. The composition of the alkene and polar lipid fractions will be reported in a later paper. The chromatograms of an extract of adults of mixed ages and sex are shown in Figure 1. The chromatogram is of the branched alkanes, only, after removal of the *n*-alkanes by molecular sieve. The structural identities of the compounds from the labeled GC peaks in Figure 1, as characterized by their mass spectra, are listed in Table 1. The amount (ng/weevil) and percent contribution to the total alkane mixture of each chromatographic peak are also listed in Table 1. No qualitative difference was found between the chromatograms of males and females. Because GC peaks were often composed of unresolved mixtures of mono- and dimethylalkanes having similar retention indices, the exact amounts of individual components were not known. Thus the values reported for mixtures in Table 1 are for the mixture only.

The *n*-alkanes comprised 39.25% of the total alkane mixture with $C_{25}H_{52}$, $C_{27}H_{56}$, and $C_{29}H_{60}$ present in equal amounts. Monomethyl (42.24%) and dimethyl (17.8%) alkanes made up the remainder (Table 1).

In the stem weevil, the monomethyl alkane fraction which contributed the greatest percentage to the alkane mixture was characterized by several single-component GC peaks. Thus, for example, 2-methyltetracosane, 2-methylhexacosane, and 2-methyloctacosane (peaks c, h, and n, respectively, Figure 1, Table 1) each contributed 7.0, 3.48, and 0.5% respectively, to the overall composition of alkanes, while 10-methylhexacosane (peak g), 10-methyloctacosane (peak m), and 10-methyltricosane (peak r) contributed 0.6, 0.9, and 0.46%, respectively. There were three homologous 3-methylalkanes found viz., 3-methylpentacosane (peak f), 3-methylheptacosane (peak k), and 3-methylnonacosane (peak p); only peak f was composed of a single component (0.7%). Peaks k and p had mixtures containing a 4-methylalkane (in peak k) and mixtures of isomeric dimethyl alkanes (peaks k and p). Another homologous series of hydrocarbons composed of monomethylalkanes was that containing three component mixtures of 9-,

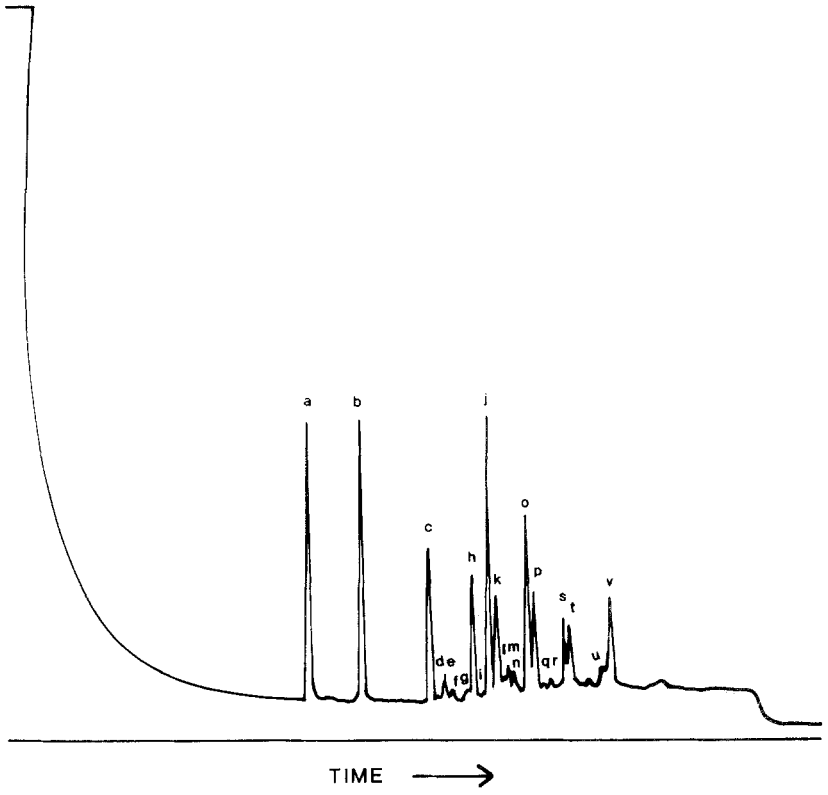


FIG. 1. Gas chromatogram of the methylalkanes from the surface lipids of the sunflower stem weevil, *C. adspersus*. Letters identify peaks whose compositions are listed in Table 1. Peaks a and b represent detector response of 1 μg each of added standard methylalkanes.

TABLE 1. NORMAL AND BRANCHED ALKANES OF ADULT SUNFLOWER STEM WEEVIL, *Cylindrocopturus Adspersus* (MIXED SEXES)

Structural identity of alkane from GC-MS[a]			Quantity relationships	
Compound(s)	Peak letter[b]	*Ki*	Alkane per weevil (ng)	Percent of total alkane mixture
n-Tetracosane	[c]	2400	64	2.7
2-Methyltetracosane	c	2470	166	7.0
Unknown	d	2485	Trace	
n-Pentacosane	[c]	2500	252	11

TABLE 1. Continued

Structural identity of alkane from GC-MS[a]			Quantity relationships	
Compound(s)	Peak letter[b]	*Ki*	Alkane per weevil (ng)	Percent of total alkane mixture
9-Methylpentacosane 11-Methylpentacosane 13-Methylpentacosane	e	2545	41	1.7
3-Methylpentacosane	f	2575	16	0.7
10-Methylhexacosane	g	2635	14	0.6
2-Methylhexacosane	h	2670	82	3.48
Unknown	i	2685	11	0.5
n-Heptacosane	[c]	2700	269	11
9-Methylheptacosane 11-Methylheptacosane 13-Methylheptacosane	j	2745	346	15
4-Methylheptacosane 9,19-Dimethylheptacosane 9,21-Dimethylheptacosane 3-Methylheptacosane	k	2765 2785	115	4.9
n-Octacosane	l	2800	13	0.55
10-Methyloctacosane	m	2835	22	0.9
2-Methyloctacosane	n	2870	12	0.5
n-Nonacosane	[c]	2900	252	11
9-Methylnonacosane	o	2940	209	8.9
9,19-Dimethylnonacosane 9,21-Dimethylnonacosane 3-Methylnonacosane	p	2975 2985	104	4.4
Unknown	q		14	0.59
10-Methyltricosane	r	3035	11	0.46
Unknown			Trace	
n-Hentriacontane	[c]	3100	71	3.0
9-Methylhentriacontane 11-Methylhentriacontane	s	3145	71	3.0
9,21-Dimethylhentriacontane 11,21-Dimethylhentriacontane	t	3185	71	3.0
Unknown			Trace	
Unknown			Trace	
9-Methyltritriacontane 11-Methyltritriacontane	u	3345	Trace	
11,21-Dimethyltritriacontane	v	3385	130	5.5
Unknown			Trace	—
Sum			2356	100.38

[a]In order of chromatographic elution.
[b]From Figure 1
[c]Not shown in Figure 1. Characterized by GC-MS before molecular sieving.

11-, and 13-methylalkanes such as found in peaks e and j or two component mixtures of 9- and 11-methylalkanes (peaks s and u). However, a third GC peak belonging to this series contained a single component, peak o (9-methylnonacosane), which comprised 8.9% of the alkane mixture and was the greatest amount contributed by any single-component methylalkane GC peak.

The presence of the homologous 10-methylhexacosane, 10-methyloctacosane, and 10-methyltriacontane suggested the utilization of propionyl-CoA rather than acetyl-CoA as the "starter piece" for biosynthesis of these compounds. Although propionate is not a common primer of hydrocarbon biosynthesis, it had been shown to be the "starter piece" for biosynthesis of fatty acids of odd carbon number which were subsequently decarboxylated, biochemically, to the alkane with an even carbon numbered backbone; thus Buckner and Kolattukudy (1976) demonstrated that propionyl-CoA was incorporated into bird wax hydrocarbons via the corresponding fatty acids. Recent insect studies which used carbon-13-labeled propionate as substrate and C-13 NMR to locate the position of the label in the hydrocarbon after biosynthesis showed that the housefly also utilized propionate as primer for fatty acid and hydrocarbon synthesis (Dillwith et al., 1982).

The mass spectrum of 10-methyloctacosane (peak m) is shown in Figure 2. Interpretation of the fragmentation was straightforward (McCarthy et al., 1968; Pomonis et al., 1978) with pairs of fragment ions at m/z 154 : 155 and m/z 280 : 281 diagnostic of the location of the methyl substituent. The M-15 ion peak is seen at m/z 393. The spectrum shown in Figure 2 had been amplified a little over three times because the quantity of sample present gave a weak spectrum which nevertheless was easily interpreted.

It is likely that in the stem weevil the homologs 2-methyltetracosane, 2-methylhexacosane, and 2-methyloctacosane (peaks c, h, and n, respectively) may have originated from valine. It has been shown for two species of cricket that 2-methylalkanes with an even-numbered carbon backbone have as their biosynthetic primer isobutyryl-CoA from valine (Blailock and Blomquist, 1976). The 2- and 3-methylalkanes are common to insects but usually have odd number of carbon backbone alkanes (Blomquist and Jackson, 1979). Some 2- and 3-methylalkanes have been found in the cuticular extracts of four castes of a termite (Howard et al., 1982); pupal tobacco budworms (Coudron and Nelson, 1978); the house fly (Nelson et al., 1981); and the Japanese beetle (Nelson et al., 1977).

The stem weevil dimethylalkanes were not the typical complex mixtures of compounds found in most other insects (Table 1). Often the major dimethyl compounds of the componenet mixtures from insect cuticle

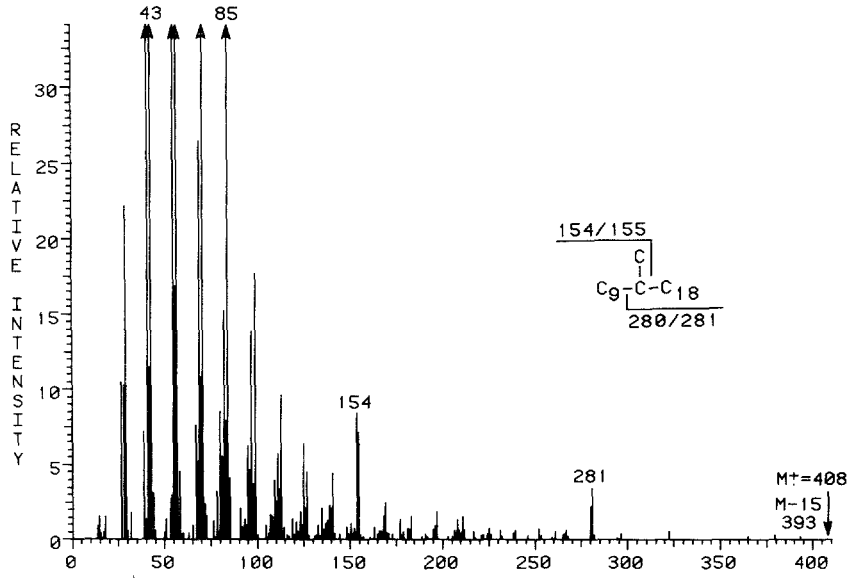


FIG. 2. EI mass spectrum of 10-methyloctacosane; peak m.

have been those in which the dimethyl substituents were separated by three methylene units (Nelson, 1978; Blomquist and Jackson, 1979). Minor amounts of other dimethylalkanes may accompany the major ones, thus, compounds having methyl branches separated by 5 or 7 methylene units are not uncommon (Coudron and Nelson, 1978; Nelson et al., 1981; Lockey, 1982a, b), and most recently minor amounts of dimethylalkanes having 9 and 11 or more methylene units separating the methyl branches have been reported to accompany the usual 3 methylene interrupted dimethylalkanes (Lockey, 1982c; Jackson, 1983). However, in the stem weevil only seven dimethylalkanes were isolated and characterized from the stem weevil, and in all cases the compounds had the methyl branches separated by 9 or 11 methylene units only (Table 1, Figure 2, peaks k, p, t, and v). These compounds comprised 17.8% of the alkanes.

Peak k (4.9%) was composed of a mixture of 4-methylheptacosane, 9,19-dimethylheptacosane, 9,21-dimethylheptacosane, and 3-methylheptacosane. The amount contributed by each component was not determined because complete resolution of each compound by GC was not possible with our column. However, partial resolution of the compounds was observed by GC-MS, so that interpretable mass spectra were obtained. When peak k was scanned by the mass spectrometer, the data revealed that the leading edge of the peak was composed of 4-methylheptacosane (Figure 3A, GC-MS scan

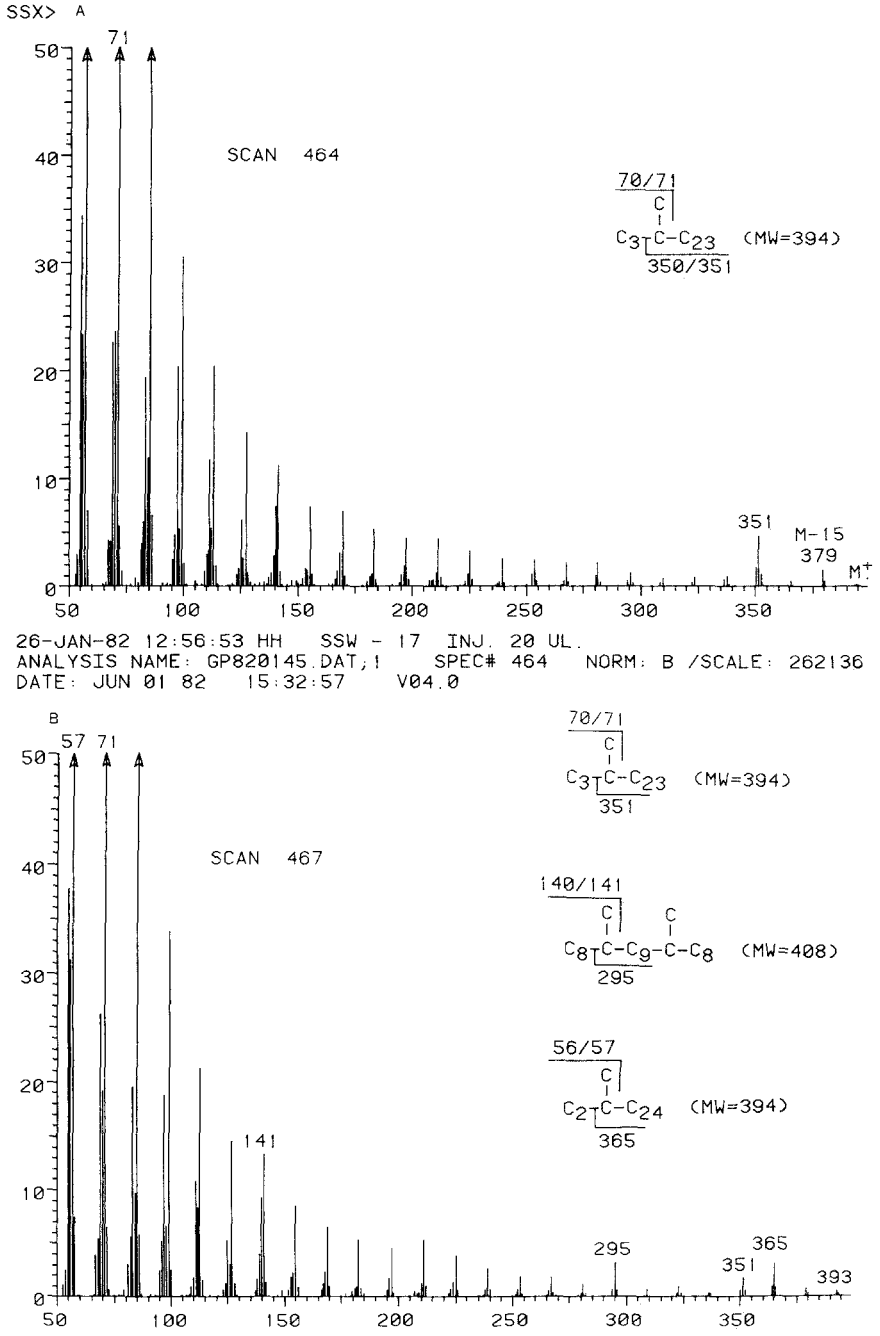
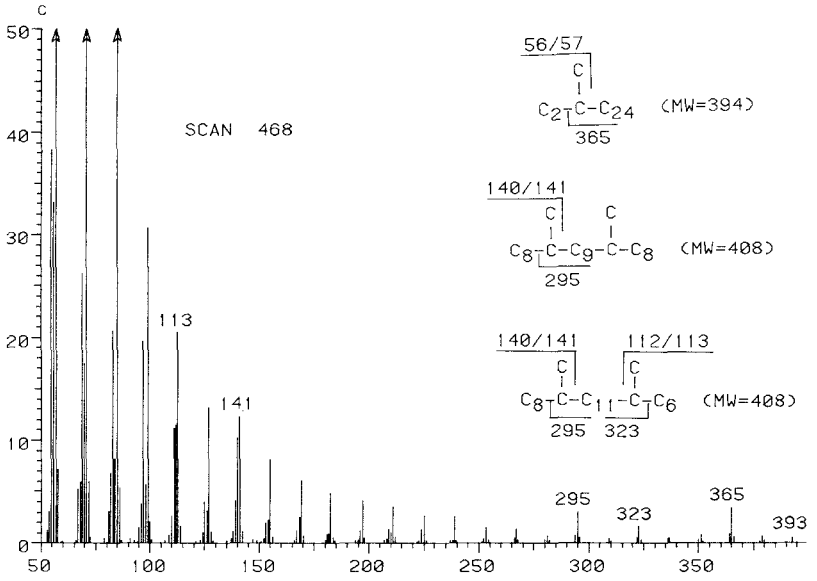


FIG. 3. EI mass spectra of component hydrocarbons of peak K. (A) 4-methylheptacosane. (B) 4-methylheptacosane plus 9,19-dimethylheptacosane plus 3-methylheptacosane. (C) 3-methylheptacosane plus 9,19- and 9,21-dimethylheptacosane. (D) 9,19- and 9,21-dimethylheptacosane.



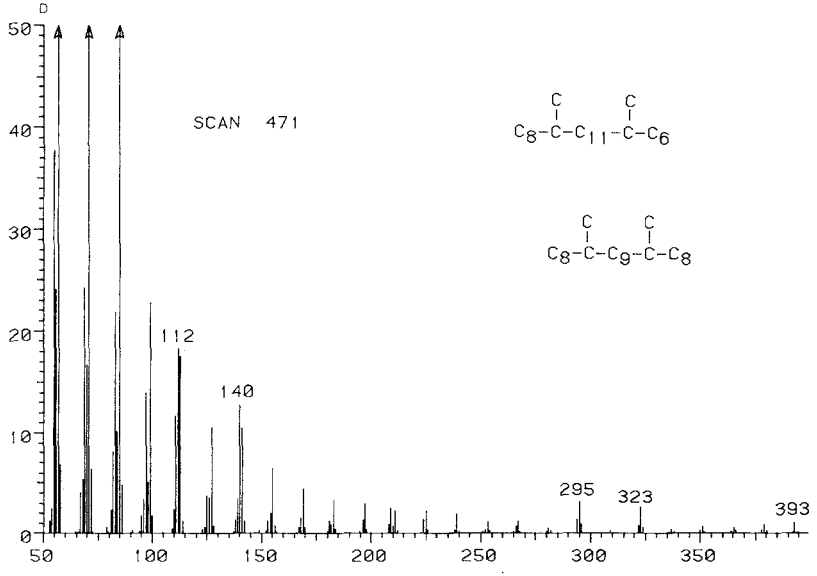


FIG 3. Continued.

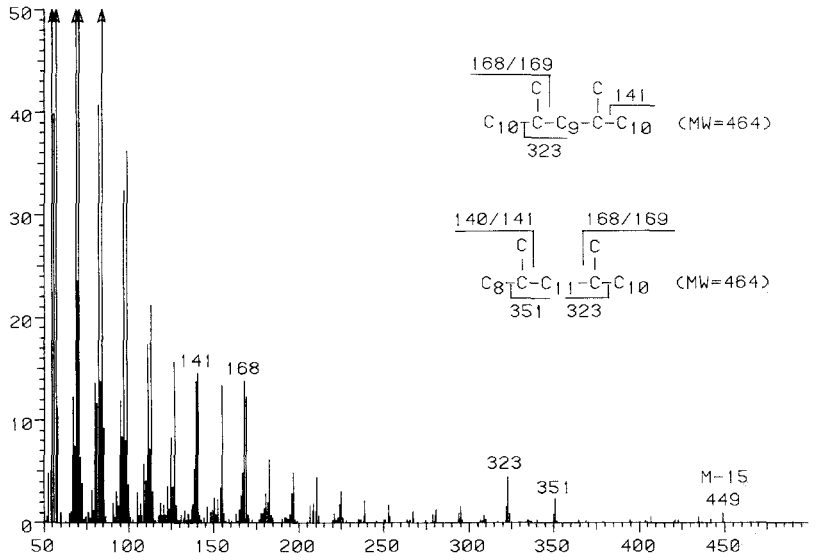


FIG. 4. EI mass spectrum of 9,21- and 11,21-dimethylhentriacontane (peak S).

464). Generally, it has been difficult to distinguish between 4-methylalkanes and 2-methylalkanes by their mass spectra only. Both 4-methylheptacosane and 2-methylheptacosane would give diagnostic peaks at m/z 350 : 351 in Figure 3A. However, in 4-methylheptacosane, one should find peaks at m/z $71 > 70 > 69$, which is what was found (Figure 3A). In 2-methylalkanes one would find m/z $71 > 70 < 69$. The former relationship between peaks is also observed in mass spectral scans 465 and 466 (not shown) but was not observed in scan 467 (Figure 3B). Furthermore, comparison of the mass spectrum of a synthetic standard 4-methylpentacosane to that of 4-methylheptacosane corroborated our interpretation vis-á-vis the fragmentation to produce an enhanced fragment ion at m/z 70.

As the mass spectrometer continued to scan across peak k, scans 467 and 468 were generated (Figures 3B and C, respectively). The interpretation of the mass spectra were consistent with the structural characteristics of the compounds shown in the figures (McCarthy et al., 1968). A small but prominent set of peaks at m/z 351 : 350 suggested that some 4-methylheptacosane was still present as well as 3-methylheptacosane (m/z 365; $C_{26}H_{53}^{+}$). Also present was 9,19-dimethylheptacosane (m/z 141 : 140, 295) which yielded two sets of peaks that were diagnostic of the branching points of a symmetrical molecule. Finally the two dimethylheptacosanes, which comprised the trailing edge of peak k, yielded a mass spectrum of the binary mixture shown in Figure 3D. The structural formulas in Figure 3C show the

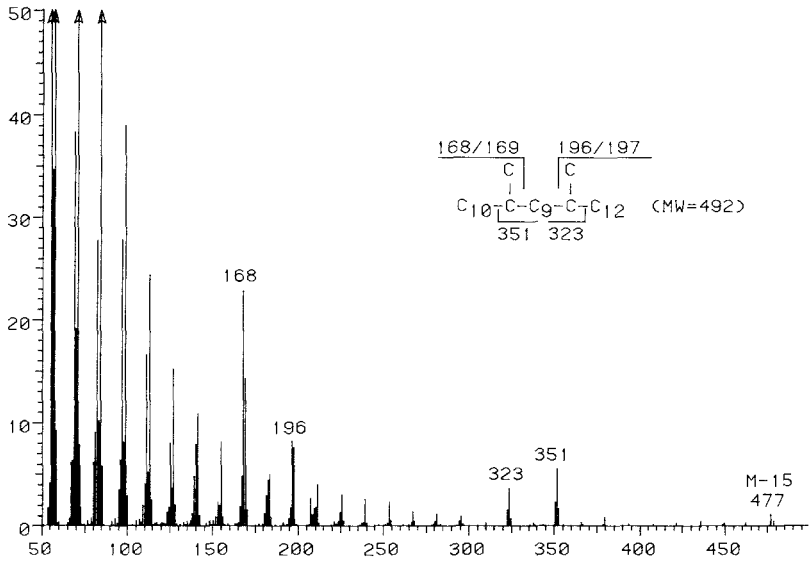


FIG. 5. EI mass spectrum of 11,21-dimethyltritriacontane.

points of fragmentation and the resulting masses obtained from scission of bonds about the branching carbons (Pomonis et al., 1980) for the two compounds 9,19- and 9,21-dimethylheptacosane.

Peak p was composed of a three-component mixture: 3-methylnonacosane, and 9,19- and 9,21-dimethylnonacosane, while peak t had only two compounds: 9,21- and 11,21-dimethylhentriacontane. Figure 4 shows the mass spectrum of this mixture. The molecular ion at m/z 464 is implied from the K_i of the GC peak and the prominent M-15 peak at m/z 449.

Finally, peak v was found to be composed of a single component: 11,21-dimethyltritriacontane (Figure 5). Characteristic fragments for the branching points were found at m/z 168 : 169 and 196 : 197 and also at m/z 323 and 351 as shown in the figure. The 11,21-dimethyltritriacontane contributed 5.5% to the total alkane mixture, thus making it the most abundant dimethylalkane in the cuticular extract.

SUMMARY

The alkane fraction of the stem weevil comprised 0.2% of the total body weight and 3% of the surface lipid fraction. The *n*-alkanes contributed 39.5% to the total alkane mixture, while branched alkanes contributed 60%. The branched alkane fraction was characterized by a significant number of

single-component GC peaks (9 of 25). The compounds were identified by GC-MS. Only seven dimethylalkanes were isolated and characterized from the stem weevil and in all cases the compounds had the methyl branches separated by 9 or 11 methylene units. These dimethyl alkanes were unique in that they were not accompanied by other dimethylalkanes having the more common spacing frequency of 3 methylene units. Three-methylene interrupted dimethylalkanes have always been in the majority concentration in literature reported cases. As the chain length increased (increase in molecular weight), the number of isomers in any mixture decreased so that the later eluting peaks were either two- or single-component.

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INTERCONVERSION OF VERBENOLS AND VERBENONE BY IDENTIFIED YEASTS ISOLATED FROM THE SPRUCE BARK BEETLE *Ips typographus*

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Abstract—Six yeast strains have been isolated and identified from the spruce bark beetle, *Ips typographus*. We have studied the ability of the yeasts to interconvert *cis*-verbenol, *trans*-verbenol, and verbenone. (1*S*)-*cis*-Verbenol is an active component in the aggregation pheromone of *Ips typographus*. The isolated *Candida molischiana*/*Hansenula capsulata* strain can convert both (1*R*)- and (1*S*)-*cis*-verbenol to verbenone. The *Candida nitratophila* strain converts (1*R*)-*cis*-verbenol to *trans*-verbenol and (1*S*)-*cis*-verbenol to verbenone. Some of the yeast strains produce 3-methylbutanol, 2-methylpropanol, and 2-phenylethanol after growth in Sabouraud medium.

Key Words—Microbial transformations, aggregation pheromones, microorganisms, yeasts, bark beetle, Coleoptera, Scolytidae, *Ips typographus*, *cis*-verbenol, *trans*-verbenol, verbenone, 3-methylbutanol, 2-phenylethanol.

INTRODUCTION

The pheromones of bark beetles include several compounds derived through oxygenation of monoterpene hydrocarbons from the host tree (cf. Wood, 1982). Hendry et al. (1980) have shown that *Ips paraconfusus* can produce two of its pheromone components, ipsenol and ipsdienol, from the host-tree compound myrcene. The ability of the beetle to produce *cis*-verbenol, the

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third of its pheromone components, from the host monoterpene hydrocarbon, α-pinene, has been shown by Renwick et al. (1976a). Pheromone components of bark beetles are found in the hindgut, but the site of their production has not been determined (Brand et al., 1975a; Byers, 1983).

Bark beetles are often associated with different types of bacteria, yeasts, and other fungi (see Barras and Perry, 1975, for a review of the literature from 1965 to 1974). Some associations may be more or less strict and some beetles even have special structures, termed mycetangia or mycangia (Am.), on various parts of the body which are used for carrying the microorganisms from one host to another (Francke-Grosmann, 1967). It is possible that the associated microorganisms take part in the synthesis of behavior-regulating chemicals used by bark beetles. Some work has been done to test this hypothesis. Brand et al. (1975a) isolated *Bacillus cereus* from the gut of *Ips paraconfusus* and showed that this bacterium was able to convert α-pinene to *cis*-verbenol, *trans*-verbenol, and myrtenol in vitro. A symbiotic fungus from the mycangium of *Dentroctonus frontalis* oxidizes *trans*-verbenol to verbenone (Brand et al., 1976). Byers and Wood (1981) noticed an inhibition in the sex-specific production of ipsenol and ipsdienol from myrcene in *Ips paraconfusus* beetles that had been fed the antibiotic streptomycin. However, synthesis of *cis*-verbenol from α-pinene was not inhibited. These works suggest that microorganisms have the ability to play a role in the pheromone synthesis in bark beetles.

The present work is an attempt to study the possibility of microorganisms associated with the spruce bark beetle, *Ips typographus*, taking part in pheromone synthesis. Any microbial synthesis of pheromone components may take place either in the beetle or on the walls of the beetle galleries. As mycangia are not found in all *Ips* beetles (Hodges et al., 1968), the gut of the beetle is one likely place to look for associated microorganisms. The components of the spruce bark beetle's attraction pheromone have been reported by Bakke (1976). They include *cis*-verbenol, ipsdienol, and 2-methyl-3-buten-2-ol. Our preliminary tests showed differences in the *trans*-verbenol–verbenone ratio after incubation. We therefore chose to start our microbial conversion studies on the oxygenated monoterpene compounds *cis*-verbenol, *trans*-verbenol, and verbenone. The population of *Ips typographus* used in this study has all three compounds in the hindgut. *cis*-Verbenol and *trans*-verbenol are major compounds, while verbenone is detected as a minor, or trace, compound (Birgersson et al., 1984).

METHODS AND MATERIALS

Biological Material and Isolation of Microorganisms. Ips typographus beetles were caught during their attraction to pheromone traps, in the prov-

ince of Värmland, Sweden. Their sex was determined according to Schlyter and Cederholm (1980). The guts were taken out with a pair of tweezers, put in a few drops of physiological saline and macerated with a glass rod. Aseptic techniques were used, but no attempt was made to disinfect the beetle surface, hence some of the isolated microorganisms may emanate from the abdominal part of the cuticula. Part of the saline solution containing possible microbes was streaked on three standard bacteriological media and one special medium with a platinum loop. Sabouraud agar, horseblood agar, and phloem agar at ambient temperature and Drigalski agar at 30°C were used. The phloem medium consisted of spruce phloem and bark cut in small pieces and extracted for approximately 4 hr in tap water. Before autoclaving, the suspension was filtered through paper and agar was added. Approximately 250 g phloem and bark was used per liter of water. Colonies that resulted were studied after three days of incubation. The isolates were deposited with the Culture Collection, University of Göteborg (CCUG). Six yeast cultures were kindly identified by the Centralbureau voor Schimmelcultures, Baarn, The Netherlands (CBS) or the National Collection of Yeast Cultures, Norwich, United Kingdom (NCYC) or by both laboratories.

Chemical Conversion Experiments. For studying the production of nonterpenoid metabolites, i.e., substances produced without monoterpenoids from spruce, 50 ml of liquid Sabouraud medium in Erlenmeyer flasks was heavily inoculated with the isolated microorganisms. After two days aerobic incubation with gentle shaking, 4 ml of the incubation mixture was extracted with 2 × 1 ml of pentane containing 2 μg heptyl acetate/ml and concentrated to a suitable volume. The added heptyl acetate was used for quantification of compounds by comparison of gas chromatographic (FID) peak areas.

Verbenol conversion experiments were carried out in Erlenmeyer flasks containing 50 ml of liquid Sabouraud medium. The terpene to be studied was added dissolved in 95% ethanol, 3–4 mg terpene/ml. Immediately after heavy inoculation with the respective microorganism, 250 μl of the ethanolic solution was added. After one day at ambient temperature with slight shaking, another 250 μl ethanol solution was added. Final ethanol concentration in the incubation medium was 1% (v/v). After a total incubation period of two days, 5 ml of the incubation mixture was typically extracted with 3 × 1 ml of pentane containing 2 μl heptyl acetate/ml. (1*R*)-*cis*-Verbenol (>95% 1*R*) and (1*S*)-*cis*-verbenol (>95% 1*S*) were both obtained from Fluka AG.[3] (1*R*)-*trans*-Verbenol (>70% 1*R*) was obtained from SCM Organic Chemicals, Florida, (1*S*)-*trans*-verbenol (>95% 1*S*) from Borregaard AS, Norway. The *trans*-verbenols used were contaminated with approximately 15% *cis*-

[3]Throughout the text we use the recommended IUPAC numbering system giving numbers 1 and 5 to the asymmetric carbon atoms in the four-membered ring and number 4 to the hydroxylated carbon atom.

verbenol. (*R*)- and (*S*)-verbenone were obtained from Borregaard. The method of König et al. (1982) was used for determination of the enantiomeric composition of the verbenols.

Chemical Analysis. Quantification of extracted terpenoids and other compounds was done with a Hewlett-Packard 5830A gas chromatograph equipped with a Superox FA coated glass capillary column (25 m × 0.25 mm ID, $df = 0.4\ \mu m$, 2,500 tp/m). Identification of compounds was made by comparison of mass spectra and gas chromatographic retention times with those of authentic reference compounds. The mass spectra were obtained on a Finnigan 4021 GC-MS system. The column used was 50 m ID 0.25 mm Superox FA $df = 0.54\ \mu m$, 2,500 tp/m. Identical temperature programing was used for both quantification and identification. Initial temperature was 60°C, held for 2 min, and then programed at 5°C/min up to 200°C and held for 20 min. Both columns were prepared at the Department of Ecological Chemistry, Göteborg.

RESULTS

Isolation and Identification of Microorganisms. The aerobic, heterotrophic microbial flora of hindguts from 20 bark beetles was investigated. Relatively few colonies grew on the four solid culture media. More colonies grew on the phloem agar and Sabouraud agar media than on the two other media, suggesting the absence of medical bacteria but the presence of yeasts. No striking differences between colony forming unit (CFU) counts in male and female beetles were noticed.

Primarily 28 colonies from different beetle samples and from different media were selected. These 28 isolates were recultured on all four solid media, checked for pure culture, culture morphology, micromorphology, and Gram reaction. Eighteen of the isolates were considered to have different phenotypes and were retained for the conversion experiments. These strains were deposited with the CCUG.

Microorganisms that had altered the pentane-extractable terpenoid blend in liquid phloem medium after 48 hours incubation, were considered of interest for further conversion experiments. Table 1 shows the isolated yeast strains used for conversion experiments. The *Hansenula holstii* strain was determined as nonsporogenous by CBS. Wickerham and Burton (1961) states that *Candida molischiana* is the imperfect form of *Hansenula capsulata.* Thus, there is no discrepancy between the results from CBS and NCYC regarding this strain.

Incubation Experiments. GC-MS analysis of pentane extracts from yeast incubations in Sabouraud medium without added monoterpenes often re-

TABLE 1. YEAST STRAINS ISOLATED FROM HINDGUT OF *Ips typographus*[a]

Strain in CCUG	Identification by CBS	Identification by NCYC	Designation in this publication
EF 11125	—	*Cryptococcus laurentii* (Kufferath) Skinner var. *magnus* Lodder et Kreger-van Rij	Cl
EF 11128	*Hansenula holstii* Wickerham	—	Hh
EF 11139	*Candida nitratophila* (Shifrine et Phaff) Meyer et Yarrow	*Candida nitratophila* (Shifrine et Phaff) Meyer et Yarrow	Cn
EF 11140	*Candida molischiana* (Zikes) Meyer et Yarrow	*Hansenula capsulata* Wickerham	Cm/Hc
EF 11141	*Cryptococcus albidus* (Saito) Skinner var. *diffluens* (Zach) Phaff et Fell	—	Ca
EF 11142	—	*Candida diddensii* (Phaff, Mrak et Williams) Fell et Meyer	Cd

[a]CCUG = Culture Collection, University of Göteborg, CBS = Centraalbureau vor Schimmelcultures, NCYC = National Collection of Yeast Cultures.

vealed 3-methylbutanol and 2-phenylethanol. Different amounts were produced by the different yeast strains. *Candida diddensi*, *Candida molischiana*/*Hansenula capsulata*, *Cryptococcus albidus* var. *diffluens* and *Cryptococcus laurentii* var. *magnus* produced medium or small amounts and, in some cases, especially for 2-phenylethanol, not detectable amounts. *Candida nitratophila* and *Hansenula holstii* produced more of both 3-methylbutanol and 2-phenylethanol under our incubation conditions than the other isolated microorganisms. *Hansenula holstii* made the largest amounts of these compounds and also produced 2-methylpropanol in minor amounts, which showed up as a distinguishable chromatographic peak (c.f. Figure 1, below). Ethanol and propanol were sometimes detected in extracts from different incubations, including controls without added microorganisms.

Figure 1 shows two gas chromatograms obtained from pentane extracts of Sabouraud medium. The upper chromatogram (Figure 1A) is from an incubation with *Hansenula holstii* and the lower chromatogram (Figure 1B) is from a control without added microorganisms. Peaks 1 (ethanol) and 2 (propanol) are probably impurities. Peak 6 is a known impurity from the solvent. Peak 5 corresponds to added heptyl acetate, and peaks 3, 4, and 7 correspond to 2-methylpropanol, 3-methylbutanol, and 2-phenylethanol, respectively. The amounts of extractable 3-methylbutanol and 2-phenylethanol, produced by *Hansenula holstii* after two days incubation in 50 ml of

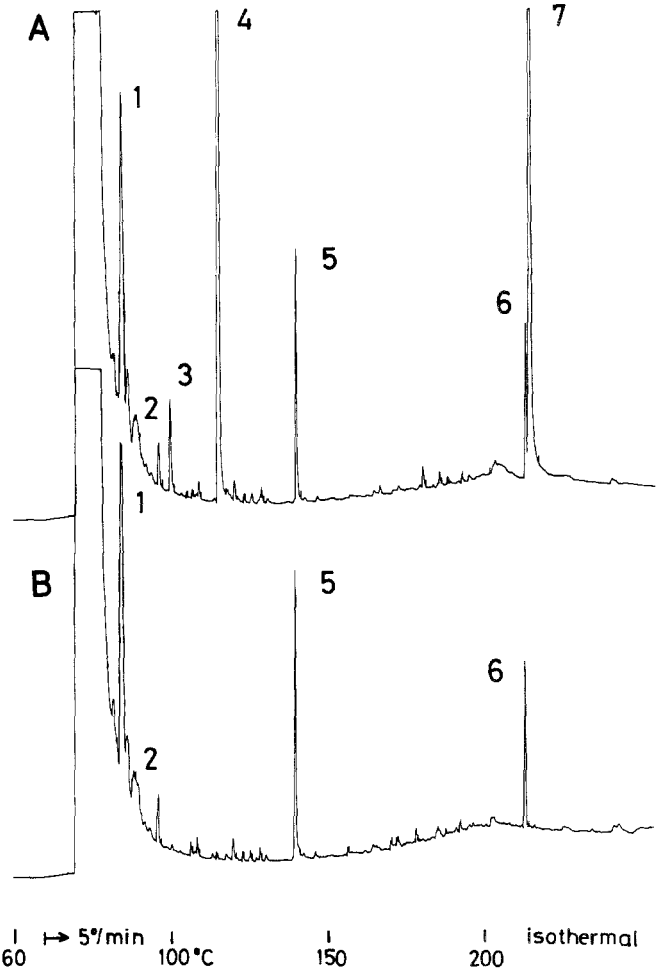

FIG. 1. Gas chromatograms of pentane extracts from Sabouraud medium. (A) Inoculated with *Hansenula holstii*; (B) control without microorganisms. Analytical conditions: 60°C isothermal for 2 min 60–200°C at 5°/min, from 200°C isothermal. (1) Ethanol, (2) propanol, (3) 2-methylpropanol, (4) 3-methylbutanol, (5) heptyl acetate, added reference, (6) impurity from the solvent, (7) 2-phenylethanol.

Sabouraud medium (Figure 1), have been estimated by comparing the gas chromatographic peak areas of the two substances to that of added heptyl acetate. The amounts are in the range of 100–600 μg and 50–400 μg, respectively.

Conversion experiments have been carried out with all of the yeast strains summarized in Table 1. (1*S*)- and (1*R*)-*cis*-verbenol, (1*S*)- and (1*R*)-*trans*-verbenol and a mixture of (*S*)- and (*R*)-verbenone have been used as sub-

strates for the conversions. The relative amounts of *cis*-verbenol, *trans*-verbenol, and verbenone in pentane extracts from Sabouraud medium alone and the medium inoculated with different yeasts, have been examined, after two days of incubation, by GC and GC-MS. The sum of the gas chromatographic peak areas from *cis*-verbenol, *trans*-verbenol, and verbenone is about the same after all incubations but in some cases other oxygenated monoterpenes, for instance myrtenol, have been noticed. Some of these compounds may originate from the added monoterpene. The results from the conversion experiments are given in Figure 2. The amounts of *cis*-verbenol, *trans*-verbenol, and verbenone are assumed to be equal to the amount of oxygenated monoterpene added. The difficulties in the extraction procedure, explained in the discussion part, are so severe that no attempts have been made to report the absolute amounts of these three substances. None of the isolated yeasts produce either *cis*- or *trans*-verbenol from a mixture of (*S*)-and (*R*)-verbenone under the incubation conditions used (Figure 2A). Incubation of the yeasts with *trans*-verbenol did not produce any striking differences in the proportions of *cis*-verbenol, *trans*-verbenol, and verbenone. *Candida molischiana*/*Hansenula capsulata* (Cm/Hc) showed somewhat higher relative amounts of verbenone compared to *trans*-verbenol after the incubation. *Candida nitratophila* (Cn) seems to convert (1*S*)-*trans*-verbenol to verbenone more readily than (1*R*)-*trans*-verbenol (compare Figure 2B and C). More pronounced differences are shown after incubations with *cis*-verbenol. All the isolated yeast strains are able to convert *cis*-verbenol. In some cases no detectable amount of *cis*-verbenol was found after the incubation (compare Figure 2D and E). *Cryptococcus laurentii* var. *magnus* (C1), *Hansenula holstii* (Hh), and *Cryptococcus albidus* var. *diffluens* (Ca) partly convert *cis*-verbenol to *trans*-verbenol and partly to verbenone. *Candida diddensii* (Cd) produces more verbenone than *trans*-verbenol from added *cis*-verbenol. More verbenone than *trans*-verbenol is also produced from *cis*-verbenol in incubations with *Candida molischiana*/*Hansenula capsulata* (Cm/Hc), especially, if (1*R*)-*cis*-verbenol is used. Stereoselective conversion is achieved with *Candida nitratophila* (Cn). (1*S*)-*cis*-Verbenol is converted to verbenone, and (1*R*)-*cis*-verbenol is converted to *trans*-verbenol (Figure 2D and E).

DISCUSSION

All of our identified yeast strains have been previously isolated from environmental sources, cf. Lodder (1970) and Callaham and Shifrine (1960). Most of them have been isolated from bark beetles or frass from coniferous trees. The interesting species *Candida nitratophila* has been isolated from *Dendroctonus monticolae* (Shifrine and Phaff, 1956). *Hansenula holstii* and *Hansenula capsulata* have often been isolated from bark beetles, probably

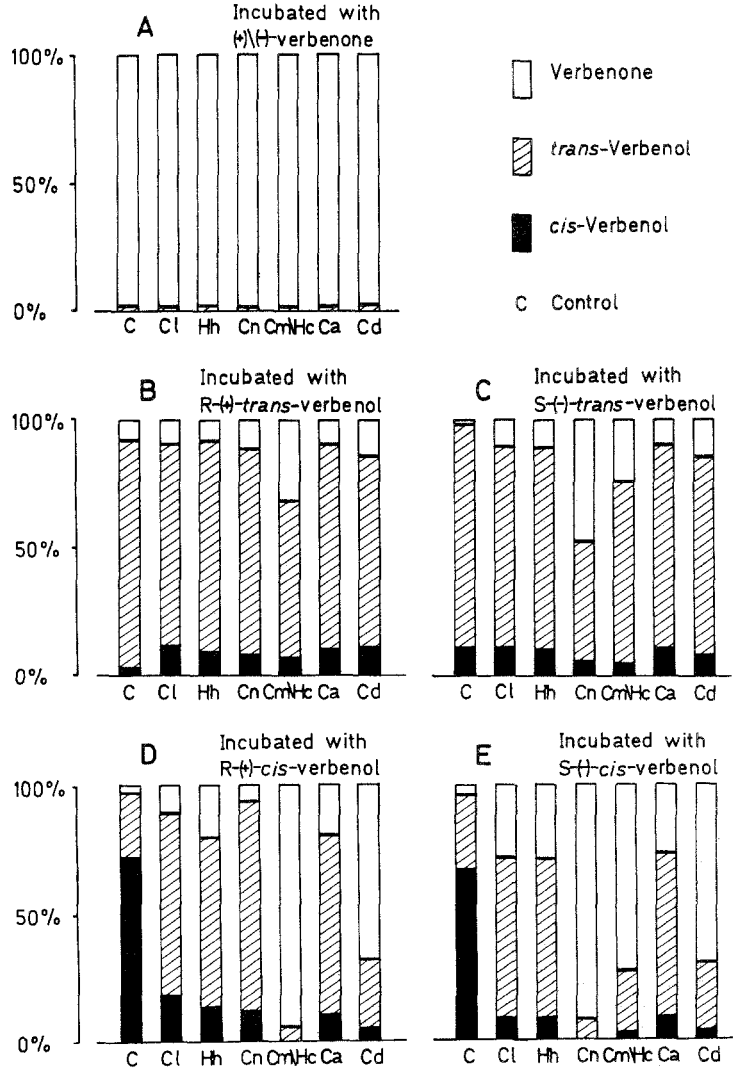

FIG. 2. Summary of incubation experiments with the six isolated yeasts, given in Table 1. The relative proportions between verbenone, *trans*-verbenol and *cis*-verbenol, after incubations with the corresponding chiral compounds, are given for each yeast.

because these yeasts produce phosphomannans which make them adhere to the beetles (Wickerham and Burton, 1961). Finding the yeasts species we have isolated associated with *Ips typographus* is therefore not surprising. Whether the yeasts always occur together with the beetles is not clear at this point. Several of the isolated yeast strains can assimilate methanol (B. Kirsop,

personal communication). This is in accordance with the findings of Lee and Komagata (1980).

The production of 2-phenylethanol in yeast incubations has been reported earlier by several authors, for instance Drews et al. (1966) and Narayanan and Ramananda Rao (1974). Soumalainen (1969) has reported the production of 2-phenylethanol and 3-methylbutanol from sucrose by fermenting yeasts. 3-Methylbutanol has been reported as one of the major volatile products by a basidiomycete associated with *Dendroctonus frontalis* (Brand and Barras, 1977).

Brand et al. (1977) reported the production of 3-methylbutanol, 2-phenylethanol, ethanol, *n*-propanol, and 2-methylpropanol by a *Hansenula holstii* strain isolated from *Dendroctonus frontalis*. Of our isolated strains, *Hansenula holstii* produces the largest amounts of 3-methylbutanol and 2-phenylethanol. 2-Methylpropanol is also produced by this strain. Whether our strain also produces ethanol and propanol has not been ascertained. Ethanol is used for cleaning the injection syringes, and it sometimes shows up as a peak in the gas chromatograms. Propanol is an impurity in the ethanol. Brand et al. (1977) also report an enhancing effect from combinations of 3-methylbutanol and 2-phenylethanol on the response of walking male *Dendroctonus frontalis* beetles to their pheromone components. Renwick et al. (1976b) report higher attraction of *Ips paraconfusus* to male infested logs in olfactometer tests if 2-phenylethanol is added. 2-Phenylethanol, but not 3-methylbutanol, has been found in guts of male *Ips typographus* from the same populations that were used for isolation of our yeasts (Birgersson et al., 1984). *Ips typographus* uses 2-methyl-3-buten-2-ol as a pheromone component (Bakke et al., 1977). *Ips cembrae* has 3-methyl-3-butenol in its pheromone blend (Stoakley et al., 1978). Both 2-methyl-3-buten-2-ol and 3-methyl-3-butenol have structural similarities with 3-methylbutanol, which might be a precursor for the production of these short-chain alcohols.

Quantifying the amount of 3-methylbutanol and 2-phenylethanol produced by the different yeast strains is accompanied with several difficulties. One may be different growth rates and amounts of yeast produced at different incubations, but the extraction procedure probably imposes the most severe restriction on any accurate quantification. The partition of compounds between the water and pentane phases is uncertain, and the recovery of added pentane after extraction is sometimes as low as 5–10%, probably because of extensive "foaming."

A schematic representation of the isolated yeasts' ability to interconvert *cis*-verbenol, *trans*-verbenol, and verbenone is given in Figure 3. The thicker the lines, the more complete are the transformations. It is interesting to note that the amount, and in some cases even the type, of substance produced is dependent on the absolute configuration of the starting material used in the incubation. The most striking example is the ability of the isolated *Candida*

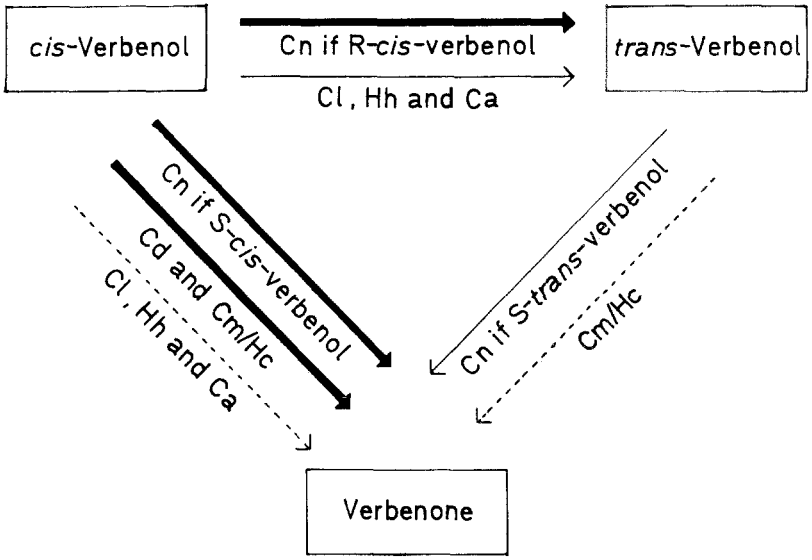

FIG. 3. Schematic representation of the microbial transformations between *cis*-verbenol, *trans*-verbenol and verbenone. Arrows indicate which substances are produced from the single compound added in each incubation. The scheme does not imply pathways involving more than one step. Thick lines denote more complete transformations. For designations, see Table 1.

nitratophila (Cn) strain to convert the optical isomers of *cis*-verbenol to different products. (1*S*)-*cis*-Verbenol is converted to verbenone and (1*R*)-*cis*-verbenol is converted to *trans*-verbenol. The *trans*-verbenol with the same geometry at carbon atoms 1 and 5, i.e., (1*S*)-*trans*-verbenol, as in (1*S*)-*cis*-verbenol is also converted to verbenone to a higher extent by the *Candida nitratophila* strain than is the (1*R*)-isomer. Interpretation of the results from incubations with *trans*-verbenol is complicated by 15% of *cis*-verbenol present as an impurity in the *trans*-verbenol used. While both (1*S*)-*cis*-verbenol and (1*S*)-*trans*-verbenol are converted to verbenone by the *Candida nitratophila* strain, one may perhaps assume that the configuration at carbon atom 4, the carbon atom carrying the hydroxy group, is less important for the transformation than the configuration at carbon atoms 1 and 5. *cis*-Verbenol, and perhaps *trans*-verbenol, can also be converted to verbenone by the *Candida molischiana*/*Hansenula capsulata* (Cm/Hc) strain, but the dependence on the absolute configuration is not clear. The (1*S*)-enantiomer is reported by Krawielitzki et al. (1977) as the biologically active form of *cis*-verbenol in the pheromone of *Ips typographus*. Verbenone, on the other hand, is considered by Bakke (1981) to be an inhibitor of the response of *Ips typographus* to their aggregation pheromone. The role of *trans*-verbenol in the aggregation of *Ips typographus* is more doubtful (Dickens, 1981).

Our results thus show that a yeast (Cn) isolated from *Ips typographus* has the ability to stereoselectively convert the active form of *cis*-verbenol, used by the beetle as an attracting substance, to verbenone inhibiting the response to the attraction pheromone. The optical antipode of *cis*-verbenol is not converted to the inhibiting verbenone under identical laboratory conditions. Whether this conversion occurs in nature is not known. Oxygenation of α-pinene to *cis* and *trans*-verbenol, reported by Klimetzek and Francke (1980), may be a means of detoxification (White et al, 1980) used by the beetles and associated microorganisms to overcome the toxic properties of the resin used as a means of resistance by the tree. As suggested by Brand et al. (1976), microorganisms introduced into the galleries by the beetle may convert the detoxification product (1*S*)-*cis*-verbenol, used by the beetles as an attractant, to the inhibiting product verbenone. This conversion might reach its maximum role when the resistance of the tree is sufficiently weakened, i.e., when the microorganisms have multipled, and then the higher level of verbenone would function to reduce attraction and cause the termination of the attack on the tree.

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DITERPENE COMPOSITION OF DEFENSE SECRETION OF FOUR WEST AFRICAN *Trinervitermes* SOLDIERS

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Abstract—The diterpene composition of the defensive secretions of minor and major soldiers is described for four sympatric *Trinervitermes* from the Ivory Coast. The degree of intra- and interspecific similarity between the secretions is assessed and related to available information on soldier behavior. A significant chemical dimorphism is observed between minor and major soldiers of *T. geminatus* and *T. togoensis*. Both species build extensive foraging trails in the open air. During these excursions, the workers are protected by numerous minor soldiers, but only a few major ones. Internest comparison in *T. geminatus* suggests that the differences between minor and major soldiers is as large within a nest as between different nests. By, contrast, in *T. trinervius* and *T. oeconomus* both types of soldiers produce very similar secretions. The foraging habits of those species are far more cryptic. The first species builds earthen tunnels, and the second one forages in close proximity to its nest under cover of vegetation.

Key Words—Termites, Isoptera, Termitidae, *Trinervitermes*, defense secretion, trinervitane, diterpenes.

INTRODUCTION

The frontal gland secretion of advanced nasute termite soldiers (Termitidae, Nasutitermitinae) consists of a mixture of cembrane-derived diterpenes dissolved in monoterpene hydrocarbons. Recently, important progress has been

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made in the structural determination of individual diterpenes (Prestwich, 1979a,b; Dupont et al., 1981). In particular, the structure of eight trinervitane diterpenes isolated from *Trinervitermes gratiosus* (Prestwich, 1977, 1978) and *T. bettonianus* (Prestwich and Chen, 1981) have been elucidated.

Intra- and interspecific variations of the diterpenoid contents of the secretions of these two species have also been studied (Prestwich, 1978; Prestwich and Chen, 1981). Moreover, Prestwich (1979a) pointed out, without details, that the *Trinervitermes* species, *T. rapulum*, *T. geminatus*, *T. occidentalis*, *T. oeconomus*, *T. togoensis*, and *T. trinervius* all possess C_{10} hydrocarbons and trinervitane derivatives in varying ratios "often with greater difference between major and minor soldiers of the same species than among major soldiers of different species." It has been suggested that these chemical variations may be viewed as indirect measures of the genetic variance within the genus (Prestwich and Chen, 1981). They are probably due to different selective regimes and evolutionary opportunities and do not necessarily reflect different levels of fitness (Prestwich and Collins, 1981).

In the course of our chemical investigations of the defense secretion of termites, we have examined the diterpene content of major and minor soldiers from West Africa *Trinervitermes*, namely: *T. geminatus*, *T. oeconomus*, *T. togoensis*, and *T. trinervius*. The degree of similarity in the diterpene contents of soldier secretions, from different nests of the same population or from different sympatric species, is assessed and discussed in the light of available biological information on foraging behaviors.

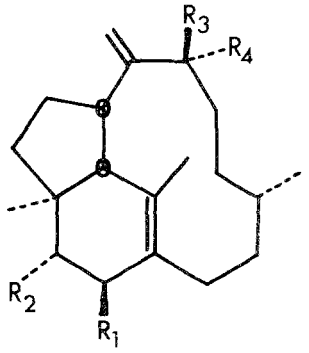
METHODS AND MATERIALS

Collection Procedures. All the materials were collected at Lamto, Ivory Coast, 6°13′N, 5°02′W, about 100 m altitude, in a derived savanna, Savane à *Borassus aethiopum*. Soldiers were collected early in the morning, when a large part of the population was in the epigeous parts of the nests. A small hole (1–2 cm^2) was made in the wall, close to the top of the nest; then scratching and blowing made the soldiers rush out of their nest. Major and minor soldiers were collected separately by means of two aspirators ("pooters"). This worked for about half an hour. If more soldiers were required, it was necessary to return to the same nest on successive days. The soldiers were preserved in pure methanol or ethanol.

Analytical Methods. Each sample was crushed and extracted exhaustively with dichloromethane. The extract was filtered on silica gel (eluent: hexane–ethyl acetate 7:3) and the filtrate analyzed by glass capillary GLC and TLC on precoated silica gel plates using hexane–ethyl acetate 7:3 as eluent. The GLCs were performed on a Varian 3700 gas chromatograph equipped

with a flame ionization detector and a WCOT capillary glass column (OV-1, 25 m × 0.5 mm, high load, N_2, T = 230°C). The relative proportions of the different diterpenes were determined by triangulation.

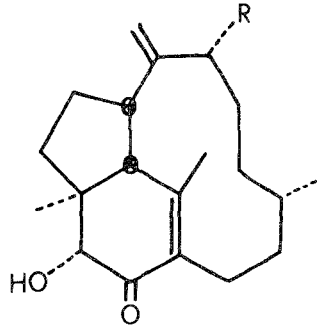
The identity of the diterpenes I, III–VII (Figure 1) was established by comparing retention times and R_f with those of authentic samples. Moreover, the pooled extracts of *T. geminatus* were chromatographed on silica gel (eluent: hexane–ethyl acetate 8:2). This led to the isolation of compounds I, IV, V, VI, and VII, the identities of which were confirmed on the basis of their spectral properties ([^{1}H]NMR, MS, and IR). The novel diterpene II was isolated, besides the derivatives I, IV, V, and VI, by successive silica gel



I R_3 = OH, R_1 = R_2 = R_4 = H

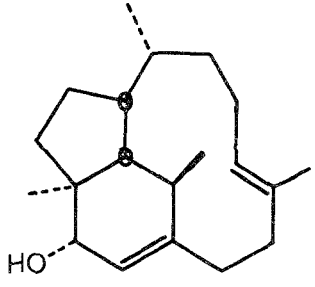
IV R_1 = R_2 = OH, R_3 = R_4 = H

VI R_1 = R_2 = OH, R_3 = H, R_4 = OAc

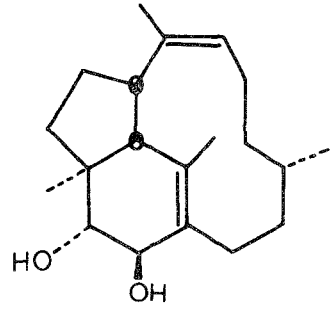


III R = H

VII R = OAc



II



V

FIG. 1. Trinervitane diterpenes isolated from the four West African *Trinervitermes* species studied.

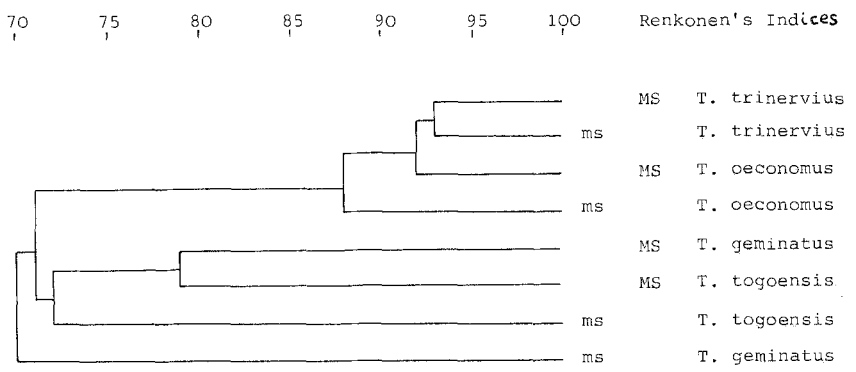


FIG. 2. Similarity between major (MS) and minor (ms) soldiers secretions of the four sympatric West African *Trinervitermes* species studied.

column chromatographies (eluent: hexane–ethyl acetate 8:2, then chloroform–ether 9:1), from pooled extracts of *T. oeconomus* and *T. trinervius*.

Intra- and Interspecific Comparisons. The level of similarities between the percentages of diterpenes found in two different samples was evaluated by calculating the Renkonen (1938) similarity indices (Figure 2) also referred to as the Raabe indice (Southwood, 1971). Statistical significance of similarities (or dissimilarities) observed between minor and major soldiers of the same species was checked by comparing relevant series of Renkonen similarity indices using the Mann-Whitney U test (Siegel, 1956). Such analysis was only possible in *T. geminatus*, of which enough samples were available.

Intra- and interspecific similarities were also visualized by constructing a dendrogram (Sneath, 1957), grouping castes and species according to their level of similarity, calculated by the Renkonen indices. In this analysis, a mean composition for each soldier caste is first calculated when more than one sample is present.

RESULTS

Seven different trinervitane diterpenes have been identified in the defense secretion of the four *Trinervitermes* examined. Three minor components (U_1, U_2, U_3) remain unidentified. Five of the diterpenes (I, III, IV, V, and VI) have already been reported from two previously studied species of *Trinervitermes: T. bettonianus* (Prestwich and Chen, 1981) and *T. gratiosus* (Prestwich, 1977). The enone VII is already known from *Nasutitermes princeps* (Dupont et al., 1981), but it is the first time that it has been isolated from a *Trinervitermes* species. Compound II is a novel derivative, the structure of which has recently been established by X-ray diffraction analysis (Braekman et al., 1983). The

distribution of these diterpenes in the different secretions is reported in Table 1.

Comparison of the diterpene content of major and minor soldiers of five different mounds of sympatric *T. geminatus* shows that all these secretions are qualitatively closely related; quantitative variations are observed between the two types of soldier. For example, in the minor soldiers, the derivative VI is the principal diterpenoid constituent of the secretion, while in the major soldiers its concentration is about the same as those of I and IV. Statistical analysis on Renkonen's indices indicates that: (1) Between different nests, the highest similarity is found within the major soldiers (Table 2, line 1). (2) When comparing the different nests, minor soldiers are more similar to minor soldiers than to major ones (Table 2, line 2). (3) The difference between minor and major soldiers is as large within a nest as between different nests (Table 2, line 3).

From these observations it follows that soldier caste prevails over society membership in *T. geminatus.* This is in agreement with the observation made on the East African species *T. gratiosus* and *T. bettonianus* (Prestwich, 1977; Prestwich and Chen, 1981). Nevertheless, considerable overlapping exists between the composition of the secretions of major and minor soldiers from different nests. For example, the secretion of minor soldiers from nest d corresponds to the secretion of the major soldiers from nest b more than any other minor soldier secretion.

This conclusion cannot be generalized to all *Trinervitermes* species as demonstrated by the dendrogram depicted in Figure 2. Indeed, *T. togoensis* resembles I. *T. geminatus* in that the secretions of major and minor soldiers are markedly different. On the contrary, the secretions of both castes are similar in *T. trinervius* and *T. oeconomus* which resemble each other in the chemistry of their defensive secretions.

DISCUSSION

The diterpene content of the *Trinervitermes* defensive secretion appears both conservative in its general principle and quite diverse in its details. Indeed, all six chemically studied species contain closely related trinervitane derivatives, essentially differentiated by the nature of the substituents at C-2, C-3, and/orC-9, as well as by the position of the double bonds. However, if the relative proportions of these diterpenes are considered, a great diversity of content is observed. Variations are noted not only between the different species, but also between allopatric populations of the same species (Prestwich, 1978; Prestwich and Chen, 1981) and between minor and major soldiers of the same nest. Moreover, since these quantitative variations are confined to a small number of related derivatives (about 10), some fortuitous resemblances may

TABLE 1. DISTRIBUTION OF DITERPENES IN DIFFERENT SECRETIONS[a]

Species	Nest	Soldiers		Percentages of diterpenes									
		Size	Number	I	II	U_1	III	IV	U_2	V	VI	VII	U_3
T. geminatus	a	M	2511	21.9			0.4	42.2		7.6	27.8		
	a	m	2563	7.4			0.6	10.5		0.6	61.7	4.3	14.8
	b	M	6702	22.6			0.4	31.9		4.8	40.3		
	b	m	4945	7.4			0.6	22.2		2.8	56.8	5.1	5.1
	c	M	1083	8.9			0.4	40.0		6.2	44.4		
	c	m	2260	4.9			0.7	9.2		3.5	70.4	7.0	4.2
	d	M	1618	25.2			0.5	45.0		6.3	23.3		
	d	m	1466	14.4			3.3	34.6		3.7	41.2	2.9	
	e	M	693	28.4		0.5	0.5	15.4		5.5	49.8		
	e	m	506	13.1		0.5	4.0	24.7		3.0	50.5	4.0	
T. trinervius	f	M	1123	25.1	20.2	3.8	1.1	18.0		27.3	4.4		
	f	m	517	27.3	19.1	4.4	3.8	16.9		23.0	5.5		
T. oeconomus	g	M	242	22.2	21.7	1.5	0.3	25.3	3.3	22.2	3.5		
	g	m	1258	21.2	20.4	11.8	0.3	13.9	2.7	29.5	0.3		
	h	M	385	19.4	19.7	10.1	0.3	16.1	2.1	29.9	2.4		
	h	m	315	11.6	15.6	5.8	2.4	18.4	5.4	34.0	6.8		
T. togoensis	i	M	266	24.3			0.4	36.8		23.5	15.1		
	i	m	517	24.3		7.1	8.5	19.5	0.2	14.1	13.6	3.6	9.0

[a]M = major soldiers; m = minor soldiers; U_1, U_2, U_3: unidentified compounds.

TABLE 2. RESULTS OF MANN-WHITNEY U TESTS APPLIED TO SIMILARITY RENKONEN'S INDICES BETWEEN DITERPENE COMPOSITIONS FROM *Trinervitermes geminatus* SOLDIERS[a]

A	B	C	Number (and range) of Renkonen's indices. $N_{AB}(R_{AB})$	$N_{AC}(R_{AC})$	U	P
Major soldiers	Major soldiers from other nests	Minor soldiers from other nests	10 (70–94)	20 (41–91)	38.5	<0.01
Minor soldiers	Minor soldiers from other nests	Major soldiers from other nests	10 (62–88)	20 (41–91)	57	<0.05
Major soldiers	Minor soldiers from same nest	Minor soldiers from other nests	5 (47–82)	20 (41–91)	47.5	>0.05

[a]Null hypothesis (H_0): the Renkonen's indices first degree between A and B and second degree between A and C have the same distribution. Alternative hypothesis (H_1): the Renkonen's indices between A and B are higher than those between A and C. If $P < 0.05$, H_0 is rejected in favor of H_1.

appear: (1) Very similar patterns are observed between major soldiers of our *T. togoensis* sample and samples of major soldiers of *T. bettonianus* (Prestwich and Chen, 1981) and minor soldiers of *T. gratiosus* (Prestwich, 1978). (2) The diterpene contents of the major soldiers of *T. geminatus* and *T. gratiosus* nests are almost identical (Prestwich, 1978).

According to Prestwich and Chen (1981), greater differences are observed between the two types of soldiers of the same species than between major soldiers of different species. In light of the present results, it is clear that this statement cannot be generalized to all *Trinervitermes* species. They seem to fall in two groups: (1) One group shows a significant difference in the composition of diterpenes between minor and major soldiers (e.g., *T. gratiosus*, *T. bettonianus*, *T. geminatus*, and *T. togoensis*). It is worth noting that in those species showing a strong caste difference, the secretion of the minor soldiers is always more complex than that of the major ones. (2) In the other group, both types of soldiers produce very similar secretions (e.g., *T. trinervius* and *T. oeconomus*). Interestingly, the secretions of these two species are also very similar to each other.

West African *Trinervitermes* can also be divided into two groups characterized by their foraging habits. *T. oeconomus* do not store grass fragments in their mounds, do not build extensive foraging trails, and collect grass in close proximity to their nest. *T. trinervius* is known to store grass fragments (Sands, 1961) but not at Lamto (Josens, unpublished observation). Their foraging columns may extend over several meters, but are covered by earth tunnels. By

contrast, *T. geminatus* and *T. togoensis* store grass in their nest. Both are well known to carry out foraging excursions at night in the open air, extending as far as 10 m from the nest. The soldiers participate together with the workers in these excursion, the minor ones being in large majority. They stand along both sides of the column, approximately one every centimeter, their noses pointing towards the outside of the column (Sands, 1961). It is remarkable that a strong chemical dimorphism in soldier defensive secretions is observed in those species which exhibit a marked behavioral specialization in the soldiers during foraging. Moreover, the secretion is more complex in the small soldiers which participate in the foraging excursions in the open air and which will then face a large array of enemies.

Unfortunately, no ethological information is available on the foraging habits of *T. bettonianus* and *T. gratiosus*. More data are needed to assess the general nature of this correlation and to investigate its possible biological meaning.

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SEX ATTRACTANT FOR CURRANT CLEARWING MOTH *Synanthedon tipuliformis* (CLERCK) (LEPIDOPTERA: SESIIDAE)

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Abstract—The currant clearwing moth, *Synanthedon tipuliformis* (Clerck) (Lepidoptera: Sesiidae), is a pest in many parts of the world. In field tests it was found that (*E*,*Z*)-2,13-octadecadien-1-ol acetate attracts males of this species. The synthesis of this compound and of its geometrical isomer (*Z*,*Z*)-2,13-octadecadien-1-ol acetate is described.

Key Words—Attractant, *Synanthedon tipuliformis*, Lepidoptera, Sesiidae, (*E*,*Z*)-2,13-octadecadien-1-ol acetate.

INTRODUCTION

The currant clearwing moth, *Synanthedon tipuliformis* (Clerck) (Lepidoptera: Sesiidae) is a serious pest of red and black currant and gooseberry not only in Europe, but also in Asia, North America, and Australia. The caterpillars penetrate the shoots, where they live for one year, then make openings, pupate, and emerge. Shoots die when they are severely infested.

Traps baited with a sex attractant would be a useful means for selectively following the flight activity of this pest insect to establish the optimal time for control measures and assess the efficacy of such measures.

Males of the many species of clearwing moths are attracted by (*E*,*Z*)- or (*Z*,*Z*)-3,13-octadecadien-1-ol, or by mixtures of these substances and/or their acetates. Several combinations of these compounds in different ratios

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have been tested in currant orchards in The Netherlands and in Hungary without success. In electroantennographic tests, male antennae responded strongly to (*E*,*Z*)-3,13-octadecadien-1-ol acetate (abbreviated *EZ*3,13-18:Ac), but it was only slightly attractive in the field.

Recently, Schwarz et al. (1983) discovered that the positional isomer (*E*,*Z*)-2,13-octadecadien-1-ol acetate (abbreviated *EZ*2,13-18:Ac) is the pheromone of the grape root borer, *Vitacea polistiformis* (Harris) (Lepidoptera: Sesiidae). With the same compound, they caught large numbers of male *Synanthedon acerrubri* (Engelhardt).

These results prompted us to synthesize this compound and (*Z*,*Z*)-2,13-octadecadien-1-ol acetate (*ZZ*2,13-18:Ac) and to investigate their efficacy and that of the corresponding alcohols as attractants for *S. tipuliformis*.

METHODS AND MATERIALS

The syntheses of *EZ*2,13-18:Ac and *ZZ*2,13-18:Ac are outlined in Figures 1 and 2. The corresponding alcohols *EZ*2,13-18:OH and *ZZ*2,13-18:OH were obtained as intermediates. Details about similar reactions were described earlier (Schwarz and Waters, 1972; Voerman and Rothschild, 1978; Voerman, 1979a,b). Any surplus $LiAlH_4$ remaining after the *trans*-reduction of (*Z*)-13-octadecen-2-yn-1-ol was destroyed with EtOAc after the addition of ether. Then, while continuously stirring, n ml H_2O, n ml of 15% NaOH solution in water, and 2.5 n ml H_2O were slowly added, dropwise, in

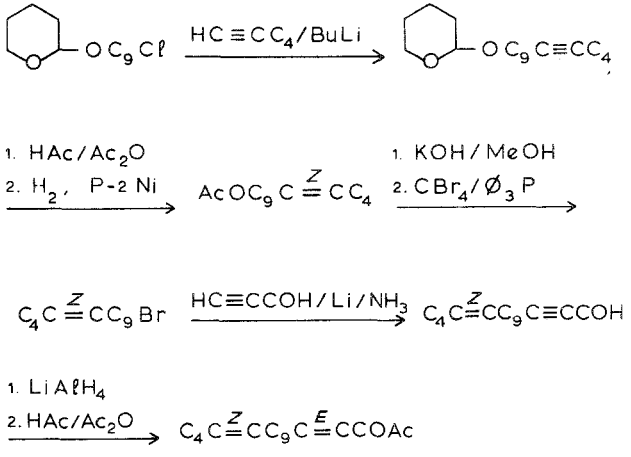

FIG. 1. Synthesis of (*E*,*Z*)-2,13-octadecadien-1-ol acetate.

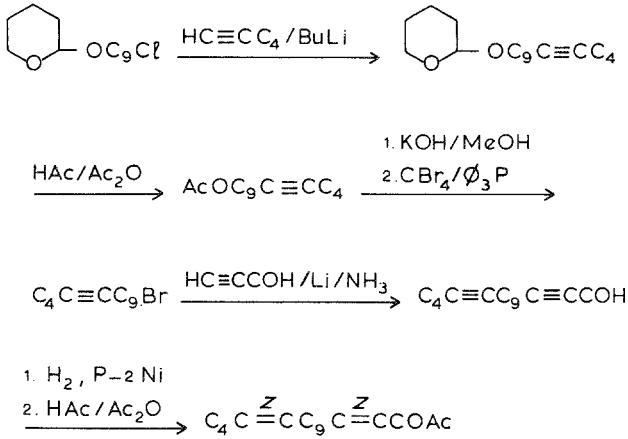

FIG. 2. Synthesis of (*Z*,*Z*)-2,13-octadecadien-1-ol acetate.

succession. The resulting granular precipitate was filtered off and washed with ether (n g $LiAlH_4$ was used) (Steinhardt, 1967).

The progress of all reactions was followed, and all products were checked by gas–liquid chromatography using a glass column, 2.1 m × 2.0 mm ID, packed with 2% SE-30 on Chromosorb W HP 80–100 mesh. The doubly unsaturated end products were ultimately purified by argentation chromatography (Voerman and Rothschild, 1978) and finally checked with HPLC (Houx and Voerman, 1976). Based on the syntheses and the analyses, their purity was estimated to be 99%. No ozonolysis or GC was performed to check for the presence of positional isomers. They were stored under nitrogen at −20° C after addition of 0.1% 2,6-di-*tert*-butyl-4-methylphenol as an antioxidant.

The main field experiments were carried out in a small currant orchard at Boven-Leeuwen, 12 km south of Wageningen. Traps were of opaque white PVC, delta model, cross-section 10 × 10 × 10 cm, with a sticky base of 10 × 20 cm. One funnel trap (Unitrap, bought from International Pheromones Ltd., Warrington, Cheshire, WA3 6QQ, England) was also used. Appropriate solutions were made in CH_2Cl_2 and applied to red rubber sleeve stoppers, 7 × 11 mm (Cat. No. 8753-D31, Arthur H. Thomas Co., Philadelphia, Pennsylvania) or placed in polyethylene vials of 1 ml (Kartell, Art. No. 10872/730) from Emergo B.V., Landsmeer, The Netherlands. The latter were closed after the solvent had evaporated. These dispensers were hung in the tops of the traps. Additional field tests were conducted in southern Germany at Starnberg, 40 km south of Munich. Tetra traps (Arn et al., 1979) with rubber-cup dispensers (serum bottle caps 18 mm diameter) were used in these tests.

RESULTS AND DISCUSSION

From Table 1 it is clear that pure *EZ*2,13-18:Ac is a potent attractant for male *S. tipuliformis* moths. The addition of small amounts of *EZ*2,13-18:OH did not increase or diminish the catches appreciably. Further tests conducted in the same area at the end of the flight period from July 1 to July 20 showed that adding 4% *ZZ*2,13-18:Ac or *ZZ*2,13-18:OH had no influence on trap captures. In four traps, baited with 1000 μg *EZ*3,13,18:Ac on a rubber sleeve stopper, five males were caught in the same period. *EZ*2,13-18:Ac was much more attractive (Table 2). Blank traps caught very few moths. When the attractant was formulated in a closed polyethylene vial, 23 moths per trap were caught, whereas only nine moths per trap were caught with the same amount (1 mg) of *EZ*2,13-18:Ac on a rubber sleeve stopper (mean of four traps, June 24-July 1). Although only one Unitrap (an omnidirectional funnel trap) was used, strong evidence was obtained that it is as good as or better than the common delta trap for this insect species. It was impossible to compare the attractiviness of the synthetic pheromone with that of virgin females because they were not available.

Single-cell recordings from antennal sensilla trichodea were conducted in June-July 1983, using wild *S. tipuliformis* males attracted at Starnberg (Bavaria) to sources of *EZ*2,13-18:Ac. These recordings revealed the presence of three types of receptor cells in these sensilla, specific to *EZ*2,13-18:Ac, *EZ*3,13-18:Ac, and *ZZ*3,13-18:Ac. There were no specialist cells for the *ZZ*2,13-18:Ac or receptors sensitive to alcohol or aldehyde analogs. Identical single-cell results were obtained on *S. tipuliformis* males collected as pupae near Vienna, Austria, in the spring of 1983.

TABLE 1. MEAN NUMBER OF MALE CURRANT CLEARWING MOTHS CAUGHT IN 4 TRAPS, BAITED WITH DIFFERENT RATIOS OF *EZ*2,13-18:Ac AND *EZ*2,13-18:OH FORMULATED ON RED RUBBER SLEEVE STOPPERS (BOVEN-LEEUWEN, JUNE 13-24, 1983)

Treatment (μg)		
*EZ*2,13-18:Ac	*EZ*2,13-18:OH	Mean catch per trap
1000	0	46
1000	30	32
900	100	34
500	500	35
100	900	9
30	1000	11
0	1000	0

TABLE 2. TOTAL NUMBER OF MALE CURRANT CLEARWING MOTHS CAUGHT IN 4 TRAPS BAITED WITH *EZ*- AND *ZZ*-2,13-18 COMPOUNDS FORMULATED ON RED RUBBER SLEEVE STOPPERS (BOVEN-LEEUWEN, JULY 1-20, 1983)

Treatment (μg)			Total catch in 4 traps
*EZ*2,13-18:Ac	*ZZ*2,13-18:Ac	*ZZ*2,13-18:OH	
1000	0	0	17
1000	40	0	15
1000	0	40	15
0	1000	0	3
0	0	1000	3

The attractivity of *EZ*2,13-18:Ac to male *S. tipuliformis* was confirmed by field trapping tests conducted in the Starnberg area. In a test series set up towards the end of the 1983 flight season, captures by this compound were found to decrease on addition of *EZ*3,13-18:Ac. This effect will be studied more closely in 1984.

These results confirm the findings of Schwarz et al. (1983) that *EZ*2,13-18:Ac is important as a sex pheromone or attractant for Sesiidae.

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POTENTIAL USES OF KAIROMONES FOR BEHAVIORAL MANIPULATION OF *Cotesia marginiventris* (CRESSON)[1]

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Abstract—The effects of kairomone pattern and preconditioning on parasitization rates of fall armyworm larvae, *Spodoptera frugiperda* (J.E. Smith) by *Cotesia* (=*Apanteles*) *marginiventris* (Cresson) were investigated in the laboratory and greenhouse using a hexane extract of frass and actual fall armyworm frass. Parasitization rates increased 55 and 26% in Petri dishes and on corn seedlings, respectively, when the entire experimental area was sprayed with the frass extract. Applying the extract in spots resulted in a 20–30% reduction in parasitization compared to treating the entire area. However, spot application produced significantly better parasitization rates compared to the control treatment. Exposing the parasitoids to actual fall armyworm frass resulted in ca. 50% greater retention in the release area and an increase of ca. 60–75% in the number of parasitoids searching. *C. marginiventris* parasitized factitious hosts topically treated with an extract of fall armyworm frass.

Key Words—Fall armyworm, *Spodoptera frugiperda*, Lepidoptera, Noctuidae, *Cotesia*, (=*Apanteles*) *marginiventris*, Hymenoptera, Ichneumonidae, kairomone, parasitism.

[1]Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

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INTRODUCTION

Modifying insect behavior in pest management programs through the utilization of nontoxic semiochemicals is recognized as a promising alternative to conventional approaches (Ridgway and Vinson, 1977). Direct regulation of pest insects by these chemical agents is accomplished through inhibiting a correct response or eliciting an incorrect one. Kairomones, mediating host finding by parasitoids and predators, offer additional strategies for the regulation of pest populations. Modifying the behavior of entomophages requires a more restricted approach because the ultimate goal of their manipulation is to redirect or stimulate a desired response. Retention in the agroecosystem and initiation of the "find-and-attack" cycle are the primary objectives of chemical manipulation in entomophages (Lewis et al. (1976b; Vinson, 1977; Jones et al., 1976). Studies by Lewis et al. (1976a) show that the efficiency of released *Trichogramma* spp. as well as wild *Trichogramma* spp. significantly improves after the application of the host-seeking stimulus, tricosane, in target areas. Gross et al. (1975) demonstrate for *Trichogramma* spp. and *Microplitis croceipes* (Cresson), that moth scales, tricosane, and larval frass retain and stimulate these parasitoids, thereby producing significantly higher rates of parasitization. *Cotesia* (=*Apanteles*) *marginiventris* (Cresson) (Mason, 1981), a larval endoparasitoid of the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith), initiates host-seeking behavior in response to plant damage and host-related materials (Loke and Ashley, 1984), and its host-finding sequence is defined (Loke et al., 1983). This parasitoid is frequently recovered from FAW larval collections (Ashley, 1979) and its biology (Boling and Pitre, 1970; Kunnalaca and Mueller, 1979), and its ecological associations with the FAW have been investigated (Ashley et al., 1983). These studies indicate that *C. marginiventris* has the potential for mass rearing and chemical manipulation. The following experiments evaluate whether the application of host-seeking chemicals and conditioning of the parasitoids at the time of release improve their efficiency. In addition, host acceptance by *C. marginiventris* is investigated through the application of a frass extract to natural and factitious hosts.

METHODS AND MATERIALS

Items common to all phases of experimentation are described in this paragraph. FAW larvae and *C. marginiventris* were reared as described by Loke et al. (1983). Female parasitoids came from a general emergence cage where they had been in contact with males since female eclosion and were 2–4 days old. Parasitization rates were determined through larval dissections, and laboratory tests were conducted at ambient conditions (27° C, 55%

relative humidity) and ca. 30 cm beneath two 20-W fluorescent bulbs. Frass from corn-reared FAW larvae was collected and stored at −10° C in tightly capped glass vials. The extract was prepared by grinding 500 mg of frass in 10 ml of *n*-hexane for 3 min using a mortar and pestle and then filtering the mixture through Whatman No. 1 filter paper. Bioassays were conducted immediately after filtration by placing the extract on the filter paper and allowing time for the solvent to evaporate.

Petri Dish Stimulation. The experimental universe was a 2.5 × 15-cm-diam Petri dish containing a 14-cm Whatman No. 1 paper filter marked radially at six equidistant points located 5 cm from the center. The four treatment patterns consisted of applying frass extract or hexane to the entire surface of the filter paper or forming 1- or 3-cm circles of extract at each of the six locations. The same quantity of frass extract was applied in each treatment, even though the surface areas differed. An ecdysing 2nd instar FAW larva that had been further immobilized by chilling was placed at each of the six points. A parasitoid was introduced into the center of the Petri dish and allowed to remain for 10 min. The time required by the parasitoid to find the first larva and the duration of searching behavior were recorded. Treatments were replicated ten times.

Greenhouse Stimulation. Three rows of corn seedlings were grown in an aluminum pan (22 × 22 × 5 cm). Treatments consisted of applying frass extract in either blanket or spot patterns. The blanket treatment was applied by evenly spraying the plants using a DeVilbiss® atomizer 163 for 30 sec (ca. 1.5 ml). The spot treatment was carried out by spraying the plants through a cardboard shield having five 3-cm holes (one at each corner and center). Control pans were sprayed with hexane and rates of application were equivalent for all treatments. Three 2nd instar larvae were placed onto each seedling after which 4 female parasitoids were released. Behavioral observations were made for 10 min and at the end of 60 min all FAW larvae were collected. Experiments were conducted between 0100 and 1300 EST, and each treatment was replicated six times. The temperature and humidity were ca. 27° C and 70% relative humidity.

Prerelease Conditioning. Uncovered Petri dishes and FAW larvae were prepared as described for the stimulation test with the exception that a small amount of frass was spotted around each larva. Each parasitoid was transferred to the center of the Petri dish in a 0.25-dram shell. Either frass extract or fresh frass was rubbed over the lip of the vial so that the parasitoid contacted the material as it exited the vial. Behavioral observations were made for 10 min, and five trials were conducted for each treatment with each treatment replicated on five different occasions.

For greenhouse tests, corn seedlings were grown in cake pans as previously described. Three 2nd instar larvae were placed on each seedling, and parasitoids were released in the center of the pan from a 1-dram vial that

had its lip treated with either fresh frass, frass extract, or left untreated. Parasitoid behavior was observed for 10 min, and after 30 min, all FAW larvae were removed and subsequently checked for parasitization. Each treatment was replicated five times.

Natural and Factitious Hosts. Frass extract (ca. 1 μl) was used to topically treat highly acceptable FAW larvae, moderately acceptable cabbage looper, *Trichoplusia ni* (Hübner), and normally unacceptable velvetbean caterpillar, *Anticarsia gemmatalis* Hübner, and greater wax moth, *Galleria mellonella* (L.), larvae. Cabbage loopers and the velvetbean caterpillars were reared on a diet similar to that of the FAW (King and Leppla, 1983), and wax moth larvae were propagated on a modified pablum diet (Singh, 1977). Assays were conducted in the Petri dish unit described for stimulation tests. Larvae were exposed to a single *C. marginiventris* female for 30 min, after which they were dissected to determine parasitization rates. FAW larvae were classified as parasitized if a single developing parasitoid was found and superparasitized if more than one parasitoid was present. Tests were replicated five times for each host species.

RESULTS AND DISCUSSION

Petri Dish Stimulation. The pattern of kairomonal extract significantly affected percent parasitization, time required for first host contact, and the amount of time spent searching (Table 1). The blanket treatment displayed the highest percent parasitization, shortest time for first host contact, and the greatest amount of searching time. The spot treatments were generally intermediate between the control and blanket treatments. More hosts were parasitized and overall searching time was longer for the 3-cm spot treatment

TABLE 1. EFFECTS OF HEXANE EXTRACT OF FALL ARMYWORM FRASS ON PARASITIZATION RATES AND SEARCHING BEHAVIOR BY *C. marginiventris* IN PETRI DISHES[a]

Treatment	Percent		Mean time for first contact (min)	Mean overall search time (min)
	Parasitization	Superparasitization		
Control	25.0 a	16.7 a	3.47 ± 0.15 a	2.83 ± 0.23 a
1-cm spot	40.0 b	23.3 ab	2.27 ± 0.16 b	3.99 ± 0.11 b
3-cm spot	56.7 c	33.3 b	2.26 ± 0.15 b	4.83 ± 0.15 c
Blanket treatment	80.0 d	25.0 ab	0.86 ± 0.05 c	6.49 ± 0.24 d

[a]Means in the same column followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple-range test.

than for the 1-cm spot treatment. In the control treatment, parasitoids remained principally on the underside of the Petri dish cover. Parasitoids in close proximity to kairomone spots or in actual contact with the treated filter paper displayed excitement and an intense searching behavior. Thus, frass extract stimulated the parasitoids to higher levels of parasitization and increased time spent in the "find-and-attack" cycle.

Similar results were reported for *Trichogramma* spp. by Lewis et al. (1975). The highest percent of superparasitization was probably an artifact of parasitoid confinement in a small arena with a small number of hosts. Beevers et al. (1981) also recorded an increase in parasitization rates in Petri dishes where the treatment pattern covered the entire surface area and considered this to be a function of parasitoid retention. Our results supported this conclusion, but also demonstrated a significant increase in searching time for blanket treatments. In contrast, Lewis et al. (1979) demonstrated higher rates of parasitization in spot treatment patterns at low host densities. Our results, therefore, may have been different if we had decreased host densities and enlarged the experimental universe.

Greenhouse Stimulation. The corn seedlings receiving the blanket spray treatment retained the highest number of parasitoids, as well as yielding the highest percentage of parasitized FAW larvae while the control treatment had the lowest values (Table 2). The lower rates of parasitization in these treatments compared to the Petri dish experiments may be attributed to differences in experimental design and environmental conditions. Parasitoids were artificially confined in the Petri dishes, but in the cake pan arrangement they could leave the corn seedlings and were observed to do so, especially in untreated pans. However, the overall trends in both experiments were similar.

Prerelease Conditioning. Parasitoids preconditioned with frass at the time of release into a Petri dish displayed significantly higher retention, searching, and parasitization levels compared to nonconditioned females

TABLE 2. EFFECTS OF SPRAYING CORN SEEDLINGS WITH DIFFERENT PATTERNS OF HEXANE–FRASS EXTRACT ON PARASITIZATION OF FALL ARMYWORM LARVAE BY *C. marginiventris*[a]

Spray pattern	Parasitoids retained (%)	Percent	
		Parasitization	Superparasitization
Control	8.3 a	1.9 a	0.0
Spot sprayed	29.2 b	13.8 b	0.0
Blanket sprayed	50.0 c	28.0 c	0.6

[a]Means in the same column followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple-range test.

TABLE 3. EFFECTS OF CONDITIONING *C. marginiventris* WITH FALL ARMYWORM (FAW) FRASS OR HEXANE EXTRACT OF FAW FRASS ON POSTRELEASE RETENTION, SEARCHING BEHAVIOR, AND PARASITIZATION RATES IN LABORATORY AND GREENHOUSE ENVIRONMENTS[a]

Location and Treatment	Percent		
	Retained	Searching	Parasitization
Laboratory (Petri dish)			
Control	24.0 a	0.0 a	0.0 a
Extract	64.0 b	60.0 b	32.6 b
Frass	76.0 b	76.0 b	34.8 b
Greenhouse (corn seedlings)			
Control	10.0 a	5.0 a	1.5 a
Extract	35.0 b	35.0 b	9.6 b
Frass	65.0 c	65.0 c	15.5 c

[a]Means in the same column within each location and followed by the same letter are not significantly different (5% level) as determined by Duncan's multiple-range test.

(Table 3). Frass was consistently more effective than frass extract, even though no significant differences were present for the Petri dish tests. This was probably because frass represented an unaltered, more natural source of host-seeking stimuli. Greenhouse results displayed the same trends as the Petri dish experiment. Significant differences were present between all treatments, with the highest values recorded for frass-conditioned parasitoids. These results indicated that a portion of the tendency for released parasitoids to disperse may be overcome by conditioning at the time of release. Parasitoid conditioning combined with application of host-seeking chemicals is

TABLE 4. PERCENT PARASITIZATION AND SUPERPARASITIZATION BY *C. marginiventris* OF NATURAL AND FACTITIOUS LARVAE TOPICALLY TREATED WITH HEXANE EXTRACT OF FALL ARMYWORM FRASS[a]

Larvae tested	Parasitization (%)		Superparasitization (%)	
	Control	Treated	Control	Treated
Fall armyworm	53.3 Aa	80.0 Bb	3.4 Aa	6.7 Aa
Cabbage looper	36.7 Aa	73.3 Bb	6.0 Aa	3.3 Aa
Velvetbean caterpillar	6.7 Ac	63.3 Bb	6.0 Aa	6.7 Aa
Wax moth	0.0 Ad	33.3 Bb	0.0 Aa	3.3 Aa

[a]Means in the same row and within each percent category followed by the same upper-case letter, and means in the same column followed by the same lower-case letter are not significantly different (5% level) as determined by Duncan's multiple-range test.

probably mutualistic and may result in improved parasitoid efficiency in biological control programs.

Natural and Factitious Hosts. Topical treatment of larvae with the frass extract significantly improved parasitization rates (Table 4). Even the unnatural hosts *A. gemmatalis* and *G. mellonella* were accepted after topical treatment with the extract. Although eggs were deposited in these two unnatural hosts, subsequent rearing of host larvae did not result in successful parasitoid emergence. This indicated that these unnatural hosts were either not nutritionally suitable or possessed defense mechanisms that precluded successful parasitoid development. Vinson (1975) derived similar results with the parasitoid *Cardiochiles nigriceps* Viereck, and two factitious hosts, *G. mellonella* and *S. frugiperda.*

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SEX PHEROMONES IN *Culicoides nubeculosus* (DIPTERA, CERATOPOGONIDAE): POSSIBLE SITES OF PRODUCTION AND EMISSION

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Abstract—This study was performed on virgin females of *Culicoides nubeculosus* to determine sites of production and emission of sex pheromone. The ventral abdominal surface is the site of emission of the pheromone. Morphologically there is evidence of the presence of abdominal atrichial areas. The abdominal areas, eight per segment (four ventral and four dorsal), are covered by a cuticle devoid of micro- and macrotrichiae. The dorsal and ventral areas differ considerably in their structure. The dorsal atrichial areas have an unperforated cuticle covering a normal epidermis, whereas the ventral atrichial areas have a cuticle perforated by numerous tubular structures, with a loosely organized procuticle and no underlying epidermal cells. A pair of voluminous cells with the classical ultrastructural characteristics of enocytes is invariably located in the vicinity of the ventral atrichial areas. These cells undergo ultrastructural modifications tending towards involution according to the age of the insects, being correlated with the decrease in pheromone emission by older females. The hypothesis is put forward that enocytes are involved in pheromone production.

Key Words—Sex pheromone, enocyte, site of emission and biosynthesis, *Culicoides nubeculosus,* Diptera, Ceratopogonidae.

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INTRODUCTION

Numerous publications, including some very recent ones, have been devoted to identifying the sites of biosynthesis of pheromones in insects (review of Percy and Weatherston, 1974); these studies have essentially concerned Dictyoptera, Isoptera, Mecoptera, and Lepidoptera. In contrast, there are very few studies of this type on Diptera (Dillwith et al., 1981; Lang, 1977; Schleinet al., 1980).

In a previous study, we demonstrated the existence of sex pheromones in virgin females of *Culicoides nubeculosus* (Kremer et al., 1979). We were also able to show that these pheromones are produced by the abdomen (Ismail and Kremer, 1983). This study concerns the histology and cytology of areas of the ventral abdominal cuticle devoid of micro- and macrotrichiae and the tissues contiguous to these areas.

METHODS AND MATERIALS

Biological Material. The strain of *Culicoides nubeculosus* was provided in 1973 by Dr. Boorman (Animal Virus Research Institute, Pirbright, England) and reared according to his protocol (Boorman, 1974).

Processing of Tissues for Electron Microscopy. For scanning electron microscopy (SEM), the insects were anesthetized with ether, coated with gold, and observed with a Philips 501 SEM.

For transmission electron microscopy (TEM), the insects were anesthetized with ether and decapitated. The last two abdominal segments were then cut off, and the insects injected with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. They were left in contact with this solution for 16 hr at 4° C. The abdomens were postfixed with 1% osmium tetroxide in 0.2 M cacodylate buffer, pH 7.4, supplemented with 0.8% potassium ferrocyanide for 2 hr. After ethanol dehydration, the specimens were embedded in epon–araldite mixture. Ultrathin sections, contrasted with alcoholic uranyl acetate and lead citrate, were examined with a Siemens I A Elmiskop electron microscope. Semithin (1 μm thick) sections were stained with borared toluidine blue.

Rendering the Cuticular Surface Impermeable. In order to localize sites of pheromone emission, we have proceeded to make the dorsal and ventral abdominal wall of 2-day-old female virgins impermeable. Females at this physiological stage are at their maximal attractiveness to males presented to them. Impermiability is accomplished by depositing a film of paraffin (mixture of paraffin and gelucire 1 : 1) on the surface of the cuticle. The paraffin is deposited with the aid of a copper wire cauter whose tip is bent to form a sharp V. The point of the V is heated to 40°C and placed against the

solid paraffin which melts at the point of contact. A small droplet of paraffin is collected between the two arms of the V and is deposited either on the dorsal abdominal surface (after the wings have been lifted) or on the ventral abdominal surface. The operations are observed with a binocular microscope.

RESULTS

The insects were fixed at different times after eclosion. The pheromone levels in females at different ages are illustrated in Figure 1. This figure summarizes the results previously published by Ismail and Kremer (1980).

Study of 2-Day-Old Virgin Females. The cuticle is covered with a dense felt of microtrichiae (3.5 μm long) and also displays a small number of macrotrichiae. However, certain areas of the cuticle (which we shall call from now on atrichial areas) correspond to the stigmatic regions and are very precisely located dorsad and ventrad and are devoid of both micro- and macrotrichiae. The atrichial areas are located on the first eight abdominal segments: four areas on the sternites and four on the tergites.

Each tergite has two small ellipsoidal atrichial areas (24 $\times$ 15 μm) near the sagittal plane, situated on the posterior third of the segment and two

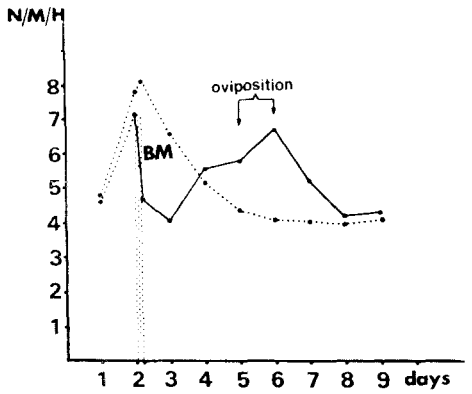
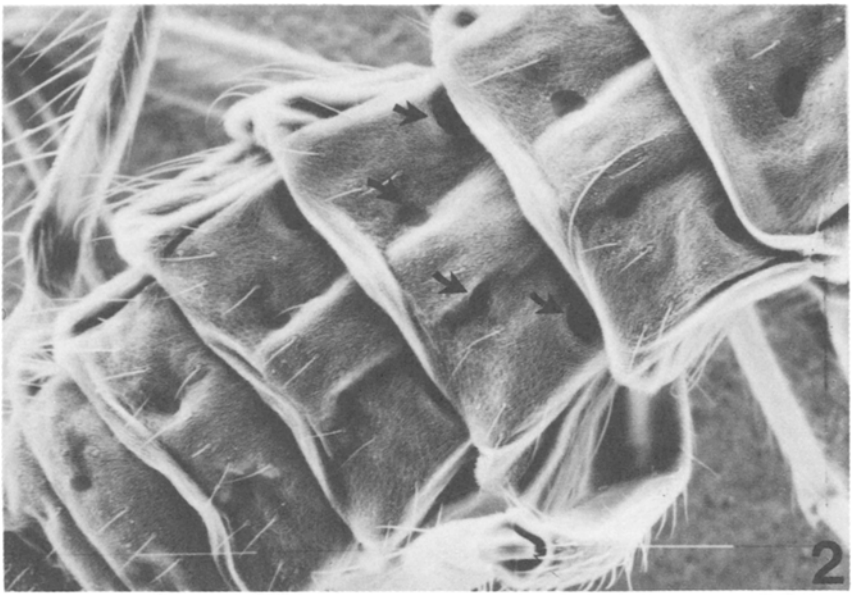
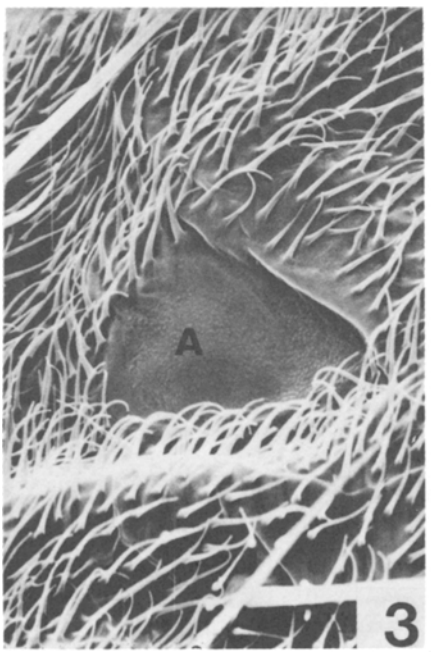


FIG. 1. Evolution of pheromone levels emitted by virgin females of *Culicoides nubeculosus,* expressed as the "number of matings per male per hour" (NMH) in couples subjected to a flow of air previously passing over unfed virgin females (. . .) or fed virgin females (———). In unfed virgin females, pheromone secretion peaks on the 2nd day and then gradually diminishes with age. In contrast; in fed virgin females, pheromone emission decreases until 24 hr after the blood meal, then rises during egg maturation, especially during oviposition, and again diminshes. BM = blood meal. (From ISMAIL and KREMER, 1980).





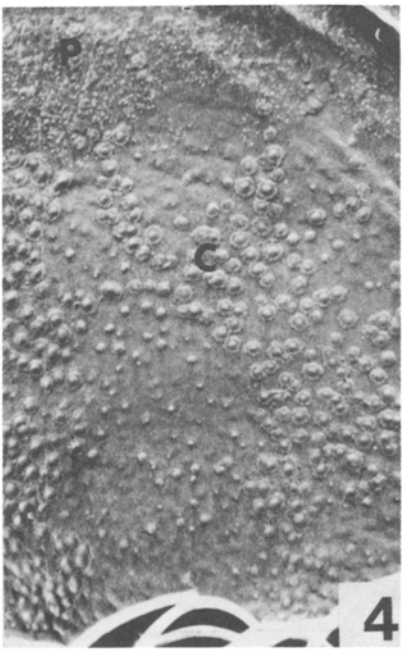


FIG. 2. *Culicoides nubeculosus* female, dorsal region, dorsal view. SEM. → atrichial area (X160).

FIG. 3. *Culicoides nubeculosus* female, ventral parasagittal porous area. SEM. Note

larger atrichial areas (40 × 24 μm) situated more laterally on the anterior third. The two bigger atrichial areas are often masked by the overlapping of the preceding segment (Figure 2). Similar atrichial areas are observed on the abdominal sternites.

The surface of the dorsal atrichial areas is devoid of ornamentation, in contrast to the ventral areas which are covered with a multitude of minute, discoid-shaped, raised buttons, each with a central papilla (Figures 3 and 4). At the periphery, these discoid-shaped buttons are mixed with protrusions of smaller size.

A careful study of serial sections of *Culicoides nubeculosus* abdomens revealed the constant presence of a pair of large cells (15 μm longer axis) in the vicinity of and always laterad to each ventral atrichial area (Figures 5 and 6). They are located either in direct contact with the epidermis or, more frequently, within clumps of subepidermal adipose cells. Such pairs of cells were never found in the vicinity of the dorsl atrichial areas.

The abdominal cuticle, about 4 μm thick, is composed of a simple epicuticle and procuticle; the procuticle has the usual appearance of coiled fibrous layers. The epidermal cells, very flat (about 4 μm thick) and with abundant rough ergastoplasmic reticulum (RER), in the form of long, more or less dilated cisternae, do not show any ultrastructural peculiarity when compared with the epidermal cells usually described in insects.

At the atrichial regions, the procuticle shows a looser fibrous structure, apparently less organized; its thickness is clearly less than that of adjacent regions (about 2 μm). Numerous electron-dense tubular structures penetrate these areas and these ducts open at the top of short discoid-shaped buttons on the surface of the cuticle (Fig. 7). These buttons probably correspond to the structures observed on the surface of the atrichial areas by SEM (Figure 4). The term "ventral porous areas" will be used to designate these areas.

An important point is that no well-preserved epidermal cells could be observed facing the ventral porous areas. However, membranous structures are constantly found on females at emergence; these structures are associated more or less loosely with the internal surface of the cuticle; cytoplasmic elements, still recognizable as ribosome-coated vesicles, can be observed. These elements can be interpreted as residual structures of epidermal cells at the end of degeneration (Figure 8).

←

the dense felt of microtrichiae outside the atrichial area and the presence of some macrotrichiae. A = atrichial area (X2500).

FIG. 4. *Culicoides nubeculosus* female; ventral parasagittal porous area, detail. The central part (C) of the area is covered with round protuberances with a central nipple. These are intermingled with smaller protrusions at the periphery (P) (X10,000).

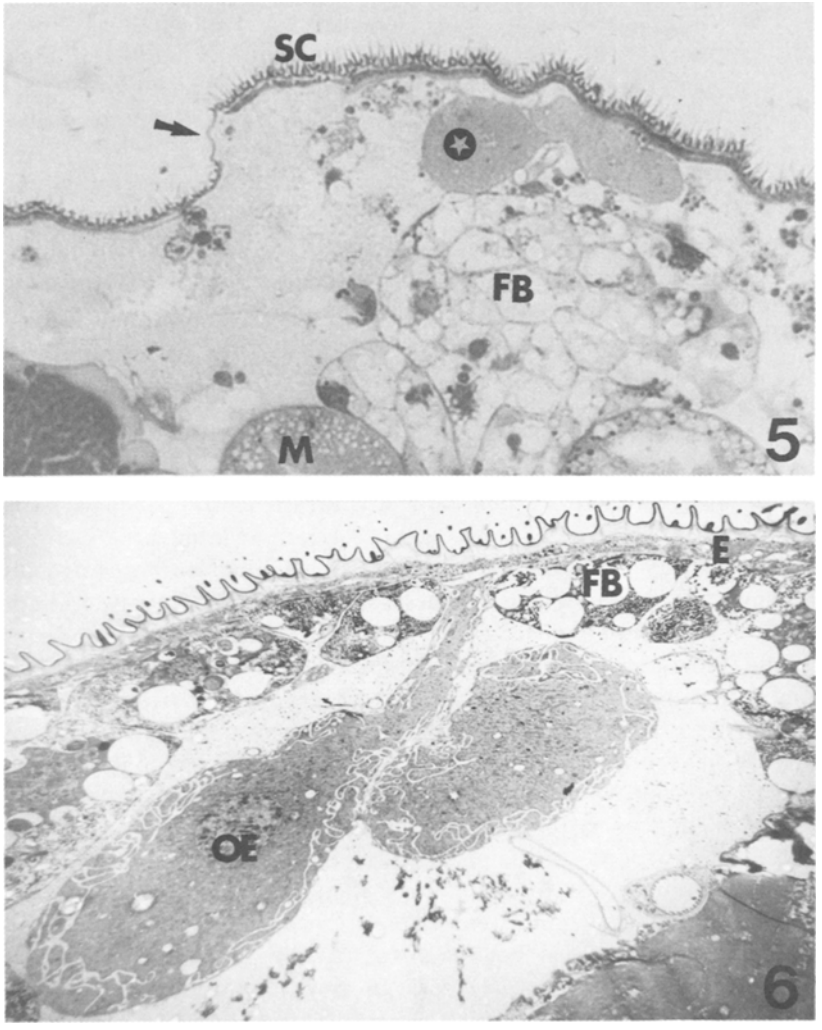


FIG. 5. Two-day-old *Culicoides nubeculosus* female, transverse section of abdominal sternite, 1 μm thick section, toluidine blue. Note, in the vicinity of the ventral porous area (→), the two voluminous cells (✪) in subepidermal position. FB = fat body; SC = sternal cuticle with trichiae, M = section of malpighian tube (X800).
FIG. 6. Two-day-old *Culiocoides nubeculosus* female, transverse section fo abdominal sternite detail. The columinous enocyte-like cells (OE) exhibit a deeply dissected peripheral cytoplasm, and one of the cells has a bridge in direct contact with the subepidermal region. FB = fat body; E = (X2500).

The large paired cells show, in practically all the sections observed, a cytoplasmic prolongation, a kind of peduncle, binding the cell directly to the subepidermal region (Figure 6). The cells have a typical structure: they are surrounded by a thin basal membrane and their plasma membrane displays numerous invaginations, pentrating deeply into the marginal cytoplasm (Figures 6 and 10b). The cytoplasm of these cells is characterized by the great abundance of SER, taking the form of a compact tangle of anastomosed tubules. The RER is sparse and the Golgi complexes, essentially vesiculate, are discrete (Figure 10a). Numerous mitochondriae with a dense matrix can be seen and autophagic bodies are often present. These cells have an ultrastructural appearance quite similar to that of the classical enocytes described in various insects (Cassier and Fain-Maurel, 1972; Delachambre, 1966; Rinterknecht et al., 1969; Locke, 1969).

The dorsal atrichial areas are similarly characterized by the absence of cuticular ornamentation such as a microtrichiae. In contrast, no tubular structures can be observed within the procuticle. The underlying epidermis has the usual structure and is continuous with the epidermis surrounding the atrichial areas. No cellular degeneration can be observed here (Figure 9). Moreover, as already observed by optical microscopy, no enocyte-like cells can be seen near these dorsal atrichial areas.

Study of 3-Day-Old Virgin Females. In fed,-3-day-old, virgin females, a clear decrease in the level of pheromones can be seen (Figure 1). Obvious ultrastructural modifications are observed in the large enocyte-like cells[3]. These have a greatly reduced volume compared with the analogous cells in 2-day-old females, and at the same time the peripheral invaginations, highly developed at day 2, display a spectacular regression. The SER, although still very abundant, frequently takes the form of a mass of more or less arched tubular structures joined together. Free ribosomes, either grouped in polysomes or associated with short ergastoplasmic cisternae, are clearly more numerous than on day 2 (Figure 11).

Experimental Demonstration of Pheromone Emission at Abdominal Sternites. We have compared the level of emission of sex pheromones of females of the same physiological stage (virgin females 2 day old). These females were separated into two groups: those whose abdomen was paraffined on the dorsal surface and those having the ventral surface paraffined (see Methods and Materials). For this experiment we used four glass chambers in pairs: the first chamber is joined to the second and the third to the fourth (the experimental procedure is described in detail in Kremer et al., 1979).

Into chamber number 1 are introduced 50 females with paraffined

[3]In 7-day-old females, which also have a very low level of pheromone (Figure 1), the enocytes show an even more marked involution of the SER, expressed by the abundance of more or less complex membranous structures.

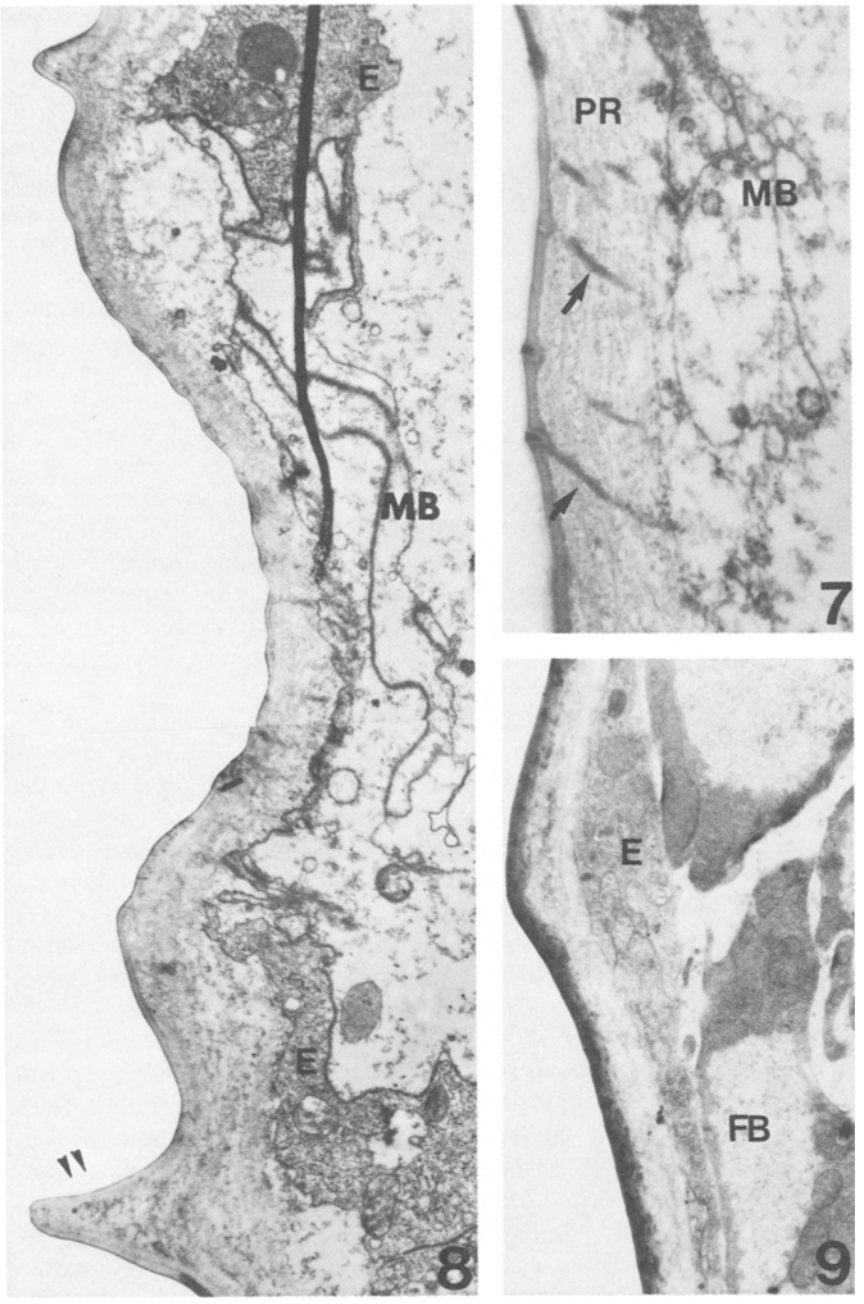


FIG. 7. Two-day-old *Culicoides nubeculosus* female, ventral porous area: detail of cuticular region. Note the loose structure of the procuticle (PR) which is penetrated by tubular structures ending in the protuberances on the epicuticle. MB = residual membrane structures. (X30,000).

dorsal abdominal surfaces and in chamber number 3, 50 females with paraffined ventral abdominal surfaces. In each of the observation chambers 2 and 4 are placed 15 males aged 24 hr and 15 virgin females aged 2 days. A flux of air is made to pass from chamber 1 to chamber 2 and from chamber 3 to chamber 4. The level of pheromone is measured by counting the number of matings and attempted matings between males and females in chambers 2 and 4. for each experiment we made six successive counts, each of 5 min duration. The number of matings thus observed is expressed as the number of matings per male per hour. The experiment was repeated four times with different lots of insects (Table 1).

The number of matings per male per hour observed in chamber 2 is higher than that observed in chamber 4 by a factor of 2.7. It is sufficient to note that the number of matings per male per hour observed in chamber 4 (average 4.3) is of the same order of magnitude as that observed in our previous experiments (Ismail and Kremer, 1980) where there was an absence of females in chamber 3. These results indicate, therefore, that the emission of pheromones by females occurs when the dorsal abdominal surface is paraffined and not when the ventral surface has been paraffined.

DISCUSSION

This study enables us to reveal the presence of unusual sternal abdominal cuticular areas (ventral porous areas) in females of *Culicoides nubeculosus*. The procuticle, structurally very loose in the areas, is perforated by numerous electron-dense tubular structures, opening at the surface in the discoid buttons scattered over the surface of these areas. A point to be emphasized is the absence of epidermal cells under these ventral areas. Residual structures, observed in females at emergence, point to the previous presence of epidermal cells at these sites. Owing to the degeneration of the epidermal cells underlying the cuticle at the time of the study, we were unable to follow the morphological continuation between the membrane of these cells and the tubular structures crossing the cuticle. Groups of cells displaying the ultrastructural characteristics of classical enocytes are invaraibly associated with these areas and are located exclusively in their vicinity.

FIG. 8. Newly metamorphosed *Culicoides nubeculosus* female, ventral porous area: general view. Note the disappearance of the subcuticular epidermis, reduced to residual membranal structures (MB); normal epidermal cells are present at the periphery of the atrichial area (E). ◢ = microtrichiae. (X15,000).

FIG. 9. Two-day-old *Culiocoides nubeculosus* female, dorsal atrichial area. The greatly flattened epidermal cells show no sign of degeneration. FB = fat body. (X10,000).

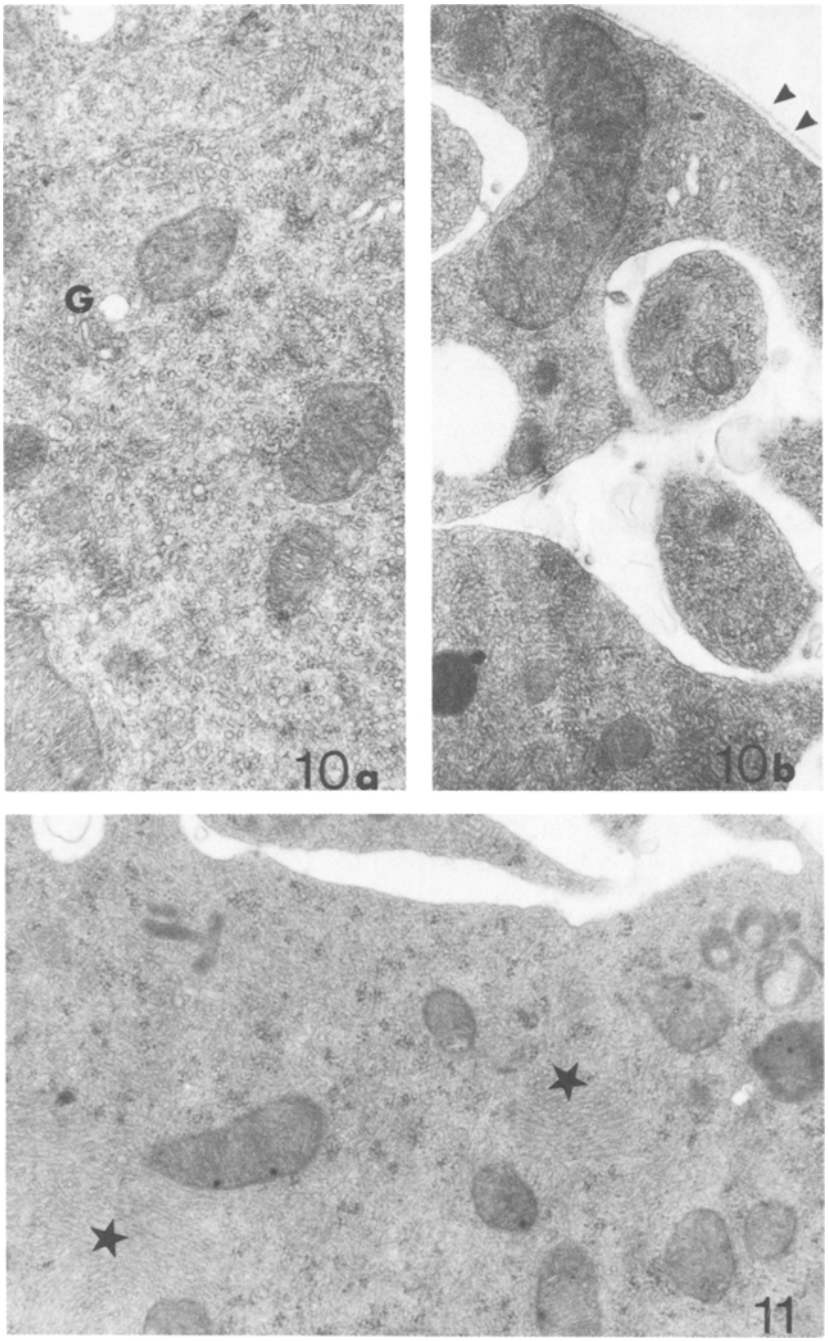


FIG. 10. (A) Two-day-old *Culicoides nubeculosus* female, enocyte-like cell. Note the abundant SER. G = Golgi complex. (X25,000). (B) Two-day-old *Culicoides nubeculosus* female, enocyte-like cell, peripheral area showing a deeply cytoplasmic invagination.▼▼= basal membrane. (X25,000).

TABLE 1. EFFECT OF RENDERING IMPERMEABLE THE DORSAL OR VENTRAL ABDOMINAL CUTICLE ON PRODUCTION OF PHEROMONE)

	Observation chamber							
	2				4			
Experiment number	1	2	3	4	1	2	3	4
	4	15.2	10.4	8.8	2.4	3.2	1.6	1.6
Number of matings	10.4	14.2	8	8.8	3.2	5.6	1.6	3.2
of each count[a]	7.2	11.2	12	12.8	4.8	6.4	4.8	7.2
	8	15.2	9.6	13.6	0.8	4.8	4	5.6
	7.2	15.2	10.4	13.6	4	4	5.6	7.2
	13.6	12	12	19.2	4.8	4	4	8.8
Average of each experiment	8.4	13.8	10.4	12.8	3.3	4.7	3.6	5.6
Overall average	11.4 ±2				4.3 ±0, 9			

[a]In chamber 2, the flux of air passed previously through chamber 1 containing virgin females aged 2 days having the dorsal abdominal surface paraffined. In chamber 4, the flux of air passed previously through chamber 3 containing virgin females aged 2 days having the ventral abdominal surface paraffined.

From the morphological observations reported in this work, we feel that the following hypothesis can be proposed: the sex pheromones are elaborated in the female *Culicoides nubeculosus* by enocytes, transported in the hemolymph, and emitted through ventral porous areas. We bring forward, in the following discussion, diverse arguments in favor of the proposed hypothesis.

The sexual pheromones of *Culicoides nubeculosus* are produced, as we have shown in a preceding work, by the abdomen of the females (Ismail and Kremer, 1983). Our work does not corroborate Lang's (1977) in *Culiseta inornata* and that of Schlein et al. (1980) in *Musca domestica* and *Glossina morsitans*. These authors place the site of emission of sex pheromone in the legs of the insects.

When the abdomen of the female of *Culicoides nubeculosus* is coated with paraffin, the legs are left uncovered and no stimulatory effect on mating is observed (Ismail and Kremer, 1983). The experimental results that we obtained in this work (by covering dorsal or ventral cuticular surfaces with paraffin) provide confirmation of the location of pheromone emission sites at the ventral abdominal surfaces.

We never found, in the abdomen, structurally complex glandular cells, either single or associated in glands, comparable to those observed by

FIG. 11. Three-day-old fed *Bulicoides nubeculosus* female, enocyte-like cell, peripheral region; Note that the SER is condensed into packs of symmetrically incurved cisternae (X18,000).

several authors in various insect groups and thought to be involved in the elaboration of pheromones (Crossley and Waterhouse, 1969; Clearwater and Sarafis, 1973). On the contrary, the enocyte-like cells observed next to the sternal areas can be considered as the potential site of pheromone. Indeed the proliferation of the SER is a characteristic of cells involved in the synthesis of small nonprotein molecules (for adrenalin glands, prothoracic glands, etc., see Wattebled et al., 1978). On the other hand, a recent study provides evidence supporting the participation of enocytes as well as epidermal cells in the synthesis of sex pheromones in *Musca domestica* (Dillwith et al., 1981).

The signs of incipient involution of the enocytes (clear regression of the peripheral digitation and SER coalescence that we have observed in females manifesting a decreased emission of pheromones (see Figure 1) are also arguments in favor of the hypothesis that the pheromones are synthesized by the enocytes.

From numerous studies in Diptera (see review in Howard and Blomquist, 1982), it may be concluded that all pheromones discovered so far in this order of insects are hydrocarbons.

Apart from the present study, a chemical investigation of the pheromones produced by *Culicoides nubeculosus* is in progress in Strasbourg, in the laboratory of Dr. Owisson. We have detected a clear difference between the hydrocarbon compounds found on the cuticular surface of males and females. Only the female hydrocarbon compounds show a mating-stimulating effect.

Biochemical arguments can be proposed in support of hydrocarbon synthesis. Diehl et al. (1975) showed that the enocytes of *Schistocerca* are able to synthetize in vitro and release into the culture medium hydrocarbons of similar structure to those found in cuticular waxes. Moreover, the level of release of these products into the medium is related to the amount of hemolymph present in it, and the authors suggest that they may be bound to components of the hemolymph. In agreement with this work, Romer (1980), in *Tenebrio,* demonstrated that isoalted enocytes efficiently and specifically incorporate [1-^{14}C]acetate into hydrocarbon.

The presence of numerous tubular structures in the cuticle of the ventral porous areas and the degeneration of the underlying epidermal cells (which is not evident in the tergal areas lacking enocytes) corroborates the hypothesis that the porous areas constitute preferential transit zones for certain substances.

Figure 12 summarizes the hypothesis proposed on the basis of observations concerning the elaboration and site of emission of sex pheromones in *Culicoides nubeculosus* females. Clearly, a rigorous demonstration on the molecular level is still required to prove that, in this insect, the site of synthesis is indeed the enocytes. Such a study is now

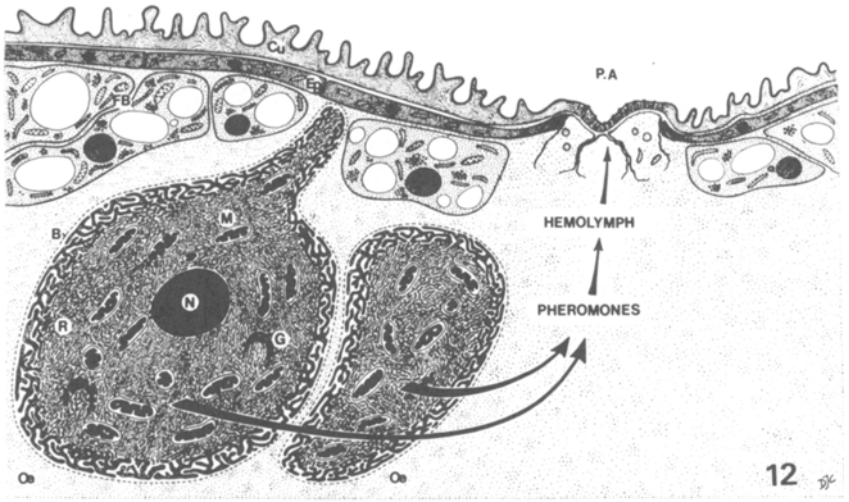


FIG. 12. Schematic summary of the hypothesis of the elaboration and sites of emission of pheromones by *Culicoides nubeculosus* female. EP = epidermal cells; FB = fat body; M = mitochondria; R = smooth endoplasmic reticulum; PA = ventral porous area; B = basal membrane; and N = nucleus.

planned, keeping in mind the possibility that other cells might also be involved in pheromone production.

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HOST ACCEPTANCE AND DISCRIMINATION BY *Comperia merceti* (COMPERE) (HYMENOPTERA: ENCYRTIDAE) AND EVIDENCE FOR AN OPTIMAL DENSITY RANGE FOR RESOURCE UTILIZATION

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Abstract—The cement used by females of *Supella longipala* (F.) (Orthoptera:Blattellidae) to bind their oothecae to substrates acts as a kairomone for host acceptance by the parasitoid *Comperia merceti*. *C. merceti* discriminates parasitized from unparasitized oothecae and oviposts at reduced levels in the former. Low survival rates for parasitoids reared from oothecae receiving fewer than four ovipositions suggests that an "optimal density range" for resource utilization by *C. merceti* exists.

Key Words—*Supella longipalpa*, Orthoptera, Blattellidae, *Comperia merceti*, Hymenoptera, Chalcidoidea, Encyrtidae, host acceptance, discrimination, optimal density range, kairomone.

Comperia merceti (Compere) is a gregarious endoparasitoid of oothecae of the brown banded cockroach, *Supella longipalpa* (L.) (Lawson 1954). No other species of cockroaches are known to be attacked by *C. merceti* and attempts to induce parasitism of other species under laboratory conditions have been unsuccessful (Lawson, 1954; Gordh, 1973). Kairomones present on or adjacent to hosts have frequently been reported as being involved in eliciting host acceptance by parasitoids (Arthur, 1981). Similarly, epideictic pheromones have been reported for many parasitoids as a means of avoiding egg wastage and loss of foraging efficiency (van Lenteren, 1981). We undertook a study of *C. merceti*'s interactions with *S. longipalpa* oothecae to determine:

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(1) the basis for the observed host specificity, (2) whether or not parasitoids discriminated against previously parasitized hosts, and (3) if discrimination occurred, whether or not an optimal density range (Prokopy, 1981) for parasitoid utilization of oothecae existed.

METHODS AND MATERIALS

Female parasitoids and cockroach oothecae for experiments were taken from laboratory cultures originally established in 1979 from cockroaches collected from a wild population in Fernald Hall at the University of Massachusetts and parasitoids obtained from the culture of A. Slater, University of California at Berkeley. Cultures were reared continuously at 23° C, 16:8 light–dark, and 40% relative humidity. Cockroaches were housed in a 74-liter aquarium and fed a diet modified from one developed for Indian meal moths (Silhacer and Miller, 1972). Parasitoids were maintained in a 11 × 23 × 31-cm plastic box on water and honey droplets. Host oothecae were collected weekly and transferred in numbered Petri dishes to the parasitoid colony container. Parasitoids that subsequently emerged from batches of hosts were returned to the parasitoid colony or used in experiments as needed. This system allowed cockroach oothecal age, immature parasitoid developmental stage, and age of female parasitoids used in experiments to be controlled.

Kairomonal Experiments. S. longipalpa is distinctive among domestic cockroaches in the United States in that it cements its oothecae to surfaces. This glue was hypothesized by Gordh (1973) to have kairomonal value for host recognition by *C. merceti.* To test this, freshly emerged (within 48 hr) *C. merceti* females were exposed in groups to sets of oothecae in 9-cm-diam. disposable plastic Petri dishes. Oothecae in initial experiments (1 and 2) were of two types: "unwashed" and "washed." Unwashed oothecae were unmodified oothecae removed from the laboratory cockroach colony and left naturally cemented to strips of paper toweling. Washed oothecae were ones from the same source that were placed in a 10% solution of household bleach (Clorox®) for 30 min, this being the minimum time capable of dissolving the cement binding the egg to the toweling. Other solvents (cold water, hot water, acetone, hexane, 70% ethanol, and 95% ethanol, were ineffective in dissolving cockroach cement. Washed eggs were rinsed in distilled water and then remounted onto clean strips of paper toweling with squares of double sticky adhesive tape sufficiently small so that they did not protrude from beneath the oothecae.

In experiment 3, a third treatment, "washed and reglued," was included. These oothecae were washed in 10% bleach solution as described above and then cockroach cement from other oothecae was reapplied. This was accomplished by removing from the colony twice the number of oothecae as

those to be "reglued" and physically scraping the glue deposits off of them with a razor blade in a water-filled watch glass under a dissecting microscope. The glue does not dissolve in water, but occurs as flakes or chunks. The water was then evaporated to dryness and a measured amount of water added back. A glass rod was then used to crush the dried cockroach cement and create a uniform water suspension of the material. Each experimental ootheca to be "reglued" then received a measured droplet of this suspension onto its upper surface. The quantity applied was such that each experimental ootheca received the kairomone that could be recovered from two scraped oothecae. The rate of recovery was not estimated, so the exact quantity of glue applied is unknown. When these droplets dried a kairomonal deposit was achieved on bleach-washed oothecae.

In experiments 1, 2, and 3, treatments were presented separately in Petri dishes (no-choice design) with 10 oothecae and 10 *C. merceti*/dish for a 24-hr exposure. In experiment 2, a 48-hr exposure was also tested. In experiment 4, treatments were presented together in Petri dishes (choice design) with a total of 15 eggs (five per treatment) and 15 female parasitoids per dish, to see if the level of distinction between treatments changed. At the end of the exposure period parasitoids were removed and each ootheca examined. The number of parasitoid egg stalks (see Lawson, 1954, for description) was determined and used as the measure of parasitoid oviposition. Experiments were conducted under the same environmental conditions as used for colony maintenance. In experiments 1, 2, and 3, oothecae were then reared (under the same environmental conditions as for the colony maintenance), and the number of emerging adult parasitoids recorded for each individual ootheca.

Epideictic Spacing Pheromone Experiments. To test parasitoid discrimination, colony oothecae of a given batch were divided into two groups. One group was exposed to newly emerged (48 hr) parasitoids in 9-cm disposable Petri dishes for 24 hr and the other was left unexposed. The oothecae exposed to parasitoids were then examined individually for parasitoid oviposition stalks, which were counted. Oothecae were then classified into groups bearing one to five stalks (low density), and those with 10 or more stalks/ootheca (high density). These two groups, plus the nonexposed (zero density) oothecae were then exposed (either separately or together according to exact experiment) for 24 hr to newly emerged (48 hr) female parasitoids in 9-cm disposable Petri dishes containing honey droplets and a small piece of moistened dental wick as a water source. Parasitoid immatures within parasitized oothecae were in the egg stage during the experiments. Ten female parasitoids were used for each replicate of 10 oothecae. In experiment 5, treatments were presented separately (no-choice design), and in experiments 6 and 7 treatments were presented together (choice design) with five oothecae of each treatment in each replicate and 15 female parasitoids. In experiment 8, treatments were presented together, but

each replicate consisted of only one female parasitoid and four oothecae, two with no previous parasitization and two with any number of previous parasitizations,

Parasitoids used in experiments emerged from isolated batches of previously parasitized oothecae. Thus newly emerged parasitoids invariably had contact with host oothecae that had *C. merceti* oviposition stalks. This experience implies that the *C. merceti* used were not naive with respect to previous contact with parasitized hosts. Whether or not the parasitoids used in experiments had actually ovipositioned on any of the unparasitized oothecae present in the batch from which they emerged is not known. Such older oothecae, while well advanced embryologically, are still acceptable for oviposition by *C. merceti* (Coler et al., 1983), in spite of statements to the contrary by Gordh (1973). After the parasitoid exposure period, each ootheca was reexamined for the number of oviposition stalks. For previously parasitized oothecae, the total number of stalks minus the number they originally bore gave the number of stalks produced during the experiment.

Optimal Density Range. To determine the average number of parasitoids produced/ootheca in wild parasitoid populations, oothecae derived from the laboratory cockroach colony were collected weekly, leaving the cement binding oothecae to the paper toweling intact. These eggs were placed at fixed locations in a fly rearing room in Fernald Hall where the percent parasitism (defined as the number oothecae attacked within a 7-day exposure period/total number of exposed oothecae) ranged from 80 to 100%. After the 7-day exposure period, these oothecae were returned to the laboratory and reared out for parasitoids under the same environmental conditions used for colony maintenance.

Oothecae from experiments 1, 2, and 3 (see *Host Acceptance* section below) were reared and the number of parasitoids produced compared to the number of stalks recorded for each ootheca. A percent survival for the parasitoids sharing each ootheca was calculated as (number reared parasitoids/number stalks) × 100. If the ratio exceeded 100% (as might happen if some stalks were broken off or obscured by the presence of numerous other stalks), it was kept at 100% since survival could not logically be any greater. The 152 oothecae from experiments 1, 2, and 3 were classified into three categories with respect to the number of stalks per ootheca (1–3 stalks, 4–7, and 8 or more) and the percent survival for each ootheca calculated. The average of these percentages was taken as the average survival for the class.

RESULTS

Host Acceptance. The mean number of parasitoid egg stalks deposited per ootheca was reduced 92% (experiments 1, 2, 3, and 4 summed, Table 1) by a 30-min wash in 10% bleach which removed the cement binding oothecae to

TABLE 1. MEAN NUMBER OVIPOSITION STALKS OOTHECA AND PERCENT ATTACKED BY *C. merceti* FOR UNMODIFIED HOST OOTHECAE AND TWO TYPES OF ALTERED OOTHECAE.

			Treatment									Statistical significance of treatment mean differences	
			Unwashed			Washed			Washed & regulated with cockroach cement				
Exp.	Variable	Design	$\bar{X}$	SD	N	$\bar{X}$	SD	N	$\bar{X}$	SD	N	Parametric (oneway ANOVA)	Nonparametric (Kruskal-Wallis Oneway ANOVA) $P \leqslant$
1	No. stalks/ootheca	Treatments separate	2.7	0.22	40	0.2	0.28	40	not included				0.020
	Percent oothecae attacked	Treatments separate	53%	20.62	40	8%	9.57	40	not included				0.190
2	No. stalks/ootheca	Treatments separate											
		24 hr	6.0	1.61	30	1.0	0.29	30	not included				0.046
		48 hr	5.0	1.41	30	0.7	0.70	30	not included				0.050
	Percent oothecae attacked	Treatments separate											
		24 hr	83%	15.28	30	33%	5.77	30	not included				0.046
		48 hr	67%	11.55	30	13%	15.28	30	not included				0.046
3	No. stalks/ootheca	Treatments separate	8.4[a]	2.69	40	1.1[b]	0.74	40	6.4[a]	2.22	40	0.0019[1]	0.018
	Percent oothecae attacked	Treatments separate	80%	21.60	40	30%	25.82	40	78%	20.62	40		0.056
4	No. stalks/ootheca	Treatments together	8.7	3.05	50	0.0	0.00	50	not included				0.005
	Oothecae attacked	Treatments together	62%	8.37	50	0%	0.00	50	not included				0.005

[a] Means in experiment 3 (stalks/oothecae) followed by different letters are statistically different by the Student-Newman-Kuels multiple-range test.

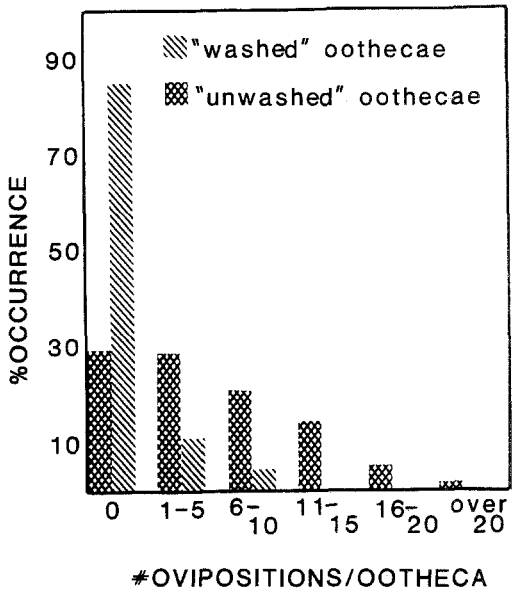

FIG. 1. Oviposition of *C. merceti* in unmodified ("unwashed") and modified ("washed") oothecae of *S. longipalpa* in laboratory choice tests.

surfaces. This reduction in mean value entailed both an increase in the proportion of oothecae receiving no parasitoid ovipositions and reductions in number of stalks per ootheca among those that were attacked (Figure 1). The proportion of oothecae attacked declined from 68% for unmodified oothecae to 15% for washed oothecae (experiments 1, 2, 3, and 4 summed, Table 1). When cockroach cement was reapplied to washed oothecae (experiment 3, Table 1) acceptability was restored, with the mean number of stalks/ootheca being 76% that of unmodified oothecae and the percent oothecae attacked being essentially the same.

These data indicate that washed oothecae were rejected because they lacked a positive factor not because the bleach imparted a negative or repellent quality to the oothecae. Parasitoids continued to distinguish between washed an unwashed oothecae for 48 hr (experiment 2, Table 1), indicating that the cement is an important behavior releaser whose significance is not quickly lost. However, when treatments were presented together in a choice design (experiment 4, Table 1), the degree of distinction between treatments increased compared to that seen in experiments 1, 2, and 3 (Table 1), suggesting that parasitoids offered only washed hosts did lose a portion of their restraint.

Host Discrimination. The mean number of parasitoid stalks deposited per ootheca was reduced 47% for previously parasitized oothecae versus unparasitized ones (Table 2, experiments 5, 6, 7, and 8 summed). This

TABLE 2. MEAN NUMBER OVIPOSITION STALKS/OOTHECA AND PERCENT ATTACKED BY *C. merceti* FOR PARASITIZED AND UNPARASITIZED HOST OOTHECAE

			Treatment									Statistical significance of treatment mean differences (Kruskal-Wallis Oneway ANOVA)
			Unparasitized capsules (0 stalks/ootheca)			Low density (1–5 stalks/ootheca)			High density (>10 stalks/ootheca)			
Exp.	Variable	Design	$\bar{X}$	SD	N	$\bar{X}$	SD	N	$\bar{X}$	SD	N	$P \leqslant$
5	No. stalks/ootheca	Treatments separate	6.6	2.13	40	2.6	0.35	30	2.8	1.61	40	0.046
	Percent oothecae attacked	Treatments separate	70%	18.26	40	37%	20.82	30	70%	14.14	40	0.145
6	No. stalks/ootheca	Treatments together	6.7	4.15	45	3.4	2.06	45	3.4	3.22	45	0.060
	Percent oothecae attacked	Treatments together	53%	10.00	45	62%	27.28	45	40%	26.46	45	0.130
7	No. stalks/ootheca	Treatments together	7.3	4.45	25	4.0	3.82	25	4.1	1.18	25	0.361
	Percent oothecae attacked	Treatments together	52%	30.33	25	52%	22.80	25	52%	10.95	25	0.928
			Unparasitized						Parasitized			
			$\bar{X}$	SD	N				$\bar{X}$	SD	N	
8	No. stalks/ootheca	Treatments together; parasitoids used singly	2.1	2.53	40				0.7	1.22	40	0.236
	Percent oothecae attacked	Treatments together; parasitoids used singly	35%	40.07	40				30%	37.70	40	0.698

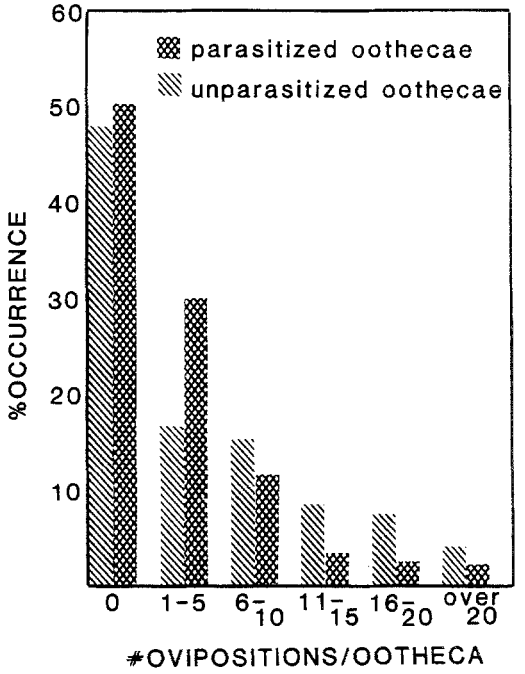

FIG. 2. Oviposition of *C. merceti* in parasitized and unparasitized oothecae of *S. longipalpa* in laboratory choice tests.

reduction was due almost entirely to many more oothecae receiving a few (1–5) eggs instead of a more substantial number (Figure 2). There was no decrease in percent oothecae attacked (Table 2), i.e., no increase in the number of oothecae receiving zero additional eggs (Figure 2). Parasitoids did not differentiate between high and low densities of previous parasitizations (Table 2, experiments 5, 6, and 7). Both levels were attacked to the same degree. For this reason, in experiment 8 they were lumped into single treatment, "previously parasitized," without regard to the intensity of parasitism. Discrimination between parasitized oothecae was not improved when treatments were presented together (experiments 6 and 7, Table 2) instead of separately (experiments 5, Table 2), indicating that parasitoid oviposition in previously parasitized hosts was not "for lack of a choice" but rather suggests that it has some positive advantage, as will be discussed under the section on optimal density range. The degree of parasitoid discrimination was similar when individual female parasitoids were used for each test (experiment 8, Table 2) as when groups of females were used (experiments 5, 6, and 7, Table 2).

Optimal Density Range (ODR). An average of 17 (SD = 5.4) parasitoids was reared per ootheca from a sample of 100 colony ootheca exposed for 7 days in a fly rearing room in Fernald Hall, with 88% of all oothecae producing between 11 and 25 parasitoids. The level of parasitism in the fly rearing room ranged from 80 to 100% of all oothecae being attacked per week. Experiments 5, 6, 7, and 8 clearly indicate that parasitoids encountering oothecae previously parasitized by other females will lay additional eggs in them. Direct observation of individual female parasitoids under laboratory conditions indicates that parasitoids vary in their oviposition patterns. In some cases, a single egg may be laid before a visit is terminated and the parasitoid moves to a new ootheca, or up to 10 eggs may be laid in quick succession (Van Driesche, unpublished data). The 17 ovipositions/ootheca characteristic of wild population oviposition are therefore most likely the result of the combined oviposition of several females during two or more visits to a given ootheca. Evidence that an optimal density range exists for parasitoid larval survival was obtained then oothecae from experiments 1, 2, and 3 were reared and the number of parasitoids produced compared to the number of stalks counted. For 46 cases where 1–3 stalks were counted survival averaged 36%; for 41 cases were 4–7 stalks were counted, survival averaged 85%; and for 63 cases where 8 or more stalks were counted, it was 70% (differences between means were significant at 0.001 using Kruskal-Walis one-way ANOVA). These data suggest that an optimal density range does exist, with too few parasitoids/ootheca being detrimental as well as the often reported detriment of too many parasitoids per host. The data do not suggest what the mechanism for higher mortality of solitary or scarce larvae (under 4/ootheca) might be.

DISCUSSION

Parasitoids must efficiently find host habitats, identify appropriate host species and stages, and evaluate quality of individual hosts. No mechanism for host habitat location by *C. merceti* has been suggested except phototaxis which was described by Gordh (1973) as reversing from positive to negative depending on reproductive readiness of females. These observations were not supported by experimental data and conflict with the observation by Lawson (1954) that parasitoids collected at windows (and hence presumably positively phototactic) would oviposit "within minutes after being caged with eggs." The distinct odors associated with cockroaches seem a more likely, although untested, means of habitat location. Host recognition and acceptance, based on our data presented here, is at least in part a response to the cement female cockroaches use to bind their oothecae to surfaces or to some substance in or on the cement.

Discrimination of host quality with regard to previous parasitization is demonstrated by our data. More interestingly, there is the suggestion that parasitoid survival is improved by the presence of a minimum of four parasitoids per ootheca. Previous references to such an optimal density range for parasitoids are rare. Flanders (1935) cites an instance for *Trichogramma* wasps in which survival in large hosts was improved by the presence of several parasitoids per host because of the drier, more favorable pupation conditions that resulted from the greater degree of larval feeding. The mechanism in *C. merceti* is unknown, but since parasitoid adults chew their way out of the ootheca, it is not likely to be a mechanical problem as faced by the cockroaches themselves, which must have a minimum number of survivors to cause the ootheca to open. A possible explanation may lie in the "yeast-like" organism that is an obligate symbiont of *C. merceti* and is injected into the host ootheca at oviposition (LeBeck, personal communication). While the exact function of this organism in parasitoid survival is unknown, presumably it may function in host regulation as has been determined for other microbial associates of parasitoids (Vinson, 1975). If this is the case, there may be a minimum level of inoculum required for success. If this level exceeds that found in fewer than four ovipositions, it would provide an explanation of the observed data.

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RESPONSES OF WILD MUSKRATS (*Ondatra zibethicus* L.) TO SCENTED TRAPS

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Abstract—Free-ranging muskrats were trapped in scented and blank traps in New York State at local ponds during all seasons and at a wildlife refuge in spring and early summer. In a total of 4839 trap-nights, 65 muskrats were caught. Trapping success was 1.34%. The overall responses to differently scented traps differed significantly. Adults preferentially entered blank and food-baited traps, whereas young showed no preference to blank, musk, or control odor. It appears that adults actively avoid musk, especially during the months May through July.

Key Words—Live-trapping, Montezuma National Wildlife Refuge, muskrats, odor response, *Ondatra zibethicus*, musk, rodents, scent.

INTRODUCTION

Both sexes of the muskrat *Ondatra zibethicus* have specialized preputial glands under the ventral skin near the external genitalia. These glands are larger in males than in females and are most active in spring. The function of their secretion is not clear. Akkermann (1975) describes territorial marking with musk and fecal piles at a high population density. Because the glands are larger in the main breeding season, musk is also believed to function as a sexual attractant (Hoffmann, 1952; Mallach, 1976).

Knowledge of the biological function of musk might be an important tool in management of muskrat pests. In the Netherlands great parts of the country are below sea level, so the burrowing of muskrats in dikes causes great concern. Since the first muskrat was captured in 1940, the number has

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increased steadily; in 1981 over 140 thousand muskrats were trapped (Commissie Muskusrattenbestrijding, 1982).

Research concerning the use of musk in trapping muskrats is very scarce. Williams (1951) found an increase in trapping success by using carrot and musk combined instead of carrot only. However, the musk and blank traps were not set in the same period, so the results are not comparable. More recently, a field experiment was carried out in the Netherlands. In this experiment, half the number of traps had a musk odor, yet they attracted 74% of the total of 38 captured muskrats (Ritter et al., 1982).

The goal of the present experiment was to investigate the response of North American muskrats to different odors, especially that of musk.

METHODS AND MATERIALS

The study took place in two locations, one at the Montezuma National Wildlife Refuge and the other near Syracuse, New York. The Montezuma National Wildlife Refuge is located about 15 km west of Auburn, New York. The area consists mainly of marshland and open pools, impounded by dikes. The main vegetation of the marsh is cattail (*Typha* sp.). The second study area at Syracuse comprised six small privately owned ponds in the villages of Jamesville, Lafayette, and Otisco.

The experiment at the refuge ran from May 24 through July 30, 1983. The muskrats were trapped in single- and double-door box traps, made of one-inch welded wire mesh. Captured muskrats were sexed according to the external genitalia exposure method of Dozier (1942), weighed to within ± 50 g, marked with a numbered metal ear-tag (National Band and Tag Co., Newport, Kentucky,) and released at the same spot they had been trapped.

One third of the 54 traps had the musk odor, and another third a control odor (we chose phenylacetic acid, a main component of the ventral gland secretion of the Mongolian gerbil). The remaining third was not scented (blanks). The musk odor was a filtered homogenate of male musk glands in isopropyl alcohol. Approximately 2.5% of one gland equivalent was used per trap.

In each area the traps were 25 m apart and arranged in a single line. Within each line the treatments were arranged in regular sequence: musk, blank, control, etc. The traps were switched twice during the experiment to counterbalance location bias. This is important as some traps happened to be closer to a muskrat house than others. The total number of trap-nights was 2894.

The small ponds south of Syracuse were trapped from August 1980 through October 1983. Equal numbers of musk-scented, food-baited, and

TABLE 1. NUMBERS OF ADULT AND YOUNG MUSKRATS CAUGHT IN TRAPS SCENTED WITH MUSK, PHENYLACETIC-ACID AS A CONTROL ODOR, AND UNSCENTED (BLANK) TRAPS AT MONTEZUMA REFUGE

	Musk	Control	Blank	Total
Adults	1	1	10	12
Young	7	13	11	31
Total	8	14	21	43

blank traps were set along muskrat trails. The ponds were trapped until no further muskrats were caught. All captured animals were removed from the ponds. The total number of trap-nights was 1945.

RESULTS

Results from Wildlife Refuge. A total of 43 muskrats were caught in the 2894 trap-nights, representing a trapping success of 1.49%. The overall distribution of the numbers of young (weighing less than 800 g) and adults caught in the three trap types (Table 1) differs significantly from chance (chi-square test, $P < 0.05$). Ten of the 12 adults (of which five were females) were caught in blank traps ($P < 0.005$), while for the young, the differences between the trap types were not significant.

Results from Ponds near Syracuse. A total of 22 muskrats was caught in 1945 trap-nights. Trapping success was 1.13%. Again adults avoided the musk-scented traps, while the young preferred them (Table 2). To increase sample size for further analysis, all data from the two experiments were pooled (Table 3). The distribution of adults and young caught in blank and musk traps is significantly different from chance (χ^2, $P < 0.005$).

TABLE 2. NUMBERS OF ADULT AND YOUNG MUSKRATS CAUGHT IN TRAPS SCENTED WITH MUSK, FOOD-BAITED AND BLANK TRAPS AT SMALL PONDS NEAR SYRACUSE

	Musk	Food	Blank	Total
Adults	0	8	6	14
Young	6	0	2	8
Total	6	8	8	22

TABLE 3. NUMBERS OF ADULT AND YOUNG MUSKRATS CAUGHT IN MUSK-SCENTED AND BLANK TRAPS[a]

	Musk	Blank	Total
Adults	1	16	17
Young	13	13	26
Total	14	29	43

[a]Pooled results from Montezuma Refuge and ponds near Syracuse.

DISCUSSION

From May through July, adult muskrats were significantly easier to trap in blank traps than in traps scented with musk or with a control odor. Altogether, adults seem to prefer blank traps, while young show no discrimination between blank and musk scented traps.

In a similar experiment in the Netherlands, musk odor was shown to be an attractant (Ritter et al., 1982). That experiment ran from September 15 through June 5 and the bulk of the muskrats were caught in autumn and spring (I. Brüggemann, personal communication). The refuge experiment was conducted in summer, and musk turned out to be repellent. This suggests seasonally different responses of muskrats to musk odors. Other studies have shown the opposite effect in small rodents. In *Perognathus*, *Dipodomys*, and *Peromyscus maniculatus*, reproductively active adults enter traps scented with conspecific odor, while nonreproductive individuals preferentially enter unscented traps (Daly et al., 1978, 1980).

Overall trapping success was rather low. At the ponds, this could be explained by the low muskrat densities. We believe in most cases the ponds were inhabited by just one pair of adults, possibly with their offspring. However, trapping was continued, even after all muskrats were believed to have been trapped, to ensure no muskrats were left.

At the refuge, trapping success could have been low because of the season. Parker and Maxwell (1980) trapped only 30 muskrats in a similar live-trapping study in New Brunswick from May through July, but 175 from August through October. Furthermore, the scent traps turned out to be repellent, so more muskrats might have been trapped if only blank traps had been used.

A comparison of the results of the Dutch experiment with ours is complicated by several factors. First, the musk odor used in our experiments was made from a different musk gland stock. Since the chemical composition of musk changes seasonally (Ritter et al., 1982), it is possible that the composi-

tion of the odor was different. The response of muskrats to the different compositions and compounds of musk is not known.

Second, in the Netherlands, mainly migrating muskrats were caught, whereas in our experiment we had saturated, resident breeding populations. It is possible that migrating and resident muskrats may respond differently to the same odors. Migrating muskrats could seek actively for conspecific odors, for they indicate the presence of other muskrats and potential mates. Residents, on the other hand, may have learned to avoid odors (=territories?) from other inhabitants to reduce intraspecific aggression.

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AGGREGATION PHEROMONE OF THE DEODAR WEEVIL, *Pissodes nemorensis* (COLEOPTERA: CURCULIONIDAE): Isolation and Activity of Grandisol and Grandisal

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Abstract—The bark weevil *Pissodes nemorensis,* a pest of pines and exotic cedars in the southeastern United States, utilizes a male-produced aggregation pheromone. The presumed pheromone components, grandisol (*cis*-2-isopropenyl-1-methylcyclobutaneethanol) and its corresponding aldehyde, grandisal, were isolated from extracts of male volatiles and male hindguts. A field test in northern Florida showed that the combination of grandisol, grandisal, and slash pine (*Pinus elliottii*) bolts acted synergistically to attract large numbers of male and female *P. nemorensis.* These components deployed in various paired combinations were not as attractive as the tripartite mixture. There was no evidence that flying weevils were attracted to unbaited pine bolts. The aggregation pheromone for *P. nemorensis* appears to be similar to that of a parapatric sibling species, *P. approximatus.*

Key Words—*Pissodes nemorensis,* deodar weevil, Coleoptera, Curculionidae, aggregation pheromone, grandisol, grandisal, synergism.

INTRODUCTION

Weevils in the genus *Pissodes* comprise an important group of coniferous forest pests throughout North America. *Pissodes nemorensis* Germar, the

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deodar weevil, occurs in the southeastern United States and breeds in all pines (*Pinus* spp.) and exotic cedars (*Cedrus* spp.) in its range (Beal, 1952). Adult *P. nemorensis* weevils chew small holes through the bark of a host tree to feed on the phloem. Females lay eggs in some feeding holes made on weakened hosts, and larvae mine under the bark, consuming the phloem and cambium tissues (Jones, 1965; Atkinson, 1979). *P. nemorensis* is considered a minor pest because the trees in which it breeds are generally predisposed by drought, mechanical injury, fire, disease, or other insects. However, heavy feeding by adults on healthy pine seedlings and saplings may cause marked loss to reforestation projects (Ollieu, 1971; Overgaard and Nachod, 1971; Baker, 1972). Adult feeding has also been correlated with increase of the pitch canker pathogen, *Fusarium moniliforme* var. *subglutinans,* on slash pine, *Pinus elliottii* Engelm., in Florida (Blakeslee et al., 1981).

Aggregation pheromones in *Pissodes* were first reported by Booth and Lanier (1974). Males of the northern pine weevil, *P. approximatus* Hopkins, that were feeding on pine bolts attracted conspecific males and females in a field test. A monoterpene alcohol, grandisol (*cis*-2-isopropenyl-1-methylcyclobutaneethanol), and its corresponding aldehyde, grandisal, were isolated from male *P. approximatus* and shown to have pheromone activity when deployed together with host odors (Booth et al., 1983). The same study isolated both compounds from males of the white pine weevil, *P. strobi* (Peck), and implicated them in the presumed aggregation pheromone of that species. Atkinson (1979) demonstrated pheromone activity of grandisol and grandisal for *P. nemorensis* in northern Florida but, in limited testing, was unable to show that males could also be attractive. Fontaine and Foltz (1982) subsequently found that male *P. nemorensis* feeding on slash pine bolts produced an aggregation pheromone. In this paper we report the isolation of grandisol and grandisal from *P. nemorensis* and the activity of these compounds in a field test.

METHODS AND MATERIALS

Adult weevils used in isolation studies were obtained from laboratory colonies in Syracuse, New York, that originated with field-collected *P. nemorensis* infesting slash pine in Gilchrist County, Florida. Weevils were reared on 4 × 10 cm bolts of red pine, *P. resinosa* Ait., under lighting of 12:12 (light–dark) at 23°C and 40% relative humidity (Phillips, 1981). To ensure that the insects were reproductively mature, newly emerged weevils were separated by sex and maintained on fresh cuttings of red pine branches under the same conditions for a period of 30–50 days before their use

(Fontaine et al., 1983). Volatiles from mature *P. nemorensis* were collected by aeration and absorption on Porapak Q (Waters Assoc., Framingham, Massachusetts) (Byrne et al., 1975). On two different occasions, separate groups of 50–100 males and females feeding on red pine bolts were aerated in large vacuum desiccators (Phillips, 1981). Aerations at 1 liter/min took place over 5- to 10-day periods under 12:12 (light–dark) lighting at 25°C amd 40% relative humidity. Porapak Q was cleaned and conditioned prior to use (Williams et al., 1981); following aeration, the volatiles were extracted from Porapak Q with distilled pentane (5 ml/g). Extracts were dried and concentrated prior to analysis by gas chromatography (GC) (Booth et al., 1983). Hindguts were removed from the weevils following aerations and crushed in small amounts of distilled pentane; the slurries were frozen, sonicated, and centrifuged, and the supernatants were used for GC analysis. Weevil bodies without hindguts were retained and similarly extracted in pentane.

The extracts were fractionated on a 6.1-m × 4-mm ID glass column containing 4% Carbowax 20 M on Chromosorb G, 60/100 mesh, oven at 100°C for 12 min, then programed to 150° at 4°/min, N_2 flow 60 cc/min, injector 140°, detector 160°. The respective retention times for grandisal and grandisol were 22 and 47 min. The fractions containing these compounds were further fractionated on a 3.05-m × 4-mm ID glass column containing 4% FFAP on Varaport 30, 100/120 mesh, N_2 flow 20 cc/min, injector 140°, detector 160°. At an oven temperature of 90°, the retention time for grandisal was 13 min; at 120°, the time for grandisol was 19.5 min. These collected compounds were each coinjected with authentic samples on a 50-m × 0.21-mm ID fused silica FFAP capillary column. The oven temperature was held at 35° for 40 sec, then to 150° at 4°/min; the N_2 backpressure was 276 kPa. The retention time for grandisal was 24.5 min; that for grandisol was 37 min. Mass spectra that matched those of authentic samples were obtained on a Finnigan 4000 instrument fitted with a 30-m × 0.21-mm ID fused silica DB-1 capillary column.

A field test of pheromone activity was conducted during November and December 1982 in a young plantation of slash pine in Gilchrist County, Florida. Hardware-cloth sticky traps were used (Bedard and Browne, 1969; Booth and Lanier, 1974), and six different treatments deployed in five randomized complete blocks were compared for their attractiveness to *P. nemorensis:* five male *P. nemorensis* feeding on a freshly cut slash pine bolt (15 × 30-cm bole section), grandisol (GOH) with a bolt, grandisal (GCHO) with a bolt, GOH and GCHO with a bolt, GOH and GCHO alone, and a bolt alone. Feral adult males used as attractive sources were collected from split slash pine billets prior to the test (Fontaine, 1981). Racemic grandisol was obtained commercially, and grandisal was synthesized directly from it

(Booth et al., 1983). Synthetic compounds were evaporated from silicone rubber chromatography septa (GR-2 septa, 9.5 mm, Supelco, Inc., Bellefonte, Pennsylvania); pheromone baits consisted of three septa for each compound at a dosage of 2.0 mg of material per septum for GOH (6 mg total) and 4.0 mg per septum for GCHO (12 mg total). The release rate of individual baits in the laboratory for GOH over a two-week period ranged from an initial rate of 45 μg/hr to 3 μg/hr at the end of the period; the range for GCHO was 30 μg/hr to 1 μg/hr (J. R. West, unpublished data).

RESULTS AND DISCUSSION

Grandisol and grandisal are produced by males of *P. nemorensis*. These compounds were never isolated from any extracts of females, nor were they found in the whole-body extracts of males from which hindguts had been removed. As in the sibling species *P. strobi* and *P. approximatus* (Booth et al., 1983), pheromone components were found only in the hindgut regions of male *P. nemorensis*. The amounts of these compounds found in aeration or hindgut extracts were not consistent between trials (Table 1). The ratio of aldehyde to alcohol in the Porapak aerations varied from about 3 : 1 to 22 : 1 (GCHO–GOH), but hindgut extracts did not show the same predominance of aldehyde. The material collected from aerations of live weevils is probably the best approximation of what is available for weevils to receive in the field, despite the difficulties that may be involved in the analytical manipulation of a sensitive compound like grandisal (Zeng, 1983).

Data from the field test indicate that grandisol and grandisal have strong pheromone activity for *P. nemorensis* (Table 2). The tripartite combination of grandisol, grandisal, and a pine bolt was the most attractive treatment in the test, and this activity can be attributed to a strong synergism among the three components. The treatment GOH + bolt

TABLE 1. ISOLATION OF GRANDISOL (GOH) AND GRANDISAL (GCHO) FROM MALE *Pissodes nemorensis*

	Porapak Q aerations (μg/weevil/hr[a])		Hindgut extracts (μg/weevil)	
Trial	GOH	GCHO	GOH	GCHO
1	0.0023	0.0070	0.04	0.05
2	0.0005	0.0110	0.04	0.01

[a]This is equal to the amount of material collected from one weevil in 1 hr.

TABLE 2. RESPONSE OF *Pissodes nemorensis* TO GRANDISOL (GOH), GRANDISAL (GCHO), AND NATURAL ATTRACTANTS IN A FIELD TEST, NOVEMBER 12–DECEMBER 8, 1982

Treatments[a]	Number of weevils captured[b]		
	Females	Males	Total
5 males + bolt	16a	4a	20a
GOH + bolt	1a	1a	2a
GCHO + bolt	54b	44b	98b
GOH + GCHO + bolt	74c	58b	132c
GOH + GCHO	12a	5a	17a
bolt	1a	0a	1a

[a]See text for details.
[b]Totals in a column not followed by the same letter are significantly different (Student-Newman-Keuls test, $P < 0.05$).

displayed virtually no activity, but the addition of GCHO resulted in a dramatic increase in response. Similarly, GOH + GCHO had low activity but gained substantial activity with the addition of a pine bolt. GCHO + bolt had moderate attractiveness that was significantly increased with the addition of GOH. Although we did not test GCHO and GOH separately without pine bolts, our data lead us to suggest that they would also have low activity. Traps baited with male *P. nemorensis* on bolts were statistically among the treatments with the lowest responses, but this can be attributed to the analysis of variance that includes many treatments, some of which have very high mean values. Other tests with fewer treatments (Fontaine and Foltz, 1982) have adequately demonstrated the pheromone activity of male *P. nemorensis.*

The relative importance of primary attraction of an insect to host material in response to host-related odors (a kairomonal response), or secondary attraction to odors from a conspecific (a pheromonal response) in host location by bark beetles (Coleoptera: Scolytidae), has been questioned many times (Heikkenen and Hrutfiord, 1965; Moeck et al., 1981; Wood, 1982). As in many Scolytidae, secondary attraction in *P. nemorensis* is manifested through an aggregation pheromone. We do not have any good evidence for the importance of primary attraction in host location by flying *P. nemorensis.* Of five traps baited with slash pine bolts on which no weevils were feeding, only one trap caught one weevil over the three-week test period (Table 2). Although host odors do not act alone in a kairomonal way, one or more volatile compounds from slash pine synergize the activity of the combined male-produced compounds. It is possible that host odors

may be important for close-range orientation of walking weevils, such as that demonstrated in a laboratory bioassay (Phillips, 1981).

The aggregation pheromone system of *P. nemorensis* that we describe here appears to be identical to that of a sibling species, *P. approximatus* (Booth et al., 1983). These species have similar ecological habits but differ in their geographic distributions and breeding seasons (Atkinson, 1979; Finnegan, 1958). In other studies (Phillips and Lanier, 1984), we have demonstrated cross-attraction between these species. Although there may be differences in the enantiomeric composition of grandisol and grandisal between these species, it appears that racemic material is very effective in a synthetic pheromone for both. The probable occurrence of identical aggregation pheromones in *P. nemorensis* and *P. approximatus* raises important questions regarding the systematic status of these presumed species.

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ALARM RESPONSE TO VENOM BY SOCIAL WASPS *Polistes exclamans* AND *P. fuscatus* (HYMENOPTERA: VESPIDAE)

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Abstract—The venoms of *Polistes exclamans* and *P. fuscatus* elicit alarm behavior and attract attacking wasps. The response is not species specific, for both hetero- and conspecific venoms elicit similar responses in both species. A test in a wind tunnel provided no support for the hypothesis that alarmed wasps release an alarm pheromone on the nest.

Key Words—Social wasp, *Polistes exclamans, Polistes fuscatus,* Vespidae, Hymenoptera, venom, alarm pheromone, alarm behavior.

INTRODUCTION

The communication of alarm by pheromones is common among the higher eusocial Hymenoptera, including the highly eusocial vespine and polybiine wasps (Maschwitz, 1964; Hölldobler, 1977; Edwards, 1980; Jeanne, 1981). Although Maschwitz (1964) found no evidence for an alarm pheromone in the primitive social wasp *Polistes dubius* Kohl in Europe, recently Jeanne (1982) discovered that the venom of *P. canadensis* (L.) in Brazil elicits alarm, reduces the threshold for attack, and acts as an attractant. Yet it is not known if venom is released on the nest by alarmed *P. canadensis,* and if nestmates thereby communicate alarm chemically. However, the fact that the sting chamber is held open during intense alarm suggests that such is the case (Jeanne, 1982).

Prior to the discovery of an alarm response to venom in *P. canadensis,* it was suggested that wing buzzing and jerky movements by alarmed wasps may communicate alarm to nestmates through vibration of the nest (West-Eberhard, 1969). However, the population of *P. canadensis* that Jeanne (1979,

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1982) studied in Brazil is peculiar in that its colonies occupy multiple combs unattached to each other. Thus the communication of alarm through the whole colony by nest vibration is not feasible, while chemical communication of alarm would be adaptive and could have an important role in colony defense. On the other hand, in species having a single comb, such as *P. dubius,* there may be little selection pressure for the evolution of an alarm response to venom and chemical communication of alarm.

In this paper we present evidence that venom elicits alarm and attracts attacking wasps of *P. exclamans* Viereck and *P. fuscatus* (F.), wasps whose nests typically consist of a single comb. Secondly, we show that heterospecific venom elicits an alarm response. And finally, we test the hypothesis that alarmed wasps release venom while on the nest, and thereby communicate alarm chemically.

METHODS AND MATERIALS

Collection and Maintenance of Wasps in Laboratory. Intact colonies of *P. exclamans* and *P. dorsalis* (F.) were collected on August 17–19, 1983, in Cleveland County, North Carolina, and colonies of *P. fuscatus* were collected on September 6–7, 1983, in Madison, Wisconsin. The colonies were transported on ice to the laboratory, where each nest was glued to cardboard mounted on screen tops of Plexiglas® cages (20 × 20 × 20 cm), and the adult wasps were allowed to reoccupy their nests. Each colony was provided with honey and water.

Field Bioassay for Alarm Pheromone. We conducted bioassays in Shelby, North Carolina on August 17 and 18 with *P. exclamans,* following the methods of Jeanne (1982). However, since colonies of *P. exclamans* normally do not nest in open areas exposed to wind, two colonies (with 30 and 28 females and no males) were collected from a building and transplanted to a 30 × 200-cm piece of 1.3-cm-thick plywood set horizontally 1.5 m above the ground in an open area exposed to prevailing wind. The nests of the two colonies were glued to the underside of the plywood crosswind to each other and 1.5 m apart. The wasps were then placed on their respective nests the night before we started conducting the bioassays.

To determine if the venom elicited alarm and attack, we presented to each colony the following stimuli in turn: (1) a venom gland and sac macerated on filter paper, (2) the internal organs of the gaster, including Dufour's gland but minus the venom gland and sac and the sting apparatus, macerated on filter paper, and (3) a clean piece of filter paper as a control. Each filter paper was presented at the end of a 2.5 × 2.5 × 100-cm stick.

We allowed the wasps to attack either dark moving, dark stationary, light moving, or light stationary models. The models consisted of four 60-ml square

plastic bottles wrapped in black or white paper, and hung on thread (moving) or stiff wire (stationary) from dowels to form a square 30 cm on a side. The models were placed 0.5 m from a nest and crosswind to the nest so that air passing over the models did not reach the nest (see description and figure in Jeanne, 1982).

For each trial, the two moving models were swung in a plane perpendicular to the direction to the nest, and then the filter paper bearing the test object was held about 15 cm upwind of the colony. We quantified the alarm and attack response by recording the number of wasps flying to the filter paper and the models. Trials were separated from each other by at least 30 min.

Since the wind speed was not constant during the trials, we conducted a second series of bioassays using a 65-cm fan placed 3 m from the colonies so as to create an airstream over the nests. We followed the same procedures as described above, except we tested the response to two (instead of one) venom glands and sacs macerated on filter paper. Responses were recorded as above.

Laboratory Bioassay for Alarm Pheromone. A wind tunnel was set up in the laboratory to direct air through two cages connected serially (Figure 1). The bioassays consisted of introducing a test substance through slits into the airstream of each tube and then recording the responses of intact colonies placed in each cage. Each test object was presented to colony B prior to colony A and at least 30 min separated each presentation to a colony. We quantified the response of each colony to each substance by recording the intensity of alarm behavior displayed by the females. In response to a disturbance near the nest, females of *Polistes* perform a sequence of behavior increasing in intensity

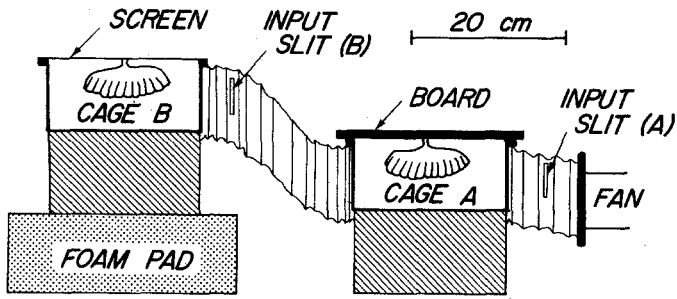

FIG. 1. Wind tunnel used in the laboratory bioassay. Two cages (A and B) were connected serially via a flexible plastic tube to a fan. Each cage had a Plexiglas® front and back and cardboard sides and bottom. The top of cage B was covered with screen, while the top of cage A was sealed with a board. Wire screen placed over the openings to the tubes prevented the wasps from escaping from each cage. Cage B was placed on a foam pad to reduce the transfer of vibrations from cage A to cage B and to eliminate visual contact between the two colonies through the tube. Test substances were introduced into the airstream through the two input slits.

until they attack or flee. Prior to attack, females turn and orient to the disturbance, spread and raise the wings, wave the forelegs, increase pumping of the gaster and bend the gaster to one side, flutter or buzz the wings, and open the sting chamber (Starr, 1981; Jeanne, 1982). During attack, the females fly off the nest toward the source of the disturbance and attempt to sting it. In our tests we divided the responses into three levels prior to attack or flee: (1) orient and wing raise, (2) gaster pump and/or gaster bend, and (3) wing flutter or buzz (during intense alarm *P. exclamans* usually did not buzz their wings, but slowly fluttered them, while *P. fuscatus* rarely fluttered their wings, but buzzed them). In addition to recording the maximum intensity of the alarm behavior of wasps on the nest, we recorded the number of females which flew off the nest.

To determine if *P. fuscatus* responds to venom, we tested (as we did with *P. exclamans* in the field) a venom gland and sac macerated on filter paper, the internal organs minus the venom gland and sac and sting apparatus macerated on filter paper, and a blank piece of filter paper (control). We conducted reciprocal interspecific tests of venom between *P. exclamans* and *P. fuscatus,* as well as a test of the responses of both species to the venom of *P. dorsalis*. In these assays we tested only the macerated venom gland and sac.

The laboratory bioassays on *P. exclamans* were conducted from August 30 to September 10, using a total of four different colonies (number of females = 113, 71, 65, and 47; each nest also contained a small number of males). The bioassays on *P. fuscatus* were conducted from September 8 to 10, using a total of five colonies (number of females = 55, 48, 36, 32, and 28; several males also were on each of these nests).

Test for Release of Alarm Pheromone by Alarmed Wasps on Nest. To test the hypothesis that alarmed wasps release an alarm pheromone on the nest, we placed two conspecific colonies in the wind tunnel (Figure 1). We then alarmed the wasps on nest A by waving a hand in front of the cage and by gently tapping on top of the cage. These stimuli could not reach colony B. The colony was aroused to the level of wing buzzing or fluttering, opening of the sting chamber, and attack. During this time we recorded the alarm response of the wasps on nest B, as described above. If alarmed wasps on nest A release an alarm pheromone, we predicted that this pheromone would be carried on the airstream to nest B, where it would elicit similar behavior by the wasps there.

To show that a pheromone released by colony A would pass over nest B, we introduced a macerated venom gland and sac into the wind tube upstream of nest A and recorded the alarm response of the wasps on both colonies. If the apparatus allowed odors to pass from colony A to B, both colonies should exhibit an alarm response. The tests were conducted using the same colonies described above.

RESULTS

Bioassays for Alarm Pheromone. In the field females of *P. exclamans* responded with alarm and attack to the macerated venom glands and sacs (Table 1). The response to venom was greatest during the second series of bioassays in which a fan and two crushed venom glands and sacs were used. Females on the nest responded to venom with a rapid movement to the upwind side of the nest, and with wing raise and flutter. On attack most wasps flew directly upwind to the filter paper containing venom and attempted to sting the paper. On the other hand, the few wasps flying to the models did not attempt to sting them but only briefly walked around on the model before leaving and then flying in zig-zag fashion around the four models. The black models attracted the most wasps, although at least one wasp flew to each of the four models.

In the laboratory *P. fuscatus* responded to its own venom with intense alarm (Table 2). Generally, most of the reacting wasps directed their responses upwind, i.e., toward the source of the venom.

The venom of heterospecifics also elicited an intense alarm response from females of *P. fuscatus,* and a less intense, although detectable, response from females of *P. exclamans* (Table 3).

TABLE 1. FIELD BIOASSAY OF RESPONSES OF *P. exclamans* TO FOUR VISUAL MODELS AND TO FILTER PAPER BEARING THE TEST SUBSTANCE[a]

Test substance	No. of trials	Filter paper	Models			
			Moving		Stationary	
			Dark	Light	Dark	Light
With natural wind						
Clean filter paper	10	0	0	0	0	0
Internal organs minus sting apparatus and venom sac	8	0	0	0	0	0
One venom gland and sac	12	6(21)	1(1)	0	0	1(1)
With a fan						
Clean filter paper	10	1(1)	0	0	0	0
Internal organs minus sting apparatus and venom sac	10	1(1)	0	0	0	0
Two venom glands and sacs	10	9(47)	3(3)	1(1)	2(2)	1(1)

[a]Data are the numbers of trials in which wasps responded with attack; the total numbers of wasps attacking each object are shown in parentheses.

TABLE 2. LABORATORY BIOASSAY OF RESPONSES OF *P. fuscatus* TO TEST SUBSTANCES ON FILTER PAPER[a]

Test substance	No. of trials	Greatest intensity of response achieved: Orient and wing raise	Gaster pump-bend	Wing buzz	Females flying off
Clean filter paper	7	0	0	0	0
Internal organs minus sting apparatus and venom sac	7	1	0	2	1(1)
Venom sac	6	0	0	6	6(20)

[a]Data are the numbers of trials in which wasps responded. The total number of females flying off the nest are shown in parentheses.

Test for Release of Alarm Pheromone by Alarmed Wasps on Nest. We found no evidence that alarmed wasps on the nest communicate alarm by a pheromone. Although the wasps on nest B responded to the control venom, they did not exhibit alarm when we physically induced alarm and attack from the wasps on nest A (Table 4).

DISCUSSION

Although venom elicits an alarm response and attracts females of *P. exclamans* and *P. fuscatus,* we found no evidence that wasps release venom, or any alarm pheromone, during intense alarm on the nest. It is possible, however, that an alarm pheromone is released in very small quantities and that our experimental procedure was not sensitive enough to detect it. Yet it is likely that in large colonies with a large number of alarmed wasps the amount of venom released would be comparable to the amount released from a single macerated venom sac, which was enough to elicit a response. Also, if a pheromone is released in ample enough concentration to diffuse over the open comb of a *Polistes* colony and if it has an important role in coordinating colony defense, the wasps on the second nest (B) should have detected it and responded with alarm. Therefore, we conclude that it is more likely that alarm is elicited in individuals detecting a disturbance near the nest and that communication, if any, of alarm on the nest likely occurs via transmission of the vibration of wing buzzing through the nest and/or the visible behavior of alarmed wasps.

This raises the question of why alarmed wasps frequently open their sting chambers if they are not releasing an alarm substance. There are at least three

TABLE 3. BIOASSAY OF RESPONSES OF *P. fuscatus* AND *P. exclamans* TO HETEROSPECIFIC VENOM[a]

Test venom	No. of trials	Greatest intensity of response achieved: Orient and wing raise	Gaster pump-bend	Wing flutter	Wing buzz	Females flying off
Response of *P. fuscatus* to						
P. exclamans venom	6	1	0	0	5	5(25)
P. dorsalis venom	6	0	1	0	5	5(17)
Response of *P. exclamans* to						
P. fuscatus venom	7	4	0	0	0	2(6)
P. dorsalis venom	6	3	1	0	0	0

[a]Data are the numbers of trials in which wasps responded. The total numbers of females flying off the nest are given in parentheses.

TABLE 4. TEST FOR RELEASE OF ALARM PHEROMONE ON NEST BY ALARMED WASPS OF *P. fuscatus* AND *P. exclamans*[a]

Treatment	Greatest intensity of response achieved: Orient and wing raise	Gaster pump-bend	Wing flutter	Wing buzz	Females flying off
P. fuscatus (10 trials for each control and test)					
Nest A					
Control venom[b]	1	0	0	8	7(21)
Test[c]	0	0	0	10	10(66)
Nest B					
Control venom	2	2	0	5	4(17)
Test	0	0	0	0	0
P. exclamans (7 trials for each control and test)					
Nest A					
Control venom	2	0	3	2	1(3)
Test	0	0	0	7	4(14)
Nest B					
Control venom	2	0	3	2	1(2)
Test	0	0	0	0	0

[a]Data are the numbers of trials in which wasps responded; the total numbers of females flying off the nest are shown in parentheses.

[b]Control venom consisted of presenting a macerated conspecific venom gland and sac upwind to colony A, which was in turn upwind to colony B, and then recording the responses by the wasps on both nests.

[c]During a test, colony A was alarmed by physically disturbing the wasps on the nest, and then the response by wasps on nest B was recorded.

possibilities: (1) It may be an inadvertent consequence of gaster bending. Perhaps the muscular contractions necessary for bending the gaster result in an opening of the sting chamber. (2) It may be a visual defensive display to potential vertebrate predators or to nestmates. (3) It may simply be a preparation for stinging.

It still remains to be discovered if the population of *P. canadensis* in Brazil releases an alarm pheromone on the nest. On the basis of their characteristic of building multiple combs, an alarm pheromone would be adaptive, since alarm cannot be communicated through nest vibration. If they do release venom as an alarm pheromone on the nest, this would explain why most of the attacking wasps flew to the models (Jeanne, 1982) and not to the filter paper as *P. exclamans* did. In *P. canadensis,* venom could have an important role in releasing alarm and attack prior to alarm by the whole colony and an attack by a wasp. Once one or a few wasps detect the venom, they in turn may release venom on the nest, making it difficult for wasps on the nest to orient to the original source of venom. Thus most of the wasps might be expected to fly more to visual disturbances than to the site of the original sting. However, a large number of wasps were attracted to the filter paper with venom, suggesting that venom also is attractive (Jeanne, 1982).

It is not clear why Maschwitz (1964) failed to elicit an alarm response to venom by *P. dubius.* It may be that not all species of *Polistes* exhibit an alarm response to venom. Alternatively, the experimental procedures or handling of the wasps may have influenced their behavior. During our bioassays, we noticed three conditions which influenced the behavior of the wasps. First, it became clear that if we tested the response of the wasps frequently over a short period of time, the wasps habituated to the odor of venom and the intensity of the alarm response declined over time. Second, the intensity of aggressiveness and alarm decreased with the amount of time the wasps spent in the laboratory cages, to the point that wasps reared in the laboratory failed to show a clear-cut response to the venom (personal observations). This probably explains why the intensity of the response of *P. exclamans* in the laboratory was low compared to that in the field tests and in the tests with *P. fuscatus.* The last bioassay with *P. exclamans* was conducted 24 days after they were collected from the field, while the bioassays with *P. fuscatus* were conducted within a few days after collection. Maschwitz (1964) does not state how long the wasps were kept in the laboratory prior to testing. Third, although we did not quantify it, colonies with few females did not respond well. Maschwitz used a colony containing 18 wasps, many fewer than the mean number of females on our colonies. Moreover, some of the 18 wasps may have been males, which do not take an active part in colony defense.

The use of defensive substances as alarm pheromones is not unusual in social insects (Hölldobler, 1977). In some social insects, such as honeybees and some ants, the alarm substance not only functions in its original role of

physical deterrence, but has been freed from the immediate context of its original role (i.e., stinging) and has evolved into use for communicating alarm to nestmates. For example, honeybees not only are alarmed and attracted to a pheromone released by a sting embedded in a predator (Ghent and Gary, 1962), but they also communicate alarm to nestmates during a characteristic alarm posture in which they raise the gaster, extrude the sting, and release a pheromone (Maschwitz, 1964). On the other hand, our results with *Polistes* indicate that in nature the alarm pheromone in the venom plays a role only in initiating alarm and attack after the object causing the disturbance has been attacked and stung. Thus the alarm pheromone would function to recruit nestmates, as well as heterospecific or conspecific females from nearby colonies, in a coordinated attack on a predator, which would be advantageous in deterring a vertebrate predator. Since there is no experimental evidence that alarmed wasps release venom except during stinging, the use of venom in alarm communication has not been freed from its originally evolved function, and therefore, compared to the alarm systems of other social insects, it probably represents a primitive state in the evolution of the complex systems of communication of alarm seen in higher social insects.

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SYNTHESIS OF HIGHLY ACTIVE JUVENILE HORMONE ANALOGS, JUVOCIMENE I AND II, FROM THE OIL OF SWEET BASIL, *Ocimum basilicum* L.

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Abstract—Juvocimene I and II are potent juvenile hormone mimics isolated from the essential oil of sweet basil, *Ocimum basilicum* L. The structures given by the formula I and II have been confirmed by synthesis with *trans*-β-ocimene and *p*-methoxycinnamyl chloride. Biological activity of the natural and synthetic juvocimenes was found to be identical.

Key Words—Juvenile hormone analog, juvocimene, sweet basil, *Ocimum basilicum*, milkweed bug, *Oncopeltus fasciatus*.

INTRODUCTION

Many chemicals are known to possess juvenile hormone activity, and although most of these are of synthetic origin, a few have been isolated from plants, including juvabione (Bowers et al., 1966) and dehydrojuvabione (Černý et al., 1967).

The presence in plants of natural insecticides (pyrethrins, rotenone, nicotine, etc.) as well as antifeedants and repellents (azadirachtin, warburganal, camphor, etc.) is well known; they are acknowledged to be important components in their defensive screen. The discovery of juvenile hormone mimics and molting hormone analogs in plants suggests additional protective strategies. The sweet basil plant (*Ocimum basilicum* L., Labiatae) has long been recognized to contain natural insecticides, including estragole and methyl cinnamate (Deshpande and Tipnis, 1977), and we now report on the

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characterization and synthesis of two extremely potent juvenile hormone analogs from the essential oil of this plant. In a prior brief report (Bowers and Nishida, 1980), we were able to determine the correct structures by spectral analysis and through the synthesis of analogous structures. We now wish to report the total synthesis of juvocimene I and II, together with their detailed chemistry and biological activity. In addition, two methylenedioxyphenyl analogs of the juvocimenes were prepared and their activities determined.

METHODS AND MATERIALS

Bioassay. The test samples were dissolved in acetone, and 1 μl of the solution was applied topically to freshly molted last (fifth) instar nymphs of the milkweed bug, *Oncopeltus fasciatus* (Dallas). After treatment, the insects were maintained in Petri dishes (9 cm ID, 5–8 individuals per dish), with milkweed seeds and water until the next molt at 29.5°C, 16:8 light–dark photoperiod (Bowers, 1968).

Instruments. Infrared (IR) and ultraviolet (UV) spectra were measured with a Perkin-Elmer 257 grating infrared spectrophotometer, and a Cary model 15 recording spectrophotometer, respectively. Nuclear magnetic resonance (NMR) spectra of natural juvocimene I and II and their hydrogenated derivatives (III and IV) were recorded with a Varian XL-100A (100-MHz, Fourier transformed), and others with a Varian HA-100 (100-MHz), in $CDCl_3$ with tetramethylsilane as an internal standard. Mass spectra (MS) were recorded with HP 5985A (GC-MS-DS) at 70 eV. (All of the samples were separated on GLC using a 15 m capillary column.) Gas chromatographic (GLC) analysis on a packed column was conducted with 3% OV-1 (2 m × 2 mm ID) at various conditions with hydrogen flame ionization detection. Adsorbents used for column chromatography were silica gel (100–200 mesh, Davison Chemical), and Florisil (60–80 mesh, Fisher Scientific Company).

Fractionation of Essential Oil of Sweet Basil. An essential oil of sweet basil (150 g, Fritzsche Dodge and Olcott Inc., New York, Oil Basil Sweet F.C.C. Extra, lot H-2324) was distilled in vacuum under a nitrogen stream. The inactive volatile fraction (142 g, to vapor temperature of 99°C, 1 mm Hg) was distilled, and the active residue was recovered as a viscous oil (6.37 g). The residual oil (6.20 g) was chromatographed on a column of Florisil (200 g, packed in petroleum ether, 320 × 35 mm ID), eluting with petroleum ether and then mixtures of increasing concentrations of diethyl ether in petroleum ether. The activity was found in two fractions. The first active fraction (fraction I, 120 mg) eluted with 1% (900 ml) and 2% (450 ml) ether in petroleum ether, after elution with 900 ml of petroleum ether. Subsequent fractions eluted with 2% (450 ml) and 5% (900 ml) ether in petroleum ether did not

contain biological activity. A second active fraction (fraction II, 310 mg) was obtained by elution with 10% ether in petroleum ether (900 ml).

Isolation of Juvocimene I. Fraction I (120 mg) was subjected to silica gel column chromatography (12 g, deactivated with 10% H_2O, packed in hexane, 240 × 9 mm ID). Activity was eluted in 24 ml of 0.3% ether in hexane (23.4 mg), after elution with 0.1% ether in hexane (60 ml), 0.2% ether in hexane (60 ml), 0.3% ether in hexane (36 ml). Portions of this active fraction were then subjected to high-performance liquid chromatography (HPLC): Lichrosorb SI-100 (5μm), 380 × 10 mm ID, 0.2% ether in heptane (2 ml/min, 25 kg/cm^2). The activity was found at R_t = 34–36 min. This fraction (~2 mg, combined from several runs) was purified further with HPLC on silica gel coated with silver nitrate [5% $AgNO_3$ on Biosil A (2–10 μm), 200 × 20 mm ID; 10% ether–heptane (6 ml/min, 10 kg/cm^2)]. From this procedure pure juvocimene I was obtained as a colorless liquid (0.75 mg). MS: m/z (%) 282 (M^+, 0.3), 93(32), 135(35), 147(100). NMR: Figure 1. IR: νcm^{-1} (neat) 3010, 2910, 1640, 1610, 1580, 1513, 1440, 1375, 1295, 1250, 1175, 1105, 1035, 985, 965, 890, 835. UV: λ_{nm}^{max} (EtOH) 237(ϵ23,000), 260(ϵ22,000). $[\alpha]_D^{23} = 0°$C (c = 0.07 in hexane).

Isolation of juvocimene II. Fraction II (310 mg) was subjected to column chromatography (25 g of 10% H_2O–silica gel, packed with hexane, 180 × 18 mm ID). The highest activity was found in a 40-ml eluate with 5% ether in hexane (41 mg) after elution with hexane (100 ml), 1% ether in hexane (100 ml), 2% ether in hexane (100 ml), 3% ether in hexane (100 ml), and

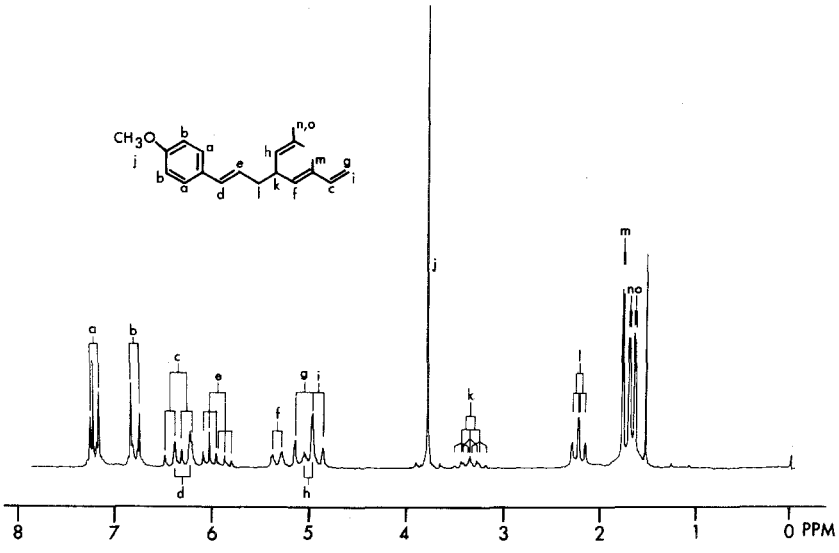


FIG. 1. NMR spectrum of juvocimene I (in $CDCl_3$, 100 MHz).

5% ether in hexane (50 ml). This active fraction (37 mg) was purified further by HPLC: Biosil A (2–10 μm), 250 × 15 mm ID, 6% ether in hexane (4 ml/min, 4.2 kg/cm). The active material was collected from R_t = 33–36 min. The same procedure was repeated with several less pure side fractions which contained juvocimene II, and the combined fractions were purified successively by HPLC [Lichrosorb SI-100 (5 μm), 250 × 10 mm ID, 8% ether in hexane (1.1 ml/min, 35 kg/cm^2), R_t = 52 min]. Final purification was accomplished by HPLC [Lichrosorb SI-100 (5 μm), 380 × 10 mm ID, 8% ether in hexane (2.2 ml/min, 63 kg/cm^2), R_t = 31.5 min] to yield pure juvocimene II as a colorless oil (0.78 mg). MS: m/z(%) 298(M^+, 0.8), 91(10), 147(100), 207(2). NMR: Figure 2. UV: λ_{nm}^{max} (EtOH) 227(ϵ29,000), 262(ϵ25,000). $[\alpha]_D^{23} = 0°$ (c = 0.06 in hexane).

Hydrogenation of Juvocimene I. Juvocimene I (250 μg) dissolved in 1 ml of ethanol was stirred for 1 hr under hydrogen atmosphere with platinum oxide (1 mg) as a catalyst. After filtration of the catalyst, the solvent was evaporated to give the octahydro derivative (III) quantitatively (Figure 3). MS: m/z(%) 290(M^+, 12), 121(100), 134(14), 147(20). NMR: δ7.10(2H, d, J = 9.0), 6.83(2H, d, J = 9.0), 3.80(3H, s), 2.52(2H, t, J = 7.0), 0.9–0.8(12H, m).

Hydrogenation of Juvocimene II. Juvocimene II (200 μg) was hydrogenated in hexane (1 ml) with platinum oxide (1 mg) by stirring the suspension for 10 min under hydrogen atmosphere. The solution was passed through 0.5 g of silica gel eluting with 10% ether in carbon tetrachloride to yield a hexa-

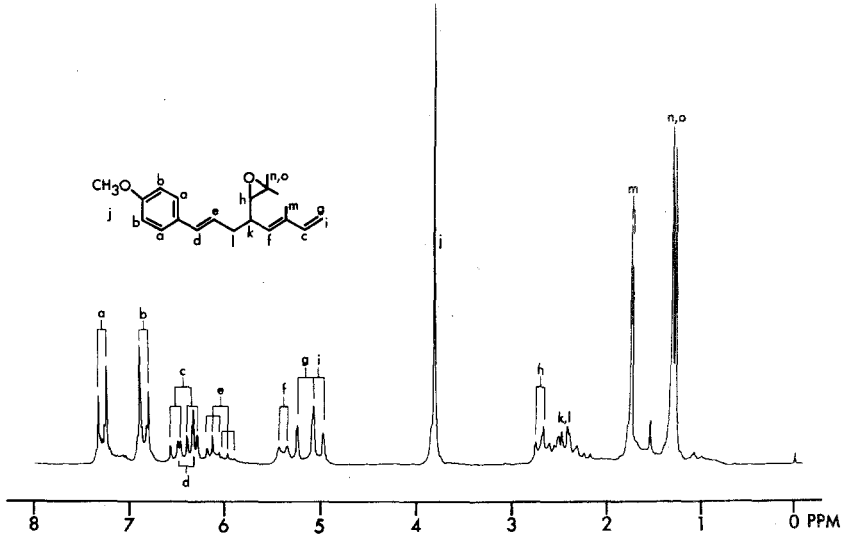


FIG. 2. NMR spectrum of juvocimene II (in $CDCl_3$, 100 MHz).

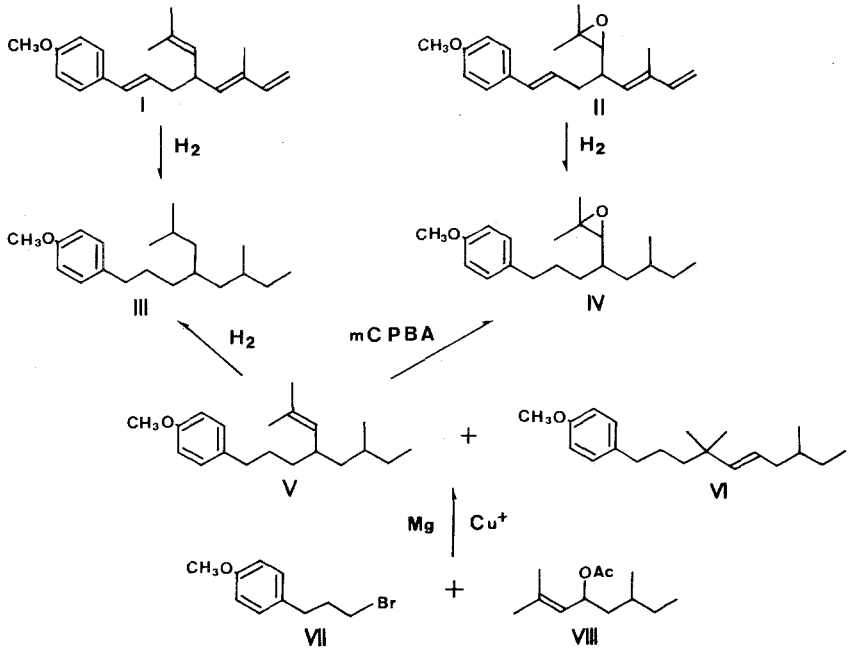


FIG. 3. Preparation of the skeletal compounds of juvocimene I and II by catalytic hydrogenation and synthesis.

hydro derivative (IV). MS: m/z(%) 304 (M^+, 2.6), 121(100), 134(54), 147(30), 163(33). NMR: δ7.12(2H, d, $J = 9.0$), 6.84(2H, d, $J = 9.0$), 3.81(3H, s), ~2.5(3H, m), 1.32(3H, s), 1.28(3H, s), ~0.9(6H, m).

Preparation of Compound VIII. 2-Methyl-1-butyl magnesium bromide was prepared from 1.2 g of magnesium and 7.6 g of 1-bromo-2-methyl butane in 40 ml of tetrahydrofuran (THF). 3-Methyl-2-butenal (4.2 g in 20 ml of benzene) was added to the Grignard reagent solution and refluxed for 30 min. The reaction mixture was treated with saturated ammonium chloride and extracted with ether. The ether solution was washed with brine and then dried over anhydrous sodium sulfate. Removal of the solvent gave a crude oil, which was purified by column chromatography (Florisil, 100 g) eluting with 15% ether in petroleum ether to give 6.70 g of 2,6-dimethyl-2-octen-4-ol as a colorless oil (yield 85%). NMR: δ5.15(1H, m), 4.45(1H, broad quartet, $J = 7.0$), 1.77(6H, broad s), ~0.9(6H, m). The alcohol (4.68 g) was mixed with pyridine (40 ml) and acetic anhydride (30 ml) and held overnight at room temperature. Usual aqueous work-up yielded a crude oil, which was further purified through a short column of Florisil (10 g) eluting with benzene, and distilled in vacuo (Kugelrohr, Aldrich) to yield a colorless oil of the acetate (VIII, Figure 3) (yield 4.81 g, 81%). MS: m/z(%) 198(M^+,

0.8), 85(100), 109(44), 127(24), 138(17). NMR: δ5.60(1H, m), 5.14(1H, m), 2.04(3H, s), 1.78(6H, broad s), ~0.9(6H, m).

Preparation of Compound V. In a 50-ml three-necked flask furnished with nitrogen inlet, dropping funnel, dry ice-acetone bath, and magnetic stirrer was placed 0.99 g of the acetate (VIII) and cuprous chloride (30 mg) in 10 ml THF. While the temperature was maintained at −20°C, a Grignard solution prepared from magnesium (0.26 g) and compound VII (2.29 g) in THF (35 ml) was slowly added. The mixture was held at this temperature for 15 hr to complete the reaction and extracted with ether, successively washing with 5% phosphoric acid, saturated sodium bicarbonate, and brine solution. The crude oil was then purified by chromatography (Florisil, 60 g). The first hexane eluate (240 ml) was composed of the isomers V and VI in a ratio of about 3:1 (Figure 3). This mixture was chromatographed on a column of 15% $AgNO_3$–silica gel (40 g) eluting with a mixture of ether and hexane. Pure compound V was obtained from 0.3% ether in hexane eluate as a colorless oil (yield 0.41 g, 28%). Compound VI (90% pure) was obtained from 0.5% ether in hexane eluate as a colorless oil (yield 0.81 g, 13%). Compound V: MS: m/z(%) 288(M^+, 17), 69(30), 121(90), 134(59), 147(100), 161(12). NMR: δ7.10(2H, d, J = 9.0), 6.83(2H, d, J = 9.0), 4.80(1H, m), 3.81(3H, s), 2.55(2H, t, J = 7.0), 1.73(3H, broad s), 1.65(3H, s), ~0.9(6H, m). Compound VI: MS: m/z(%) 288(M^+, 13), 69(40), 121(57), 134(28), 147(100), 148(32), 161(1). NMR: δ7.11(2H, d, J = 9.0), 6.84(2H, d, J = 9.0), ~5.3(2H, m), 3.82(3H, s), 2.56(2H, t, J = 7.0), 1.00(6H, s), ~0.9(6H, m).

Hydrogenation of Compound V. Compound V (100 mg) was hydrogenated in the presence of platinum oxide (3 mg) in 3 ml of ethanol under hydrogen atmosphere by stirring the suspension for 40 min. A colorless liquid (100 mg) was obtained after removal of the catalyst and solvent. MS: m/z(%) 290(M^+, 12), 121(100), 134(15), 147(20). NMR: δ7.10(2H, d, J = 9.0), 6.83(2H, d, J = 9.0), 3.81(3H, s), 2.56(2H, t, J = 7.0), ~0.9(12H, m).

Epoxidation of Compound V. To a solution of compound V (288 mg in 12 ml of dichloromethane) was added 200 mg of 85% *m*-chloroperbenzoic acid at 0°C, and the mixture was maintained for 2 hr with stirring. After the excess peracid was destroyed with 5% sodium bisulfite, the reaction mixture was extracted with hexane and washed with a solution of sodium bicarbonate followed with brine. The starting material was found to be quantitatively converted to a mixture of the epoxides, IV and its diastereomer IV′, in a ratio of approximately 2:1. These isomers were readily isolated by HPLC (Lichrosorb SI-100, 380 × 10 mm ID, 10% ether in hexane, 2 ml/min); compound IV: R_t = 24 min. compound IV′: R_t = 21 min. The hydrogenated juvocimene II gave R_t = 24 min under the same conditions. Compound IV: MS: m/z(%) 304(M^+, 3.1), 121(100), 134(42), 147(25), 163(36). NMR: δ7.12(2H, d, J = 9.0), 6.82(2H, d, J = 9.0), 3.81(3H, s), ~2.5(3H, m), 1.32(3H, s), 1.28(3H, s), ~0.9(6H, m). Compound IV′: MS: m/z(%) 304(M^+, 4.1),

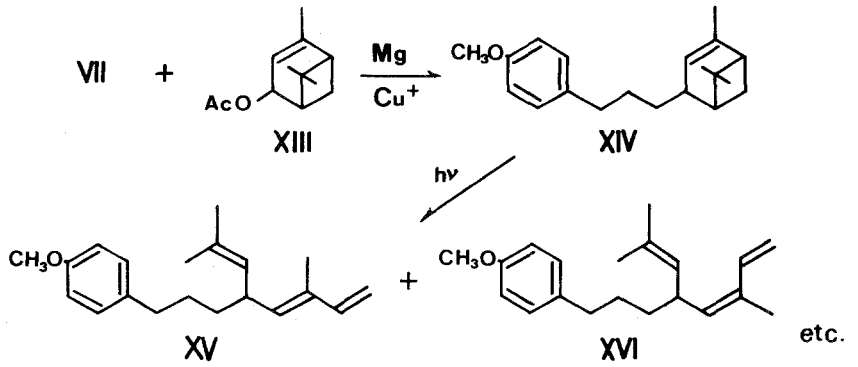


FIG. 4. Photochemical synthesis of dihydrojuvocimene I (XV) and its geometrical isomer (XVI).

121(100), 134(56), 147(31), 163(17). NMR: δ7.11(2H, d, J = 9.0), 6.83(2H, d, J = 9.0), 3.81(3H, s), ~2.5(3H, m), 1.33(3H, s), 1.29(3H, s), ~0.9(6H, m).

Preparation of Compound XIV. The (±)-*trans*-verbenyl acetate (XIII, Figure 4) was prepared from (±)-α-pinene (Aldrich Chem. Co.) through reaction with lead tetraacetate according to the method of Whitham (1961). The solution of 3-(4-methoxyphenyl)-1-propyl magnesium bromide in THF (see preparation of compound V) was added dropwise into a mixture of *trans*-verbenyl acetate (1.78 g) and CuI (60 mg) in THF at −25° C under nitrogen stream. The purplish solution was maintained at −20° C for 10 hr. Aqueous work-up followed by chromatographic separation on Florisil gave compond VII as a colorless oil (yield 352 mg, 14%). Although this reaction was less feasible than that between compound VII and VIII, no significant amounts of other isomers were observed on GC-MS. MS: m/z(%) 284(M^+, 25), 77(31), 91(34), 93(43), 105(47), 121(100), 134(49), 147(33). NMR: δ7.11 (2H, d, J = 9.0), 6.85(2H, d, J = 9.0), 5.20(1H, m), 3.82(3H, s), 2.60(2H, t, J = 7.0), 1.70(3H, t, J = 2.0), 1.32(3H, s), 0.88(3H, s).

Photochemical Synthesis of Compounds XV and XVI. The photochemical reaction (Figure 4) was carried out in a Pyrex tube (ID 12 mm) with a mixture of compound XIV (90 mg), acetophenone (180 mg), and benzene (7.5 ml). A stream of nitrogen was introduced into the reaction tube with a capillary tube, and the whole apparatus was immersed in an ice bath. A Hanovia 450-W medium pressure mercury arc was placed 5 cm distant from the reaction vessel for irradiation. The reaction was stopped after 1.5 hr, although about half the starting material remained. A mixture of the photolyzed isomers with the starting compound was separated from acetophenone with a short column of Florisil. The GC-MS indicated that four principal isomers were formed in almost equal amounts in this reaction. The desirable compounds XV and XVI were isolated by successive chromatography on

silver nitrate coated on Florisil or on silica gel. The *cis*-isomer (XVI) was eluted with 6% ether in hexane from a column of 15% silver nitrate on silica gel. The *trans*-isomer (XVI) was eluted with 10% ether in hexane together with the other isomers. Pure *trans* isomer was isolated by HPLC [5% $AgNO_3$ on Biosil A (2–10 μm), 250 × 5 mm ID, eluting with 10% ether in heptane, 1.1 ml/min, 60 kg/cm^2, R_t = 16.5 min], but the other isomers were refractory to isolation. Both compounds XV and XVI yielded an identical hexahydro derivative by catalytic hydrogenation, the NMR and GC-MS of which coincided with those of compound III (Figure 3). Compound XV: MS: *m/z*(%) 284(M^+, 13), 77(47), 78(24), 79(21), 91(53), 93(85), 105(26), 107(30), 121(81), 134(77), 135(68), 147(100). NMR: δ7.10(2H, d, J = 9.0), 6.86(2H, d, J = 9.0), 6.36(1H, double d, J = 10.5 and 17.5), 5.29(1H, broad d, J = 8.0), 5.10(1H, broad d, J = 17.5), ~5.0(1H, m), 4.93(1H, broad d, J = 10.5), 3.79(3H, s), 3.26(1H, broad quintet), 2.55(2H, t, J = 6.5), 1.75(3H, broad s), 1.69(3H, broad s), 1.64(3H, broad s), ~1.4(4H, m). UV: λ_{nm}^{max} (EtOH) 228(ϵ23,000). Compound XVI: MS: *m/z*(%) 284 (M^+, 23), 77(50), 78(22), 79(21), 91(56), 93(91), 105(36), 107(31), 121(100), 134(91), 135(64), 147(94). NMR δ7.09(2H, d, J = 9.0), 6.81(1H, double d, J = 10.5 and 17.5), 6.79(2H, d, J = 9.0), 5.30(1H, broad d, J = 7.0), 5.20(1H, broad d, J = 17.5), 5.10(1H, broad d, J = 10.5), ~5.1(1H, m), 3.80(3H, s), ~3.3(1H, m), 2.56(2H, *t*, J = 7.0), 1.84(3H, broad s), 1.72(3H, broad s), 1.66(3H, broad s), ~1.4(4H, m). UV: λ_{nm}^{max} (EtOH) 229(ϵ24,000).

Synthesis of Juvocimene I. A 50-ml three-neck flask was furnished with a nitrogen inlet tube, a rubber injector septum, and a Drierite tube. A solution of *trans*-β-ocimene (IX, 0.5 g, prepared by the method of Kropp (1969) followed by chromatographic purification with 15% $AgNO_3$ on Florisil) in 5 ml of THF, was cooled to −70° C in a Dry Ice–acetone bath with magnetic stirrer under a nitrogen stream. A mixture of *N,N,N',N'*-tetramethylethylenediamine (TMEDA) and *n*-butyl lithium (1.5 ml) [prepared from 1.2 ml of *n*-butyl lithium (1.6 M hexane solution, Aldrich) and 0.6 ml of TMEDA according to the procedure of Crawford (1972)] was slowly added from the injector. The temperature was gradually raised to −35° C during addition, and the mixture turned to a dark red color. The mixture was maintained at −35° C for 30 min and again cooled down to −70° C. A solution of 365 mg of *p*-methoxy-*trans*-cinnamyl chloride (prepared by the method of Wigfield et al., 1974) in 5 ml of THF was added, and the Dry Ice–acetone bath was removed. The reaction mixture was poured into ice-water when it reached room temperature and was extracted with a mixture of hexane–benzene (1:1). The organic layer was washed with 1% phosphoric acid, 1% sodium bicarbonate, and brine. The solution was dried over anhydrous sodium sulfate and passed through a short column of Florisil (5 g), eluting with additional benzene. The crude oil (0.7 g) was chromatographed on Florisil (25 g). The first fraction, eluted with 150 ml of hexane, contained unreacted

ocimene. The next 1% ether–hexane fraction contained a mixture of juvocimene I and XVII (272 mg) in a ratio of approximately 7:3. The yield of juvocimine I based on GLC analysis was about 30% at this step. Since juvocimine I was slightly more retained than compound XVII on a column of 15% $AgNO_3$–silica gel (gradient from 2–5% ether in hexane), repetition of the chromatography permitted the separation of both pure compounds. The synthetic juvocimene exhibited the same physical constants as those of juvocimene I isolated from oil of sweet basil (GLC, HPLC, MS, IR, NMR, and UV. Compound XVII: MS: m/z(%) 282(M^+, 1.5), 135(16), 147(100). NMR: δ7.29(2H, d, $J = 9.0$), 6.84(2H, d, $J = 9.0$), 6.34(1H, d, $J = 15.5$), 6.01(1H, double t, $J = 15.5$ and 7.0), 6.3–5.7(3H, m), 5.63(1H, d, $J = 15.0$), 5.04(1H, broad d, $J = 11.0$), 5.01(1H, broad d, $J = 17.5$), 3.81(3H, s), 2.34 (2H, d, $J = 7.0$), 1.78(6H, broad s), 1.19(3H, s). UV: λ_{nm}^{max} (EtOH) 242 (ϵ26,000), 261(ϵ24,500).

The same reaction was performed with *cis*-β-ocimene (X) instead of *trans*-β-ocimene under exactly the same conditions. The coupling products analyzed by GLC consisted of the same components, a mixture of I and XVII (7:3) (yield of juvocimene I: 22% by GLC).

Synthesis of Juvocimene II. *m*-Chloroperbenzoic acid (67 mg) was added in portions to a solution of the synthetic juvocimene (92 mg in 5 ml of dichloromethane) at 0°C under nitrogen stream. After 30 min of stirring, the mixture was extracted with benzene and washed with 1% sodium bicarbonate and then with brine. After drying over anhydrous sodium sulfate and evaportion of the solvent in vacuo, the mixture was chromatographed on silica gel (10 g). A mixture of juvocimene II and its epimer (II′) was obtained from 20% ether in hexane eluate in a ratio of 3:1(II to II′) (yield; 38 mg as the mixture). Portions of the mixture were separated on HPLC; juvocimene II was collected from R_t = 31–32 min, and II′ was collected from R_t = 28–29 min [Lichrosorb SI-100 (5 μm), 380 × 10 mm ID, 8% ether in hexane (2.2 ml/min, 63 kg/cm^2)]. The physical data of synthetic juvocimene II coincided with those of juvocimene II isolated from oil of sweet basil by GLC, HPLC, MS, NMR, and UV. The physical properties of juvocimene II′ were very similar to those of juvocimene II, but some differences were found in its behavior on GLC and HPLC, and in the NMR spectrum. Juvocimene II′: MS: m/z(%) 298(M^+, 1), 91(12), 147(100). NMR: δ7.26(2H, d, $J = 9.0$), 6.83(2H, d, $J = 9.0$), 6.39(1H, double d, $J = 17.5$ and 10.5), 6.34 (1H, d, $J = 16.0$), 5.95(1H, double t, $J = 16.0$ and 6.5), 5.45(1H, broad d, $J = 8.0$), 5.10(1H, d, $J = 17.5$), 4.95(1H, d, $J = 10.5$), 3.81(3H, s), 2.8–2.1(4H, m), 1.77(3H, d, $J = 1.1$), 1.31(3H, s), 1.28(3H, s). UV: λ_{nm}^{max} (EtOH) 227 (ϵ25,000), 262(ϵ27,500).

Synthesis of Compounds XVIII and XIX. 1-(3,4-Methylenedioxyphenyl)-2-propen-1-ol was prepared from 3,4-methylenedioxybenzaldehyde and vinyl magnesium bromide according to White and Fife (1961) and then

was reacted with thionyl chloride to give 3,4-methylenedioxy-*trans*-cinnamyl chloride (Wigfield et al., 1974). The THF solution of β-ocimene–lithium complex (2 mmol) prepared in the previous section was added into the solution of the chloride (393 mg in 5 ml of THF) under the same conditions as those in the synthesis of juvocimene I. After chromatographic purification (15% silver nitrate-loaded Florisil, eluted with 7–10% ether in hexane), 187 mg of XVIII was obtained as a colorless oil (yield 32%). MS: m/z(%) 296(M^+, 0.3), 161(100). IR: ν cm^{-1} 3030, 2970, 2915, 1642, 1610, 1507, 1492, 1448, 1377, 1363, 1254, 1192, 1124, 1098, 1041, 988, 964, 934, 895, 862, 816, 796, 777. NMR: δ6.7–6.9(3H, m), 6.38(1H, double d, J = 17.5 and 10.5), 6.36(1H, d, J = 15.5), 6.01(1H, double t, J = 15.5 and 7.0), 5.97(2H, s), 5.35(1H, broad d, J = 9.0), 5.14(1H, broad d, J = 17.5) 5.04(1H, m), 4.99(1H, broad d, J = 10.5), 3.40(1H, m), 2.28(2H, t, $J \simeq 7$), 1.77(3H, broad s), 1.72(3H, broad s), 1.66(3H, broad s).

Compound XIX (150 mg) was epoxidized with *m*-chloroperbenzoic acid (100 mg) in dichloromethane (5 ml) by the same method as that described for juvocimene I to II. An epimeric mixture was obtained after chromatographic purification (Florisil, 10 g, eluted with 15% ether in hexane) (yield as the mixture: 21 mg, 13%). MS: m/z(%) 312(M^+ 0.7), 161(100). IR: ν cm^{-1} 3010, 2970, 2910, 1640, 1680, 1505, 1491, 1444, 1374, 1362, 1248, 1190, 1122, 1097, 1044, 988, 964, 934, 895, 860, 815, 795. NMR: δ6.7–6.9(3H, m), 6.40(1H, double d, J = 17.5 and 10.5), 6.40(1H, d, J = 15.5), ~6.0(1H, m) 5.98(2H, s), ~5.4(1H, m), 5.2–5.0(2H, m), 2.8–2.1(4H, m), 1.76(3H, s), ~1.3(6H, broad s).

RESULTS

An essential oil of sweet basil was subjected to vacuum distillation to separate inactive volatile components. Chromatographic fractionation of the active residue on Florisil revealed two active fractions which were eluted with mixtures of ether–petroleum ether. Successive purification of each fraction by means of high-performance liquid chromatography (HPLC) gave each single component, juvocimene I or II, respectively, as colorless oils.

Structure of Juvocimene I. Juvocimene I showed the molecular ion peak at m/z 282. The NMR spectrum (Figure 1 showed 26 hydrogen units, exhibiting seven olefinic and four aromatic protons. Thus, the molecular formula was suggested to be $C_{20}H_{26}O$, a highly unsaturated compound. The NMR assignments of juvocimene I are given in Figure 1. The presence of a *p*-methoxycinnamyl moiety was indicated by a methoxy signal (H_j: δ3.81, 3H, s), an A_2' B_2' pattern by the four aromatic protons (H_a: δ7.25, 2H, d, H_b: δ6.85, 2H, d, J_{a-b} = 9.0), and ABX_2 pattern of the alkenyl protons (H_d: δ6.32, 1H, d; H_e: δ6.00, 1H double t, J_{d-e} = 15.5, J_{e-l} = 7.0; H_l: δ2.42,

2H, broad t, $J_{l-k} = 7.5$) in conjugation with the aromatic ring. The most abundant fragment ion m/z 147 in the mass spectrum also supported the presence of this system.

The coupling constant between H_d and H_e (15.5 Hz) indicated the double bond to be *trans*. An absorption band in the UV spectrum at λ-258 nm (ε22,000) also provided the evidence that juvocimene I possessed the *trans* configuration; *trans*-anethole: λ-258 (ε24,000). Besides this absorption, the UV spectrum showed an absorption band at λ-237 nm (ε23,000), which suggested the presence of a conjugated diene system in the molecule. The detail of this dienic structure was examined in the NMR spectrum. A vinylic moiety consisting of protons H_g(δ5.11, 1H, broad d), H_i(δ4.96, 1H, broad d) and H_c(δ6.38, 1H, double d) exhibited an ABX system ($J_{c-g} = 17.5$ and $J_{c-i} = 10.5$). This vinyl group appeared to be conjugated with another olefinic group bearing a methyl group H_m: δ1.78, 3H, d, $J_{f-m} = 1.3$) to form a 2-methylbutadienyl moiety, $CH_2{=}CH{-}\underset{\displaystyle CH_3}{\underset{|}{C}}{=}CH{-}$. A long-range coupling of H_f(δ5.37, 1H, broad d, $J_{f-k} = 9.5$) between H_c($J_{c-f} = 0.7$) and H_m($J_{f-m} = 1.3$) was verified by the decoupling experiments. The chemical shift of H_c empirically implied the geometry of the dienic system to be *trans*. The details are discussed later.

The presence of an isobutenyl moiety was given by two allylic methyl signals, H_n(δ1.72, 3H, d) and H_o(δ1.66, 3H, d); both were coupled with an olefinic proton H_h(δ5.04, 1H, double septet, $J_{h-n} = J_{h-o} = 1.5$, $J_{h-k} = 9.5$). One methine proton (H_k) was observed at δ3.37 as a broad quintet, coupling with H_f, H_h, and H_l ($J_{f-k} = J_{h-k} = 9.5$, $J_{l-k} = 7.5$). The relationship of the adjacent protons was verified by decoupling experiments. Thus, we concluded that the above-mentioned three moieties were attached to this methine carbon atom to provide structure I as shown in Figure 1.

Structure of Juvocimene II. Juvocimene II gave a molecular ion peak at m/z 298. The base ion fragment at m/e 147 suggested the presence of a *p*-methoxycinnamyl moiety as an analog of juvocimene I. The NMR spectrum exhibited signals in common with those in juvocimene I (Figure 2). The signals by a *p*-methoxycinnamyl moiety [H_a: δ7.26(2H, d, $J_{a-b} = 9.0$); H_b: δ6.85(2H, d); H_d: δ6.40(1H, d, $J_{d-e} = 16.0$); H_e: δ6.08(1H, double t, $J_{e-l} = 6.5$); H_l: δ3.81(3H, s)] and a 2-methyl-*trans*-1,3-butadienyl moiety [H_c: δ6.41(1H, double d, $J_{c-g} = 17.5$, $J_{c-i} = 10.5$); H_g: δ5.16(1H, broad d); H_i: δ5.03(1H, broad d); H_f: δ5.39(1H, broad d, $J_{f-k} = 8.0$); H_m: δ1.76(3H, d, $J_{m-f} = 1.3$)] were almost superimposable to those of juvocimine I. The presence of 1,2-epoxy-2-methylpropyl moiety, $CH_3{-}\underset{\displaystyle CH_3}{\underset{|}{C}}(\overset{\displaystyle O}{\diagup\diagdown})CH{-}$ in juvo-

cimene II was suggested instead of an isobutenyl moiety in juvocimene I [a pair of singlet methyls by H_n and H_o at δ1.33(3H, s) and 1.30(3H, s), and an epoxy proton H_h at δ2.71(1H, d, $J_{h-k} = 8.0$)]. Signals by H_l and H_k gave a highly split pattern around δ2.4. Thus, juvocimene II shown by formula II (Figure 3) is an epoxide form of juvocimene I.

Verification of Skeletal Structure. In order to verify the skeletal structure of I and II, compounds III and IV were prepared (Figure 3). Hydrogenation of juvocimene I gave a saturated compound III (M^+, m/z 290), the NMR of which exhibited four methyl groups (δ0.8–0.9). Hydrogenation of juvocimene gave a molecular ion peak at m/z 304. This compound showed a pair of geminal dimethyl signals on the epoxide ring at δ1.28(3H, s) and 1.38(3H, s), and anteisodimethyl signals around δ0.9(6H, m). These saturated compounds, III and IV, were synthesized by the route shown in Figure 3. A Grignard coupling reaction between compounds VII and VIII yielded a mixture of compounds V and VI (approximately 3:1). The mixture was readily separated by means of column chromatography (15% $AgNO_3$ on silica gel). Catalytic hydrogenation of compound V gave a saturated compound (III), the physical constants of which coincided to those of the octahydro derivative (III) from juvocimene I. The epoxidation of compound V with *m*-chloroperbenzoic acid afforded two products, compound IV and its epimer IV′, in a ratio of about 2:1, the major one of which gave identical chromatographic and spectrometric data as the hexahydro derivative of juvocimene II. Thus the structures of juvocimene I and II were unequivocally verified to be I and II, respectively.

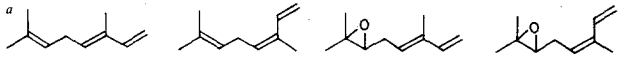
Verification of Geometry of Conjugated Diene System. Both juvocimene I and II possess a 2-methyl-1,3-butadienyl moiety in each molecule. The UV and NMR spectra are known to be useful to distinguish *cis* and *trans* in this system (Ohloff et al., 1964; Thomas, 1966). As was described previously, the chemical shifts H_c in compounds I and II (I: δ6.38; II: δ6.41) implied that both compounds had *trans* configurations. However, the UV spectrum of juvocimene I (λ-237 nm) rather suggested that juvocimene I should have possessed a *cis*-configuration by comparison with the data in the literature (Ohloff, 1964) (Table 1). The difference in the physical constants was re-examined by comparison with closely related analogous compounds. The chemical shifts of H_c, H_g, and H_i, and $\pi \longrightarrow \pi^*$ absorptions of *trans* and *cis*-β-ocimene (IX, X) and their epoxides (XI, XII) are listed in Table 1 in correlation with those of juvocimene I and II. The coincident values for juvocimene I and II were observed in the chemical shifts of their corresponding *trans* analogs.

Analogous results were also obtained in the combination of compounds XV and XVI, synthesized by a photochemical reaction of an α-pinene derivative (XIV) (Figure 4). Compound XIV was prepared by a Grignard coupling reaction between *trans*-verbenyl acetate (XIII) and compound VII (Figure 3).

TABLE 1. VALUES OF NMR CHEMICAL SHIFTS AND UV ABSORPTIONS OF *cis* AND *trans* ISOMERS OF β-OCIMENE DERIVATIVES[a]

	I	IX *trans*	X *cis*	II	XI[c] *trans*	XII[c] *cis*	XV *trans*	XVI *cis*
δ								
H_c[b]	6.38	6.38	6.84	6.41	6.42	6.77	6.36	6.81
H_g	5.11	5.10	5.23	5.16	5.16	5.27	5.10	5.23
H_i	4.96	4.96	5.11	5.03	5.02	5.15	4.93	5.10
λ_{nm}^{max}	237	232	237.5	227	228	231.5	228	229

[a]



IX X XI XII

[b] H_c, H_g, and H_i represent the terminal olefinic protons $-CH=\overset{\overset{CH_3}{|}}{C}-C=C\begin{matrix}H_g\\H_i\end{matrix}$, in which H_c is *trans* to H_g in each case.

[c] Epoxides XI and XII were prepared from β-ocimenes IX and X, respectively, by epoxidation with equimolar amount of *m*-chloroperbenzoic acid (see the synthesis of juvocimene II).

The photochemical reaction proceeded successfully according to the method for the preparation of *cis*- and *trans*-β-ocimene from α-pinene in the presence of acetophenone (Frank, 1968; Kropp, 1969). Compounds XV and XVI were formed as major products in a ratio of 1 : 1 together with some other unidentified isomers. The formation of the *trans* form (XV) in this reaction is considered to be a result of a photochemical equilibrium between the initially formed *cis*-form (XVI) (Erman, 1967). Values of H_c, H_g, and H_i of these two compounds coincided with those of β-ocimenes, respectively, whereas the values of the UV absorption gave a small difference between them. The UV absorptions seemed to be influenced by the neighboring π electrons in some degree, and the chemical shifts of the olefinic protons (H_c, $_g$, and $_i$) appeared to be diagnostic for distinguishing *trans–cis* relationships in this system. Thus, these results clearly demonstrated that both juvocimene I and II possess the *trans* configuration at the conjugated diene position.

Synthesis of Juvocimenes. The photochemical conversion of the α-pinene derivative (XIV) to the β-ocimene derivatives (XV, XVI) was successful (Figure 4); however the introduction of a double bond in conjugation with the aromatic ring appeared to make the reaction more complex. We tried several approaches to juvocimene I, and one simple method was discovered, as shown in Figure 5. When a complex of *n*-butyl lithium–*N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) (Fieser and Fieser, 1974) was added in a solution of *trans*-β-ocimene (IX) in tetrahydrofuran, it formed a dark

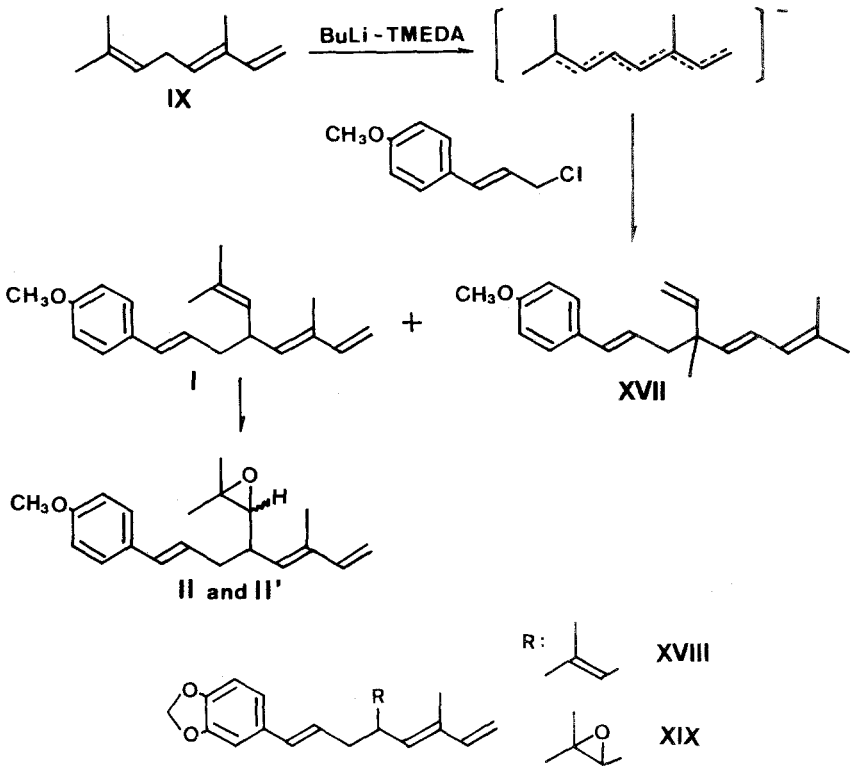


FIG. 5. The synthetic route for juvocimene I and II.

red solution of lithium salt with the triene at about −35°C. Then a solution of *p*-methoxy-*trans*-cinnamyl chloride was added to the solution to yield a mixture of the condensed products.

The products consisted mainly of juvocimene I and compound XVII in a ratio of approximately 7:3. The yield of juvocimene I was about 30% by GLC analysis of the reaction mixture. Juvocimene I was readily separated from compound XVII by repetitive column chromatography (15% $AgNO_3$–silica gel). Similar results were obtained when *cis*-β-ocimene (X) was allowed to react instead of *trans*-β-ocimene (IX). No significant amount (more than 5%) of the *cis* isomer of juvocimene I was detected in either case (by NMR analysis). Therefore, the resonance of the carbanion through the main chain of the intermediate (Figure 4) is considered to stabilize the *trans* form. First the metalation reaction of olefins with *n*-butyl lithium–TMEDA complex was demonstrated with limonene as a substrate (Crawford et al., 1971), in which one of the allylic methyls was selectively metalated. The selective elimination of a proton on the "doubly allylic" position appears to be feasible in the case of β-ocimene.

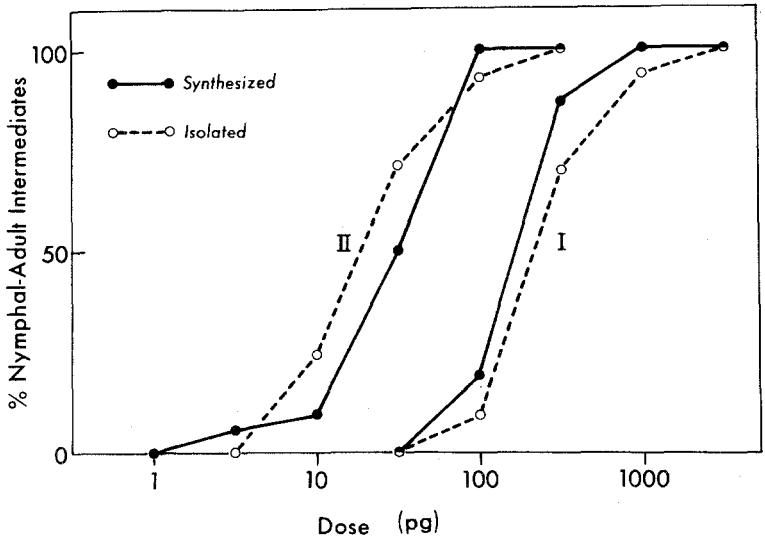


FIG. 6. Juvenile hormone activity of synthetic (solid line) and natural (broken line) juvocimene I and II in the milkweed bug juvenile hormone assay. Each value represents the average modification of 30 insects.

The synthesized juvocimene I was converted to juvocimene II by epoxidation with *m*-chloroperbenzoic acid. A mixture of juvocimene II and its epimer II′ (II–II′ = 3 : 1, approximately 35% of the yield of the mixture) was formed besides other minor products. Each isomer was distinguishable by its GLC and NMR spectra and was readily separated by HPLC. The diastereomeric relationship between II and II′ has not yet been confirmed.

Analogous compounds of juvocimenes, such as compound XVIII and XIX, were readily synthesized by a coupling reaction with β-ocimene and the corresponding halide (i.e., 3,4-methylenedioxy-*trans*-cinnamyl chloride), and subsequent oxidation to its epoxide.

Biological Activity of Synthetic Juvocimenes. As reported previously (Bowers and Nishida, 1980), juvocimene I and II induced the formation of nymphal-adult intermediates of the milkweed bug in the picogram range. Juvocimene II showed about 3000 times as much activity as that of juvenile hormone I in the milkweed bug juvenile hormone test (Bowers, 1968). The bioassay results of synthetic juvocimene I and II (Figure 6) were superimposable on the dose–response curves of juvocimene I and II isolated from oil of sweet basil. These dose–response curves show that the biological activity of the synthesized and natural juvocimenes is identical.

The methylenedioxyphenyl analogs (XVIII and XIX) were tested on the milkweed bugs. Compound XIX also exhibited activity as potent as that of juvocimene II, but compound XVIII exhibited much lower activity than that of juvocimene I as follows [dose (percent nymphal-adult intermediate)]:

XVIII [30 ng (100%), 10 ng (68%), 3 ng (60%), 1 ng (38%), 300 pg (0%)], XIX [300 pg (100%), 100 pg (71%), 30 pg (43%), 10 pg (0%)] (each value represents the average modification of 16 insects).

DISCUSSION

The structures of juvocimene I and II suggest that the skeleton may have been formed through the condensation of a monoterpene and a cinnamyl moiety. It should be noted that ocimenes, linalool, estragole, etc., are major components of the essential oil of sweet basil (Deshpande and Tipnis, 1977; Lawrence et al., 1971). It seems very probable that the sweet basil plant has taken advantage of the presence of these constituents to create the juvocimenes. Although speculative, it is possible that the morphogenetically active juvocimenes may add an additional dimension of protection to the plant's defensive screen against insects.

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IDENTIFICATION OF TRAIL PHEROMONE OF THE ANT *Tetramorium caespitum* L. (HYMENOPTERA: MYRMICINAE)

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Abstract—The trail pheromone of the ant *Tetramorium caespitum* L. is a 70:30 mixture of 2,5-dimethylpyrazine and 3-ethyl-2, 5-dimethylpyrazine. The average total amount of the two pyrazines present in the poison vesicle was found to be 3.9 ng per ant, of which 2.7 ± 0.4 ng is 2,5-dimethylpyrazine and 1.15 ± 0.25 ng is 3-ethyl-2,5-dimethylpyrazine. The pyrazines constitute only 0.03% of the volume of the poison vesicle but account for the whole of the trail-following activity. A 70:30 mixture of the respective pyrazines evoked the highest activity in artificial trail-following tests.

Key Words—Ant, *Tetramorium caespitum*, trail pheromone, 2,5-dimethylpyrazine, poison gland, venom, Hymenoptera, Myrmicinae, synergism.

INTRODUCTION

A large number of ant species are known to employ trail pheromones as a means of communication, but only in a very few cases has the pheromone been chemically identified. The first trail substance to be identified was methyl 4-methylpyrrole-2-carboxylate from *Atta texana* Buckley (Tumlinson et al., 1972). The same compound was subsequently demonstrated to be active in evoking trail following in *A. cephalotes* L. (Riley et al., 1974) and *Acromyrmex octospinosus* Reich (Robinson et al., 1974).

3-Ethyl-2,5-dimethylpyrazine has been shown to be the major component of the trail pheromone of *Atta sexdens rubropilosa* Forel (Cross et al., 1979), and the same compound has since been identified as the single component of the trail pheromone of eight species of *Myrmica* (Evershed et al., 1981, 1982). In the beginning of the present study, only the aforementioned

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two compounds were known as trail substances that originate from the poison glands. Faranal, a terpenoid that originates from the Dufour gland, has been identified as the major trail pheromone of *Monomorium pharaonis* L. (Ritter et al., 1977). There is a controversy about the composition of the trail pheromone of *Solenopsis invicta* Buren. Williams et al. (1981) have reported it to be (*Z*,*Z*,*Z*)-allofarnesene, while Vander Meer et al. (1981) describe it as a mixture of (*Z*,*E*)- and (*E*,*E*)-α-farnesenes and (*Z*,*Z*)- and (*Z*,*E*)-homofarnesenes. A mixture of C_6–C_{12} and C_{14}–C_{20} fatty acids are reported to be the active trail following mixtures for *Lasius fuliginosus* Latrielle (Huwyler et al., 1975) and *Pristomyrmex pungens* Mayr (Hayashi and Komae, 1977), respectively. In *Iridomyrmex humilis* Mayr (*Z*)-9-hexadecenal has been identified as one of the components of its trail pheromone (Cavill et al., 1979; Van Vorhis Key and Baker, 1982).

The above summary illustrates that the information available about the chemistry of trail pheromones is very limited. Some of the artificial trails laid with the above-mentioned single substances were not species specific, although the natural trails showed a much higher degree of species specificity. Although many trail pheromones had been recognized as multicomponent mixtures, the true quantitative and qualitative compositions of none of them were known at the beginning of this study. We have recently shown that the trail pheromone of *T. caespitum* L. contains two pyrazine compounds (Attygalle and Morgan, 1983) and give here the full identification of these substances and show how the synergistic action of these two compounds together completely accounts for the activity of the natural pheromone.

METHODS AND MATERIALS

Insect Rearing. Colonies of *T. caespitum* were collected from Heartland moor in Dorset. The ants were maintained in the laboratory at room temperature in a wooden box filled with moist soil and peat. The ants were fed on a diet of desiccated coconut, meal worm larvae (*Tenebrio molitor*), and sugar solution (10% w/v).

Preparation of Glandular Extracts for Bioassay. The ants were killed by exposing them to the cold vapor from liquid nitrogen. The poison glands and Dufour glands were separated by dissecting the ants in water. The glands were macerated with a solvent such as hexane or acetone (100 μl) and kept ice-cold for further use.

Bioassay of Trail-Following Behavior. The method of Pasteels and Verhaeghe (1974) was employed to measure the trail-following behavior of ants towards the test solutions. A circle of 5 cm radius was drawn with a lead pencil on a piece of white paper (13 × 13 cm). The circumference of the circle was marked with arcs (1 cm). The solution under investigation (usually 25–100

μl) was injected into a Standardgraph (Blundel Harling, Dorset) "S" funnel pen (0.8 mm) and a continuous streak was drawn on the circle. The solvent was allowed to evaporate for 2 min, and the paper was placed in the foraging area of the ant nest. The number of arcs run along the trail by each individual worker ant was recorded for 20 min. The median of the values thus obtained was used as a measure of activity. The activities of extracts of two poison glands and two Dufour glands were tested separately. Median values were obtained by repeating the tests three times. A blank bioassay using solvent only was always performed before a test to ensure no residual activity was present in the pen.

Thin-Layer Chromatography. An extract of two cleanly dissected poison glands was made in distilled acetone (50 μl). The extract was applied to the origin of a silica gel layer (20 cm × 5 cm × 0.3 mm) on a glass plate and developed with hexane–acetone (60:40). The solvent front was allowed to run 15 cm. The plate was air dried, and the silica was cut into ten bands (1.5 cm each). The bands were scraped separately into Pasteur pipets plugged with glass wool. Each fraction of silica was extracted with acetone (100 μl) directly into a Standardgraph pen. The trail-following activity evoked by each fraction was tested by bioassay. Blank bioassays using solvent only were performed before and in between each test to ensure no activity was present by contamination. The test was repeated in the same manner, except only the region between 4.5 and 9 cm was scraped with a small spatula and the width of each band was narrowed to 2 mm. All determinations were made in duplicate.

Similar experiments were performed to test for functional groups. Two poison glands were extracted separately in HCl in acetone (1%) and Br_2 in hexane (1%, v/v) respectively. The reaction mixtures were separated by TLC, and the bioassays were carried out as before. Samples of synthetic 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were chromatographed under the same conditions as above. The spots were visualized under a UV lamp and the the R_f values were calculated.

Gas Chromatography. Gas chromatography (GC) was performed with a Pye 104 gas chromatograph with a flame ionization detector using a packed column of 2.75 × 4 mm, 10% PEG 20 M on Chromosorb W (100–120 mesh) at 130°C. The nitrogen carrier gas flow rate was 50 ml/min. The ionization amplifier was used at attenuation ×50. Three poison glands were cleanly dissected without the Dufour gland and the sting, blotted dry, mounted on a small piece of glass and sealed in a glass tube (25 × 1.8 mm). The contents of the tube were chromatographed via a solid injection method (Morgan and Wadhams, 1972). A Dufour gland was chromatographed under the same conditions in order to distinguish any peaks that may arise as contaminants in the poison gland GC traces.

Trapping of GC Effluent. Two poison glands were injected onto the PEG

20 M column at 130° C. The effluent was split using an all-glass splitter (Baker et al., 1976) (95:5, trap–FID) and collected in metal U-tubes (1 mm ID) cooled in a mixture of liquid nitrogen and ethyl acetate. The collection tubes were changed at 1-min intervals. The trapped material was directly washed with acetone (50 μl) into Standardgraph pens, and the activities of various fractions were bioassayed.

Gas Chromatography–Mass Spectrometry. A Pye 104 gas chromatograph linked through a glass jet separator to an AEI MS 12 mass spectrometer was used. The poison glands of 50 ants were cleanly dissected and sealed in a glass tube for solid injection (Morgan and Wadhams, 1972). The mass spectra of the two major components of the poison gland were obtained by GC-MS using a low bleed 5% SE-30 column (1.5 m × 4 mm ID) at 130° C. The mass spectrometer was operated under the following conditions. Electron energy, 70 eV; accelerating voltage, 8 kV; multiplier voltage, 3 × 10 kV; and source temperature, 140° C. Background spectra were recorded before and after the peaks of interest and subtracted from the total spectra. The mass spectra of synthetic 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, ethylpyrazine, and 3-ethyl-2,5-dimethylpyrazine were obtained under the same conditions using solutions in hexane (500 ng/μl).

Quantification of Glandular Components. The poison glands of ten worker ants were chromatographed separately via the solid injection technique on the PEG 20 M column at 130° C. A computing integrator (DP 101, Spectra Physics) was employed to calculate the absolute quantities of material using a solution of 2,5-dimethylpyrazine (510 ng/μl) as an external standard.

Bioassay of Synthetic Substances. Mixtures of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine in hexane (to give a total of 4 ng/100 μl) were made in 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10 proportions. The trail-following behavior released by each mixture (100 μl) was bioassyed. The activity of material taken from poison gland was tested for comparison. The ability of each of the following compounds to evoke trail following behavior was tested by bioassay by presenting 4 ng in hexane per 31.4-cm trail: 2-ethylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine, 3-ethyl-2,5-dimethylpyrazine, and methyl 4-methylpyrrole-2-carboxylate. The ant species used to test the activity were *Tetramorium caespitum* L., *T. impurum* Foerster, *Myrmica rubra* L., and *M. ruginodis* Nyl.

Threshold concentrations of the two pyrazines at which the worker ants display trail-following reactions were found by presenting to them synthetic 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine separately at concentrations of 10^{-3}, 10^{-2}, 10^{-1}, 1, 10, 10^{2}, and 10^{3} ng per trail and recording activities.

To determine whether the 2,5-dimethylpyrazine and 3-ethyl-2,5-dimeth-

ylpyrazine act in synergy, bioassays were made using first the mixture at 4 ng total on a 31.4-cm trail and then 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine separately at 2.8 ng per trail and 1.2 ng per trail, respectively.

RESULTS AND DISCUSSION

Blum and Ross (1965) first reported that the trail pheromone of *T. caespitum* originates from the poison gland. As a preliminary survey, the work of Blum and Ross was repeated in the present study. Artificial trails were laid on a circular track with extracts obtained from the poison gland and the Dufour gland. The extract from the poison gland evoked high trail-following activity from the worker ants, whereas the Dufour gland extract was completely inactive. This result confirmed the observations of Blum and Ross.

The poison gland and the associated Dufour gland are attached to the sting. The poison gland contents are dispensed through the sting to lay trails as a means of communication during food gathering and change of nest sites. The two filaments of the poison-producing glands open into a spherical venom reservoir. The average diameter of the spherical reservoir was measured. The average volume was calculated (assuming it to be a sphere) to be about 14 nl.

Little or no work has been done on the chemistry of the *T. caespitum* poison gland, apart from the report by Blum and Ross (1965). They detected trace quantities of some free amino acids (aspartic acid being the major component) by paper chromatography.

The preliminary characterization of the trail pheromone was achieved by TLC. The contents of two poison glands were chromatographed on a silica gel plate. The silica gel was cut into ten bands, each representing a R_f difference of 0.1. When the bands were eluted with hexane and tested by bioassay, it was found that three bands of R_f values between 0.3 and 0.4, 0.4 and 0.5, and 0.5 and 0.6 elicited high activity. Initially, it was difficult to understand why the activity was spread over a range of R_f values. However, when the silica gel was cut into narrower bands, two regions of high activity were evident with a valley in between (Figure 1). Therefore it was possible to infer that the trail pheromone of *T. caespitum* was composed of as least two components of moderate polarity.

The chemical treatment of the glandular extracts and subsequent TLC separation and bioassay showed the trail pheromone components are basic because the activity was destroyed by acid treatment. Furthermore, the activity was unaffected by Br_2 in hexane, showing the absence of unsaturation.

GC examination of the contents of three poison glands on the PEG 20 M column showed the presence of two major components (Figure 2). Only a narrow fraction of the GC effluent containing these two components was able

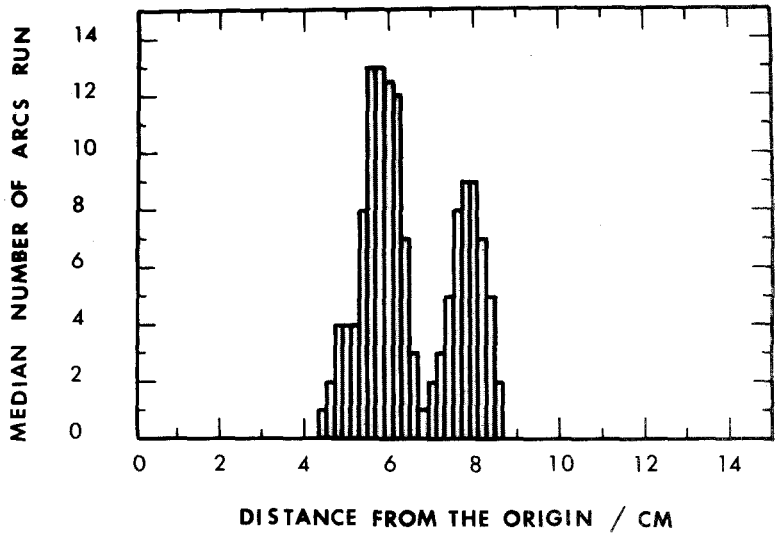


FIG. 1. TLC bioassay of poison gland contents of *Tetramorium caespitum*. An extract of two poison glands was chromatographed using hexane–acetone (60:40) as the eluent. The solvent front was allowed to run 15 cm and the silica gel was cut into narrow bands (2 mm). The trail-releasing activity of each band (stippled bars) was measured by bioassay after solvent extraction of the silica.

to evoke trail following activity when the GC effluent was trapped and bioassayed. The retention indices of the two peaks on the PEG 20 M column were 1370 and 1450, respectively. Similarly those on a SE-30 column were 859 and 1063, respectively. This indicated the compounds were moderately polar and their approximate molecular weight range to be between 100 and 150.

The identification of the two major components present in the poison gland was achieved by GC-MS. The mass spectra were obtained using 50 cleanly dissected poison vesicles sealed in a glass vial. The sample was injected by the solid injection technique on a 5% SE-30 column, and the mass spectra were recorded by GC-MS. The mass spectrometer was operated at a very high sensitivity; therefore it was necessary to record the background spectra between the peaks of interest. The mass spectra of the two major components, after the manual subtraction of the background, are given in Figure 3. The mass spectrum of the peak with lower retention time was identified as 2,5-dimethylpyrazine by comparison with published data (Deck and Chang, 1965; Stenhagen et al., 1974). Three positional isomers are possible for dimethylpyrazine; the mass spectra of synthetic samples of the three isomers were recorded under identical conditions and only the spectrum of the 2,5 isomer corresponded to that of the natural material.

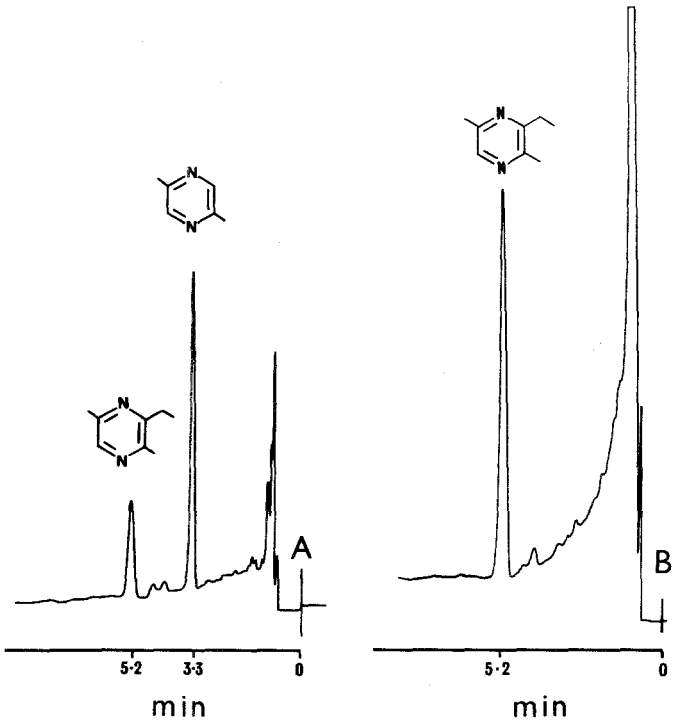


FIG. 2. Gas chromatograms of poison vesicle contents of (A) *Tetramorium caespitum* (attenuation ×50) and (B) *Myrmica ruginodis* (attenuation ×20). Three poison vesicles each were solid injected on a 2.75-m × 4-mm packed column of 10% PEG 20 M on Chromosorb W, at 130°C.

The mass spectrum of the peak with higher retention time (Figure 2) was identified as 3-ethyl-2,5-dimethylpyrazine. It was identical with that obtained under the same conditions from the synthetic material and agreed well with the published spectrum (Evershed et al., 1981; Stenhagen et al., 1974). Further confirmation of the two major components as 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine was obtained by showing they had identical retention times with those of authentic samples on three different GC phases. Furthermore, the R_f values obtained on TLC (60:40, hexane in acetone) for 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were 0.38 and 0.52, respectively. These R_f values corresponded to the active regions shown by TLC of poison gland extracts (Figure 1).

The average total amount of the two pyrazines present in the poison vesicles of individual worker ants was quantified from the GC peak areas using a computing integrator and was found to be 3.9 ng per ant, of which

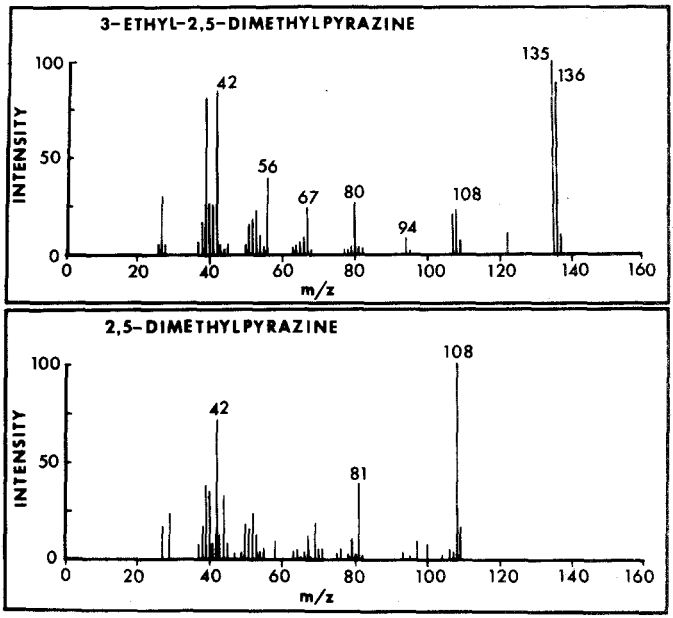


FIG. 3. Mass spectra of the pyrazines from the poison glands of *Tetramorium caespitum*.

2,5-dimethylpyrazine was 2.7 ± 0.4 ng (70 ± 4%) and 3-ethyl-2,5-dimethylpyrazine was 1.15 ± 0.25 ng (30 ± 4%).

The activities of mixtures of the two pyrazines in different proportions in releasing trail-following behavior in worker ants were tested by bioassay. A total of 4 ng of the two pyrazines was applied to the circular trail of 31.4 cm. The results are shown in Table 1. The 70:30 mixture of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine showed the highest activity. The results given are the median values obtained from three replicate determinations. Table 1 shows that 2,5-dimethylpyrazine has twice the activity of 3-ethyl-2,5-dimethylpyrazine to evoke trail-following behavior when used in identical concentrations and conditions.

The most significant fact that can be seen from Table 1 is that the 70:30 mixture of 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine makes a synergistic mixture with highest activity. When 2,5-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrazine were tested separately using concentrations of 2.8 and 1.2 ng per 31.4-cm trail, the mean activities observed were 13.5 and 5.1 cm, respectively. If the activities were additive, that of the mixture would be expected to be 18.6 cm. But the 70:30 mixture demonstrated an activity of 31 cm, clearly showing that synergism operates. Furthermore, the 70:30 mixture

TABLE 1. TRAIL-FOLLOWING ACTIVITY EVOKED BY MIXTURES OF TWO PYRAZINES IN DIFFERENT PROPORTIONS COMPARED WITH THAT OF ONE POISON VESICLE[a]

Test solution	DMP-EDMP ratio[b]	Trail-following activity			
		I	II	III	Median[c]
Pyrazine mixture	10:0	14	16	15	15
	9:1	18	16	19	18
	8:2	26	24	25	25
	7:3	31	34	29	31
	6:4	28	29	26	28
	5:5	18.5	20	17	18.5
	4:6	15	17	14	15
	3:7	14	17	13	14
	2:8	10	9	9	9
	1:9	7	9	9	8
	0:10	7	9	5	7
Single poison gland		31	36	32	32
Blank (hexane)		0	0	0	0

[a]The trails were applied as hexane solutions (100 μl) on a circle of 5 cm radius marked with 1-cm arcs. The number of arcs run along the trail by each worker ant was recorded for 20 min, and the median value was determined.
[b]The total concentration of 2,5-dimethylpyrazine (DMP) + 3-ethyl-2,5-dimethylpyrazine (EDMP) was 4 ng per trail.
[c]Median values obtained from three replicate determinations.

showed no significant difference in activity from that of the trail made with a single poison vesicle. Although at least one multicomponent trail pheromone of ants has been reported (Vander Meer et al., 1981), this is the first complete identification of the composition of such a pheromone mixture.

A range of concentrations of the two pyrazines was presented separately to the ants to determine the amount of material that evokes most efficient trail-following behavior. Concentrations between 1 and 10 ng per 31.4-cm trail released highest activity (Figure 4). The ants were able to detect concentrations as low as 0.3 pg/cm but showed difficulty in following any lower concentrations. Relatively high concentrations also were less effective. When concentrations about 3 ng/cm were applied, the ants were alarmed and confused, and they exhibited a very low trail-following behavior.

A number of related compounds were tested by bioassay to obtain some information on the structural specificity of their trail-following activity. The results are summarized in Table 2. The 2,5 substitution on the pyrazine ring appears to be important because 2,3- and 2,6-dimethylpyrazines were inactive. It was interesting to find that 2,3,5-trimethylpyrazine was able to evoke weak trail-following in *T. caespitum* and the three species of *Myrmica* tested.

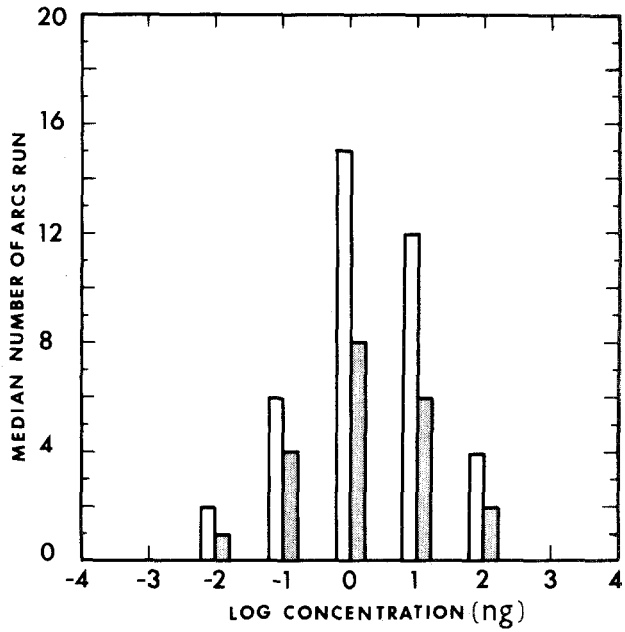


FIG. 4. Bioassay of the two pyrazines over a range of concentrations. 2,5-Dimethylpyrazine (open bars) and 3-ethyl-2,5-dimethylpyrazine (stippled bars) were applied as hexane solutions of different concentrations on a circular trail (5-cm radius). The number of arcs run along the trail by each individual worker was recorded for 20 min, and the median value was obtained.

T. impurum did not respond to any of the chemicals tested, indicating its trail pheromone to be different from any of the tested compounds.

The bioassays were always performed under identical conditions as far as possible, therefore the results obtained in any given experiment, conducted within a short period of time, were comparable. Nevertheless significant differences in absolute values can occur from time to time because the activity of ants appears to be dependent on many factors such as sunlight, temperature, humidity, time of the day, etc.

Pyrazines have been identified from a variety of sources, many of them odorous to man (Barlin, 1982). 2,5-Dimethylpyrazine has been identified as a flavor component of potato chips (Deck and Chang, 1965). It is also found in fusel oil, black tobacco, and in the smoke of nonfilter cigarettes made from these tobaccos (Barlin, 1982). 3-Ethyl-2,5-dimethylpyrazine is a component important to the aroma of baked potatoes (Buttery et al., 1973) and coffee (Goldman et al., 1967). Maga and Sizer (1973a) have published a review listing the extensive occurrence of alkylpyrazines in foods. The review published by Brophy and Cavill (1980) provides the mass spectra of a number of pyrazines.

TABLE 2. TRAIL-FOLLOWING ACTIVITY EVOKED BY VARIOUS SYNTHETIC PYRAZINES AND A PYRROLE ON SPECIES OF *Tetramorium* AND *Myrmica*[a]

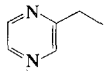
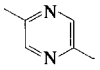
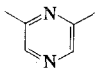
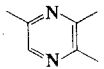
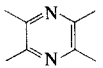
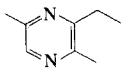
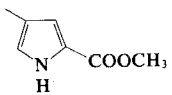
Compound	Structure	Test species				
		T. caespitum	*T. impurum*	*M. rubra*	*M. ruginodis*	*M. scabrinodis*
2-Ethylpyrazine		1	0	0	0	0
2,3-Dimethylpyrazine		0	0	0	0	0
2,5-Dimethylpyrazine		14	0	0	0	0
2,6-Dimethylpyrazine		0	0	0	0	0

TABLE 2. Continued

Compound	Structure	Test species				
		T. caespitum	*T. impurum*	*M. rubra*	*M. ruginodis*	*M. scabrinodis*
2,3,5-Trimethylpyrazine		6	0	3	4	3
2,3,5,6-Tetramethylpyrazine		0	0	0	0	0
3-Ethyl-2,5-dimethylpyrazine		8	0	10	10	12
Methyl 4-methylpyrrole-2-carboxylate		0	0	0	0	0

[a]Trails were applied as hexane solutions (4 ng/31.4 cm) on a circle of 5 cm radius marked with arcs (1 cm). The number of arcs runs along the trail by each worker ant was recorded for 20 min, and the median value was calculated.

A number of trisubstituted alkylpyrazines have been reported from the mandibular glands of some subfamilies of ants. Many other insects such as wasps (Wheeler et al., 1982), flies (Baker et al., 1982), and some beetles also have the ability to biosynthesize pyrazines.

A wide variety of alkaloids have been identified from the poison glands of ants but 3-ethyl-2,5-dimethylpyrazine was the only pyrazine that has been identified before this study. It has been shown to be present in the trail pheromone of *Atta sexdens rubropilosa* (Cross et al., 1979), *A. sexdens sexdens* (Evershed and Morgan, 1983), eight species of *Myrmica* (Evershed et al., 1981), and in small quantities in *Atta cephalotes* (Evershed and Morgan, 1983). The 2,5-dimethylpyrazine identified in *Tetramorium caespitum* is novel because it is the simplest and the only disubstituted pyrazine yet reported from ants.

Blum (1974) has postulated that the trail pheromones of myrmicine ants were originally trace constituents of the venom that were exploited as the function of the gland changed into its present form. The present study showed that the trail pheromone components of *T. caespitum* are indeed trace constituents. The pyrazines occupy only 0.03% of the volume of the poison gland. The poison reservoir contents have been reported to be rich in free amino acids (Blum and Ross, 1965), which probably can react enzymatically to form pyrazines (Maga and Sizer, 1973b). Morgan (1984) has postulated a mechanism for the biosynthesis of pyrazines from amino acid precursors.

As the same 3-ethyl-2,5-dimethylpyrazine had been identified as the trail pheromone of eight species of *Myrmica*, it was anticipated that *T. caespitum* and species of *Myrmica* may follow each other's trails. Such a transposition study has not been carried out previously for these species. Therefore cross-activity was studied with poison gland extracts of *T. caespitum* and *M. rubra*. It was found that these species indeed followed each other's trails. The results are given in Table 3. The chemical composition of the poison glands explains the observations. *T. caespitum* followed the trails of *Myrmica* moderately well

TABLE 3. TRAIL-FOLLOWING ACITIVITY EVOKED BY SINGLE POISON GLANDS OF TWO SPECIES OF MYRMICINAE SUBFAMILY[a]

Source species	Test species	
	T. caespitum	*M. ruginodis*
Tetramorium caespitum	31	14
Myrmica ruginodis	12	19

[a]The trails were applied as hexane solutions (100 μl) containing one poison gland equivalent of material, on a circle of 5 cm radius marked with 1-cm arcs. The number of arcs run along the trail by each worker ant was recorded for 20 min, and the median value was determined.

because of the presence of the 3-ethyl-2,5-dimethylpyrazine. However, the trail pheromone of *Myrmica* ants has only one component. Poison glands of *M. ruginodis* were examined carefully, and no peak corresponding to 2,5-dimethylpyrazine was found in the GC traces (Figure 2). *Myrmica* workers followed poison gland extracts of *T. caespitum* because they contained 3-ethyl-2,5-dimethylpyrazine and the effect was not marked or inhibited by the 2,5-dimethylpyrazine.

Similar observations in interspecific trail-following behavior have been reported with the leaf-cutting ants *Acromyrmex octospinosus* and *Atta sexdens* (Robinson et al., 1974). The major components of their trail pheromones are methyl 4-methylpyrrole-2-carboxylate and 3-ethyl-2,5-dimethylpyrazine respectively. However, *Acromyrmex octospinosus* will follow the trails of *Atta sexdens* because the pyrrole is also present as a trace component in the venom of the latter. But *A. sexdens* will not follow a trail of a *A. octospinosus* (Robinson et al., 1974) because the pyrazine is not present in the venom of *A. octospinosus* (Evershed and Morgan, 1983). Similarly *Atta sexdens* will not follow a trail of synthetic pyrrole (Robinson et al., 1974). On the contrary, *A. sexdens* will follow the venom of *A. texana* which contains both the pyrrole and the pyrazine (Evershed and Morgan, 1983).

Bolton (1976) has suggested on the ground of habitats and morphology that *Tetramorium* and *Myrmica* have most probably descended from the same ancestral stock. The similarities of the chemical composition of the poison glands and the trail-following behavior, as found in the present study, appear to support the postulate of Bolton, which considers the tribes Myrmicini and Tetramoriini to be closely related.

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VARIABILITY IN ACCUMULATION OF PROANTHOCYANIDINS (CONDENSED TANNINS) IN NEEDLES OF DOUGLAS-FIR (*Pseudotsuga menziesii*) FOLLOWING LONG-TERM BUDWORM DEFOLIATION

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Abstract—Long-term defoliation by budworms was associated with higher levels of soluble proanthocyanidins in the current year needles of Douglas-fir trees. The proanthocyanidin contents of needles from defoliated Douglas-fir trees were considerably more variable than those levels of undefoliated ones. The increased mean and variability of proanthocyanidin levels following defoliation may have interesting ecological consequences for Douglas-fir and its defoliators.

Key words—Proanthocyanidins, condensed tannins, plant–herbivore interactions, Douglas-fir, *Pseudotsuga menziesii*, western spruce budworm, *Choristoneura occidentalis*, Lepidoptera, Tortricidae, nested ANOVA.

INTRODUCTION

Proanthocyanidins (condensed tannins) are oligomers and polymers of hydroxy-flavan units, typically having molecular weights of 300–6000 daltons. They form complexes with and precipitate proteins, a property which probably has significant ecological consequences for plants producing them (Swain, 1979; Haslam, 1981). Proanthocyanidins may lower the nutritive value of foliage, or they can act as feeding deterrents to insect herbivores (Reese et al., 1982; Klocke and Chan, 1982; Bernays, 1981; Dethier, 1982.)

Variability in plant defense compounds is now considered to be an important obstacle for herbivores to overcome (Denno and McClure, 1983; see other articles in the same volume). Schultz (1983) and Baldwin and Schultz (1983) found sugar maple leaves to be highly variable in foliage feeding characteristics and argued that variability in plant defensive characteristics

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can be adaptive for trees defoliated by insects. On the basis of single-needle assays for proanthocyandin content of Douglas-fir *Pseudtsuga mengiesii* (Mirb.) Franco needles and a more detailed statistical analysis, we report not only an increase in the mean level of proanthocyanidins, but an increase in the variability of proanthocyanidin levels in budworm-defoliated trees. Therefore, since trees may respond to herbivory by increasing proanthocyanidin synthesis in a highly variable fashion, western spruce budworms continuing to feed upon previously exploited Douglas-fir foliage may have to contend with higher and variable amounts of these feeding deterrents.

METHODS AND MATERIALS

Trees Defoliated by Budworms. Douglas-fir (*Pseudotsuga menziesii*) samples were taken from a mixed Douglas-fir–white fir stand (elevation 1500 m) near Heppner, Oregon, in the Umatilla National Forest on February 1, 1983. Trees at this site were defoliated by western spruce budworms the previous spring and for the two springs prior to that (private communication, Bill Helfenstein, USDA Forest Service, Heppner, Oregon, and to whom we are greatly indebted for information and guidance). Sample trees were not more than 100 m from each other and were at least 190 m from the road.

Five budworm-defoliated (40–75% of the past several years' growth missing as estimated by visual inspection) and five undefoliated (less than 15% of the past three years' growth missing) trees were selected. The outer part of a branch was removed from each tree, sealed in a plastic bag, put on ice for 8 hr transit time, and stored in a freezer at −20° C. Six undamaged needles were later removed from each branch; three were from the outside of the branch (the apical region of the current year's growth), and three were from just outside the previous year's terminal bud scar (the basal region of the current growth) (Table 1). (Fresh weight approximately 15 mg/needle, dry weight/fresh weight 45%).

Extraction Procedure. Single needles were cut into approximately 3-mm sections and homogenized for 10 sec at room temperature with a Tekmar Tissuemizer in 2 × 0.5 ml methanol–H_2O (70:30, v/v). After centrifugation, both supernatant (the "methanol-soluble" fraction) and pellet (the "methanol-insoluble" fraction) were analyzed.

Proanthocyanidin Assay. Extracts and pellets were assayed for proanthocyanidins (Stafford and Lester, 1980) with modifications described below.

We mixed 1.2 ml *n*-butanol–HCl (95:5, v/v) into extract aliquots (0.05–0.2 ml) and water, for a total volume of 1.4 ml. Aliquots were chosen to give absorbances less than 0.2, since a biphasic curve has been reported (Stafford

TABLE 1. MEAN METHANOL-SOLUBLE PROANTHOCYANIDINS (ΔA_{550}/100 mg FRESH WEIGHT) OF NEEDLES FROM RELATIVELY UNDEFOLIATED TREES AND TREES DEFOLIATED BY BUDWORMS ($X \pm$ SE)

	Neighboring needles	Trees	
Relatively Undefoliated			
U1a[a]	43.4 ± 3.6		
U1b	37.9 ± 1.4	40.7 ± 2.1	
U2a	44.4 ± 1.3		
U2b	38.4 ± 2.1	41.4 ± 1.7	
U3a	83.6 ± 3.4		
U3b	69.6 ± 6.0	76.6 ± 4.4	53.1 ± 2.91
U4a	56.3 ± 2.1		
U4b	42.7 ± 2.8	49.5 ± 3.4	
U5a	63.7 ± 3.6		
U5b	51.1 ± 10.5	57.4 ± 5.8	
Defoliated			
D1a	64.4 ± 2.9		
D1b	59.4 ± 7.3	61.9 ± 3.7	
D2a	62.3 ± 5.0		
D2b	87.2 ± 23.7	74.9 ± 5.2	
D3a	75.3 ± 3.8		
D3b	75.9 ± 10.5	76.6 ± 5.0	75.8[b] ± 3.85
D4a	115.2 ± 10.2		
D4b	73.4 ± 7.7	94.3 ± 11.1	
D5a	69.1 ± 2.5		
D5b	75.9 ± 6.9	72.5 ± 3.6	

[a]Groups: U, D (undefoliated, defoliated); trees: 1–5 (5 trees/group); Locations: a, b (apical, basal).
[b]43% greater than undefoliated trees.

and Lester, 1980). Pellets were suspended in 1.0 ml *n*-butanol–HCl (95:5, v/v). The tubes were placed in a 95° C bath for 60 min. After cooling and remixing, the absorbances of the methanol-soluble proanthocyanidins were recorded. To measure the proanthocyanidin content of the insoluble fraction, tubes containing pellets were recentrifuged, and an appropriate aliquot of the supernatant was diluted with *n*-butanol–HCl (95:5), v/v) to a final volume of 1.2 ml. After mixing, the absorbance was recorded. Procyanidin values were expressed as A at 550 nm produced by an extract equivalent to 100 mg fresh weight of tissue per milliliter (ΔA_{550}/100 mg fresh weight).

Statistical Analyses. Measurements of proanthocyanidin content of single needles were analyzed with a nested factorial analysis of variance computed by an original Fortran IV plus program.

RESULTS

Long-Term Defoliation by Western Spruce Budworms. Soluble proanthocyanidin levels in the current year's foliage of budworm-defoliated trees were 45% higher than those in relatively undefoliated trees from the same site (Table 2). This difference was significant when the measurements were recorded on a fresh weight basis ($P = <0.001$), but on a per needle basis, a 67% difference was not significant at 5% level ($0.05 < P < 0.1$) due to the high individual variability of the trees.

Examination of the methanol-soluble fraction using paper chromatographic and HPLC methods (Stafford and Lester, 1980) indicated that among the lower molecular weight phenolic compounds in defoliated and undefoliated trees catechin and gallocatechin were the most common flavan-3-ol monomers; epicatechin–catechin was the most common dimer. High chlorogenic acid levels were also encountered.

Data for methanol–insoluble forms are given, but the values are difficult to interpret because they may be only artifacts of extraction, i.e., those proanthocyanidins which form complexes with other cell components during extraction and which may no longer give the same cyanidin equivalents upon heating in butanol–HCl. Proanthocyanidins are probably localized within the vacuole of the plant cell, where they may be sequestered in vesicles (Baur and Walkinshaw, 1973; Mueller and Greenwood, 1977). When the cells are disrupted during extraction, they are released from the vacuole and are free to bind with other cellular components.

On both fresh weight and per needle bases, soluble proanthocyanidin measurements from neighboring needles were considerably more variable in defoliated trees than in undefoliated ones (Table 3). There was a fivefold increase in residual variability on a fresh weight basis or a threefold increase on a per needle basis. The variability between neighboring needles represented only about 20% of the total variability in needles from undefoliated trees, but accounted for about 60% of the variability in needles from defoliated trees.

TABLE 2. PROANTHOCYANIDINS (ΔA_{550} / 100 mg FRESH WEIGHT) OF NEEDLES FROM BUDWORM-DEFOLIATED AND RELATIVELY UNDEFOLIATED TREES

	Trees[a]		
	Undefoliated	Defoliated	P^b
Methanol soluble	53.11 ± 2.91	75.81 ± 3.85	<0.001
Methanol insoluble	28.61 ± 1.40	25.38 ± 0.88	<0.1

[a]Mean ± standard error, $n = 30$.
[b]Approximate t test for unequal variances.

TABLE 3. NESTED ANALYSIS OF VARIANCE OF METHANOL-SOLUBLE PROANTHOCYANIDINS OF BUDWORM-DEFOLIATED AND RELATIVELY UNDEFOLIATED DOUGLAS-FIR TREES, WITH 3 INTACT NEIGHBORING NEEDLES FROM 2 LOCATIONS ON A BRANCH FROM EACH OF 5 DEFOLIATED AND 5 UNDEFOLIATED TREES

Source	*df*	Sum of squares	Mean square	*F*	*P*	Total variability (%)
Relatively Undefoliated						
Trees[a]	4	545814	136454	7.20	<0.05	64.9
Location[b]	5	94778	18956	2.95	<0.05	13.8
Residual[c]	20	128483	6424			21.3
Budworm-defoliated						
Tree	4	342104	85527	1.13	>0.1	3.4
Location	5	379079	75817	2.45	<0.1	31.5
Residual	20	618357	30918[d]			65.1

[a]Between trees.
[b]Between locations within tree branches.
[c]Within locations; between three neighboring needles and assay error.
[d]Greater than undefoliated. $F = 4.81$, $P < 0.001$.

Total amounts of proanthocyanidins (methanol-soluble plus insoluble) in the undefoliated trees were similar to those reported earlier (Stafford and Lester, 1981) and represented about 12% of the total dry weight of needles.

Mechanical Defoliation of a Seedling. Preliminary studies were made on single needles of a 1-year-old Douglas-fir seedling to determine whether mechanical defoliation could induce short-term changes in proanthocyanidins. A feeding response was mimicked by removal of parts or entire needles by successive snips with a scissors until approximately 50% of the current year's growth was removed. New needles were in their later stages of elongation (about 10 mg fresh weight per needle). Comparisons of the methanol-soluble proanthocyanidins before and 48 hr after defoliation showed a statistically significant increase of 56% in the uninjured leaves after defoliation. (ΔA at 550nm/100 mg fresh weight increased from 4.4 to 7.3; $P < 0.001$, $N = 36$).

A nested ANOVA comparing three intact neighboring needles from two locations (outer vs. inner part of a branch) on two branches from three regions (top, middle, and bottom of the plant) showed a fivefold increase in variability in all categories. Partitioning of the total variance was similar before and after defoliation, with about 50–60% between regions, 0–5% between branches within regions, 10% between sampling locations within branches, and 35% as residual variance (including assay variance as well as that between neighboring needles at a location). Prior to the 48-hr study, similar plants were not synthesizing significant amounts of proanthocyanidins in such short time

periods. Genotypic variation in native Douglas-fir populations and the possibility of communication between plants via volatile agents (Rhoads, 1983) will make it difficult to design effective controls for such experiments. However, the above data indicate that mechanically defoliated seedlings of Douglas-fir can be useful biological tools in attempts to understand the mechanism of induction of the increases in proanthocyanidin content upon defoliation.

DISCUSSION

The data indicating an increase in proanthocyanidin content after budworm defoliation are consistent with, but do not prove, the hypothesis that these phenolics are chemical defenses against herbivores such as the budworm and that plants can respond to such an attack by inducing the synthesis of these constitutive compounds. Our data do not indicate what mechanism is involved in this increase. The mechanism could be due to the greater availability of a limiting factor from older regions, or to internal or volatile chemical messengers arising from the injured leaves. (Rhoades, 1983; Baldwin and Schultz, 1983). The response could be specific for proanthocyanidins or a generalized response for all defense compounds. If volatile chemical messengers were produced by the budworm-defoliated trees we studied, the 43% higher proanthocyanidin levels of the defoliated trees may understimate the total effect of defoliation on a tree.

Haukioja and Niemela (1979), Schultz and Baldwin (1982), and Rhoades (1983) have reported increases in proanthocyanidins or total phenolics following defoliation or foliage injury in angiosperm trees. Published data for gymosperm trees is lacking, but mechanical defoliation resulted in increased proanthocyanidin levels for several species, including ponderosa pine (Michael Wagner, personal communication). However, Cates et al (1983) reported that proanthocyanidins, indirectly measured by the protein-precipitating ability of needle extracts, were unimportant in predicting budworm success on Douglas-firs; instead, terpenoids were the most important variables. They apparently analyzed young needles with low quantities of proanthocyanidins and emphasized the importance of qualitative defenses rather than quantitative ones in such ephemeral development stages (p. 16 in Cates et al., 1983).

Martin and Martin (1982) argue the merits of protein-precipitation assays, but protein–proanthocanidin interactions are specific (see Zucker, 1983, for a discussion), and the precipitation of a protein in vitro may not resemble the precipitation of proteins or inhibition of enzymes in vivo. Evidence that proanthocyanidins are feeding deterrents and reduce efficiency of utilization of digested food appears to be better documented than the

lessened digestibility (Bernays, 1981); no feeding experiments have yet been done with the spruce budworm. More work is needed before the relationship between the protein-precipitating effects of proanthocyanidins and their physiological effects is understood.

Both assay variability and actual variability between the three neighboring needles are contained within the residual variability, which was higher in the defoliated trees. Since the assay procedure was identical for needles from defoliated and undefoliated Douglas-firs, we attribute the higher residual variability of the former to a higher variability between neighboring needles.

We do not know what the proanthocyanidin content of the five heavily defoliated trees was prior to the recent years of heavy infestation by budworm. In order to assess this, long-term studies would be needed to permit analysis prior to infestation as well as after defoliation.

Whitham (1983) and Schultz (1983) argue that plant host variability is adaptive under conditions of insect herbivory: feeding upon less-acceptable foliage may produce nutrient deficiencies, while traveling to preferred sites may introduce competition for those sites, increased exposure to pathogens, and increased predation possibilities. Western spruce budworm larvae build feeding shelters by binding young needles together; individuals venturing from these shelters may be more easily captured by birds. Torgerson and Campbell (1982) have shown that birds can exert significant control over budworm population densities, although it is not clear that birds are more likely to catch budworms when they are not in their feeding shelters.

Variability in proanthocyanidin production may also be an economical defense strategy. Proanthocyanidins are costly for the tree to make, as their biosynthesis competes with the Krebs cycle and with protein synthesis (Hahlbrock and Grisebach, 1975). During insect outbreaks, trees effectively preventing continued herbivory by increasing proanthocyanidin levels in some of the foliage should be able to allocate more energy to growth and reproduction than those producing large amounts of proanthocyanidins throughout the foliage. Our evidence implies that in the case of soluble proanthocyanidins of Douglas-fir needles, such variability exists only when it is "required," i.e., in response to herbivory.

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SEARCH FOR POTENT ATTRACTANTS OF ONION FLIES[1,2]

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Abstract—Of various chopped vegetables tested, *Allium* spp. high in propyl-containing alkyl sulfides (e.g., *cepa* group) caught the most onion flies in trapping tests in the field. Fly catches to chopped onion increased with bait quantity. Attractancy of chopped onion changed dramatically during aging in the field; catch increased over the first few days, peaked at ca. fivefold over fresh material by 3–5 days, and then declined sharply. This age-dependent increase in attraction was not seen for garlic (known to have antimicrobial properties) nor with chopped onion mixed with chopped garlic. These data suggested that attraction of onion flies to onions was strongly influenced by microbial activity associated with decomposing onions. The bacterium *Klebsiella pneumoniae* was identified as a major colonizer of onions maximally attractive to onion flies. This increased attraction is not due to the previously reported microbially produced volatiles ethyl acetate and tetramethyl pyrazine.

Key Words—*Delia antiqua,* onion fly, Diptera, Anthomyiidae, food attractants, host attractants, microbial attractants, *Klebsiella pneumoniae,* bacteria, *Allium,* onion, garlic, chive.

INTRODUCTION

The onion fly, *Delia* (=*Hylemya*) *antiqua* Meigen, is an economically important pest of onions throughout the northern hemisphere (Loosjes, 1976). In North America, control of *D. antiqua* is achieved mainly by

[1]Diptera: Anthomyiidae.
[2]Paper No. 11047 of the Michigan State University Agricultural Experiment Station.

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organophosphate soil insecticides (e.g., Fonophos™) applied under onion seed at planting. Although the onion maggot is currently "under control," growers and pest managers are highly concerned that resistance to available insecticides (Harris et al., 1982) will increase in North American populations to the extent that crop failures (Guyer and Wells, 1959) will again occur.

We have been examining the onion fly life system from a behavioral and physiological perspective, hoping to identify points of vulnerability to human manipulation. An aspect deserving closer attention is behavior-modifying chemicals. For example, attractants offer the possibility for manipulating *D. antiqua* spatial distributions to allow population monitoring (via trapping) and increased visitation to insecticidal baits.

Since the original finding (Matsumoto, 1970) that the volatile alkyl sulfides from onion elevate trap catches, research on onion fly attractants has been directed toward finding increasingly potent baits. Major onion volatiles like *n*-dipropyl disulfide (*n*-Pr_2S_2) can yield trap catches ca. 10-fold higher than unbaited traps (Dindonis and Miller, 1981a; Vernon et al., 1981); however, these onion synthetics have not proven to be consistently attractive (Miller and Haarer, 1981; Eckenrode et al., 1975). Optimizing release rates of alkyl sulfides (Dindonis and Miller, 1981a) or employing chemical blends (Vernon et al., 1981) led to only modest increases in attraction. Furthermore, baits consisting of intact (Dindonis and Miller, 1980) or freshly cut onion tissues (Dindonis and Miller, 1981b; Vernon et al., 1981; Ishikawa et al., 1981) do not yield catches notably greater than those of synthetics. Combined with direct behavioral observations of onion fly response in the field (Dindonis and Miller, 1980b), these data suggest that, in the setting of commercial fields, onion volatiles alone are not attractive enough to be of much use in managing *D. antiqua* adults.

Attention has been directed to other sources of attractants. Miller and Haarer (1981) found that enzymatic yeast hydrolysate (EYH) is quite attractive to onion and seed flies, yielding catches 17- and 25-fold more, respectively, than unbaited traps. Although maximally attractive to females only during vitellogenesis (Miller, unpublished), EYH has proven to be a more effective and practical bait for *D. antiqua* than onion synthetics.

Another potential source of onion fly attractants is microorganisms. It is not unusual for muscid flies to be attracted to the volatiles emanating from microbially infested organic matter (Hwang et al., 1978). In addition to possible nutritional benefit to the adult onion fly, bacteria are known to speed larval development (Schneider et al., 1983, and references therein). Hence, there is ample reason to search for microbially generated attractants of *D. antiqua.*

Several groups, working independently, have documented that traps baited with decomposing onions catch more flies than do those baited with

freshly damaged onion tissues. Dindonis and Miller (1981b) found that after 4 days, bacteria-inoculated onion halves became significantly more attractive than freshly cut onion halves. Furthermore, they hypothesized that the optimal *D. antiqua* attractant would be a mixture of onion volatiles combined with volatiles from particular microorganisms. Vernon et al. (1981) also reported that onion attractancy to *D. antiqua* increased with time and showed this change was correlated with qualitative and quantative changes in the profile of volatiles as determined by GLC. Following the little-known lead of Tomioka (1977), Ishikawa et al. (1981) provided the best documentation, to date, for the increased attractancy of decomposing onion and the time course for its development.

In this paper we: (1) compare the attractiveness of fresh onion tissue with that of other alliums, (2) present further data on the time course of changes in onion tissue attractiveness as it decomposes, (3) identify a major microorganism from optimally decomposing onion, and (4) determine whether the increased attractiveness of decomposing onions is due to the microbially produced volatiles ethyl acetate and tetramethyl pyrazine as reported by Ikeshoji et al. (1980).

METHODS AND MATERIALS

Experiment 1—Relative Attractiveness of Alliums. The attractiveness of onion (*Allium cepa*) was compared to that of other alliums to determine whether *A. cepa* is uniquely attractive to *D. antiqua* and whether *A. cepa* tissue is, in fact, the best starting material upon which to try to improve attraction. The vegetables selected for this test were chopped (mean particle weight ca. 100 mg), dispensed in 100- to 150-g portions in 9-cm petri dishes covered with 9 mesh/cm cheesecloth, and deployed under cone traps (Dindonis and Miller, 1980) in post-harvest commercial onion field in Stockbridge, Michigan. The precise cultivar of these market-purchased vegetables was unknown. Onion (*A. cepa*), shallot (*A. ascalonicum*), and garlic (*A. sativum*) were purchased as bulbs. Leek (*A. porrum*) and chive (*A. schoenoprasum*) came as intact maturing plants, but only the nonchlorophyllose portions were chopped. Radish (*Raphanus sativus*) bulbs and potato (*Solanum tuberosum*) tubers were included in the test as non-*Allium* controls. The experimental design was randomized complete block, and trap spacing within the five linear blocks was ca 5 m. Baits were replaced with freshly chopped materials rerandomized every 24 hr during the first half of the test (total duration September 21, to October 3, 1979) and then every 48 hr.

Experiment 2—Fly Catch as Affected by Bait Quantity. Cone traps baited with precise quantities (0, 15, 25, 100, and 300 g) of chopped onion

(Michigan-grown Yellow Globe cultivar) and garlic (the most and least attractive alliums, respectively, from Experiment 1 were deployed to test the hypothesis that catch varies with bait quantity. Chopped materials were dispensed in 9-cm-diam × 10-cm-high aluminum-screen (7 mesh/cm) cylinders capped on the bottom and top with a 9-cm Petri dish. The top cap was painted brown to reduce solar incidence. This test was conducted from May 28, to June 1, 1980, on the edges of a commercial onion field in Stockbridge, Michigan. The design was that of experiment 1, but with four blocks. Every 24 hr, baits were replaced with fresh material and traps within blocks were rerandomized.

Experiment 3—Fly Catch as Affected by Bait Aging. The Allium materials generated during Experiment 2 were saved and set apart from experiment 1 to age. Thus, sets of chopped onion and garlic, incrementally aged from 0–1 days to 4–5 days, were readily obtained for concurrent testing for attractiveness to *D. antiqua.* Only the 300-g portions of chopped material were used in experiment 3, since this quantity of freshly chopped onion was maximally attractive in experiment 2. This test was conducted as per experiment 2 and ran from June 3 to 5, 1980. During this time, freshly chopped material was rotated into the experiment while material older than 4–5 days was rotated out.

Experiment 4—Inhibitory Effect of Garlic on Onion Attraction. In Experiment 3, the attraction of chopped onion increased dramatically with time while that of chopped garlic did not. As judged by human sight and smell, the aged onion material decomposed while garlic did not. Since garlic has strong antimicrobial properties (Al-Delaimy and Ali, 1970), we hypothesized that, over time, a chopped onion–garlic mixture would not become as attractive as chopped onion alone. This idea was tested using the procedures of experiment 3. The treatments were 150 g chopped onion, 150 g chopped garlic, 150 g of each thoroughly mixed, and 150 g of chopped onion placed directly over but not in physical contact with 150 g of chopped garlic. The five age classes of each treatment were tested concurrently on August 22–28, 1980, in Grant, Michigan.

Experiment 5—Identification of Predominant Microorganism from Optimally Decomposing Onion. Chopped onion tissue 3–5 days old and ca. five fold more attractive in the field than fresh material was immediately transported to the laboratory for microbial analysis. Fifty grams fresh weight of onion tissue were placed in 200 ml of sterile buffer (0.05 M potassium phosphate buffer, pH 7.0, containing 0.15 M NaCl) and homogenized for 5 min by using a sterile Waring blender. Serial decimal dilutions of the homogenate were made in buffer, and 0.1-ml portions of each dilution were spread on the surface of solid culture medium containing (%): nutrient agar (Difco), 2.3; glucose, 0.5; and Difco yeast ex-

tract, 0.5. Plates were then incubated aerobically at 25 ± 1°C. A bacterial isolate, derived from the dominant colony type appearing on isolation plates, was then examined by morphological, physiological, and biochemical tests (Holdeman and Moore, 1972; Lennette et al 1974). Assignment of the isolate to a taxon was made by consulting contemporary classification keys (Lennette et al., 1974; Buchanan and Gibbons, 1974).

Experiment 6—Attractiveness of Ethyl Acetate and Tetramethyl Pyrazine. Ikeshoji et al. (1980) identified ethyl acetate and tetramethyl pyrazine among the volatiles over *Klebsiella* sp. inoculated onion slices. These chemicals elevated oviposition when added to certain levels of *n*-Pr_2S_2; however, it was not clear whether these "synergists" primarily attracted more flies to decomposing onion or stimulated increased oviposition once flies had found the resource. Here, we tested whether these chemicals elevated trap catches in the field, either when dispensed alone or in combination with *n*-Pr_2S_2, ethanol, and acetic acid.

The 20 treatments are given in Table 2. Each of the *n*-Pr_2S_2, ethanol, and ethyl acetate treatments was comprised of two No. 130 Beem Capsules, size 00 (Ted Pella Co., P.O. Box 510, Tustin, California 92680), half filled with the respective liquid. Acetic acid was dispensed as a 33% aqueous solution in a 7-ml (1.6-cm-diam) glass miniscintillation vial with a 6-mm hole punched through the polyethylene snap-on cap. Tetramethyl pyrazine was dispensed as 750 mg of dry crystals in each vial with the 6-mm hole in the cap. Odors from each treatment were readily detectable by human olfaction. Treatments were placed in 10-cm-diam clay pots containing enough washed silica sand to hold all capsules and vials cap-upright. Onion was dispensed as per experiment 2.

This test was conducted July 17–25, 1981, at Eaton Rapids, Michigan, using four linear blocks along the edges of a commercial onion field in which no pesticides were used. Trap spacing was 3 m, and treatments within blocks were rerandomized three times during the test. Onion materials and acetic acid treatments were replaced every 2 days. Capsules with little liquid in reservoir were replaced as appropriate.

RESULTS AND DISCUSSION

Experiment 1—Relative Attractiveness of Alliums. This test demonstrated that onion flies are differentially responsive to plant volatiles generated by the selected vegetables (Figure 1). Onion, shallot, and leek fell into a grouping of alliums most attractive to *D. antiqua* adults. In this test, chive was less attractive than onion, and garlic was the least attractive Allium. Chopped potato and radish, as rich as the alliums in water and other nutrients, yielded catches statistically indistinguishable from the very low

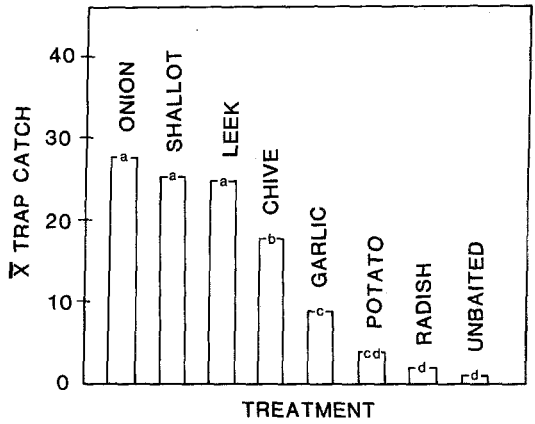

FIG 1. Onion fly trap catch as affected by the type of chopped vegetable used as bait. Means indicated by a common letter are not statistically different at $P \leq 0.05$ as determined by two-way ANOVA followed by a planned F test for mean separations of data transformed to $(x + 0.5)^{1/2}$.

catches of unbaited traps. It is notable that the volatile profiles of the most attractive alliums are similar (Saghir et al., 1966; Bernhard, 1970); all produce alkyl sulfides preponderant in propyl moieties. Chive volatiles also contain predominantly propyl sulfides, but are richer in methyl sulfides than is onion. Garlic, by contrast, generates predominantly allyl sulfides and only trace quantities of propyl sulfides. From experiment 1, we conclude that the alliums richest in propyl sulfide are or become most attractive to *D. antiqua* and, although not uniquely attractive to onion flies, *A. cepa* tissues are a justified starting point in attempting to enhance onion fly attraction.

Experiment 2—Fly Catch as Affected by Bait Quantity. Traps baited with increasing quantities of freshly chopped onion caught increasing numbers of onion flies (Figure 2). For onion, no upper plateau in the dose-response curve was visible by the highest dosage, 300 g/trap; however, we judged this quantity to be our upper workable limit. For garlic, catch plateaued by 25 g chopped tissue. Interestingly, catches to onion and garlic were virtually identical at the low dosages. Experiment 2 clearly demonstrated that catch varies with dosage of chopped alliums and provided a basis for selecting bait quantities for subsequent experiments.

Experiment 3—Fly Catch as Affected by Bait Aging. As shown in Figure 3, the attractancy of chopped onion increased dramatically as it aged in the wire-screen cylinders standing in the field. Traps baited with onion tissue aged 4–5 days caught ca 50 flies/trap/day; such catches in a Michigan commercial field with a typical non-outbreak *D. antiqua* population were a record high. These results confirm and add resolution to those of Ishikawa et al. (1981). The aged onion, which caught nearly fivefold more flies of both

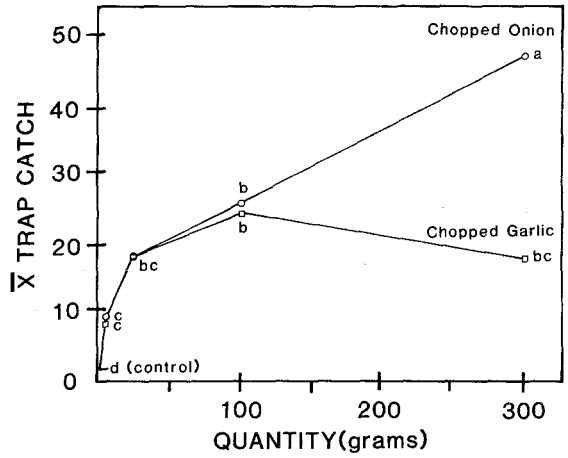

FIG 2. Onion fly trap catch as affected by bait quantity. See caption to Figure 1 for explanation of the statistics.

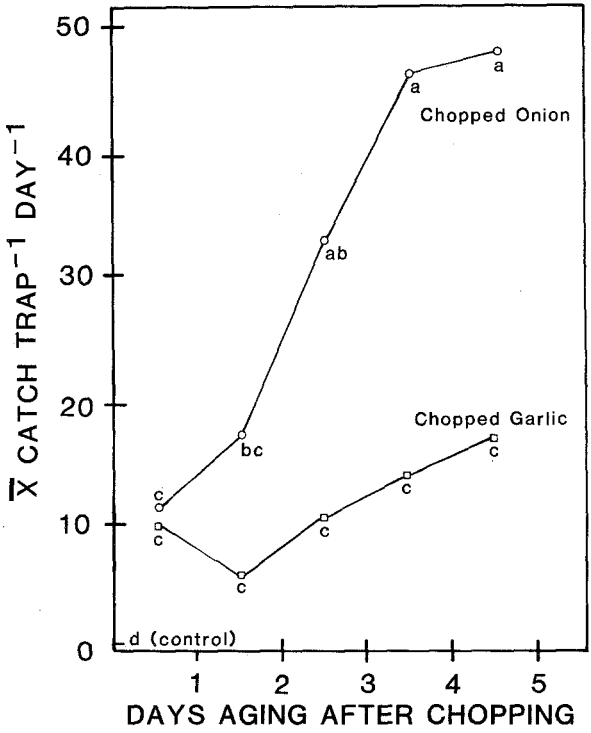

FIG 3. Onion fly trap catch as affected by bait aging after chopping. See caption of Figure 1 for explanation of the statistics. Mean separation achieved by Student-Newman-Keuls multiple range test. In light of the data of Figures 1 and 2, the mean catch for freshly chopped garlic is inexplicably high.

sexes than fresh, was judged decomposing; it turned increasingly off-white, shrank to about one half its original volume, lost its original strong propyl sulfide odor, and developed a semipleasant fruity-ethanolic aroma. Onion baits maximally attractive to *D. antiqua* also attracted large numbers of sap beetles (Nitidulidae), well-known for being attracted to souring or fermenting plant juices (Borrer and DeLong, 1964), and red admiral butterflies. Additionally, the sigmoidal profile of *S. antiqua* catch vs. onion age (Figure 3) is suggestive of a growth curve for a microbial population.

In contrast to onion, changes in chopped garlic over time were few. Freshly chopped and aged garlic had similar physical properties, and there was no statistically significant time-dependent increase in the attractiveness of garlic to *D. antiqua.* These results are readily explained by the knowledge (Al-Delaimy and Ali, 1970) that garlic allelochemicals are strongly antimicrobial.

Experiment 4—Inhibitory Effect of Garlic on Onion Attraction. The curves for *D. antiqua* catch over time for onion and garlic (Figure 4) are essentially similar to those of experiment 3 (Figure 3). However, decomposition apparently proceeded more rapidly in experiment 4, perhaps because of

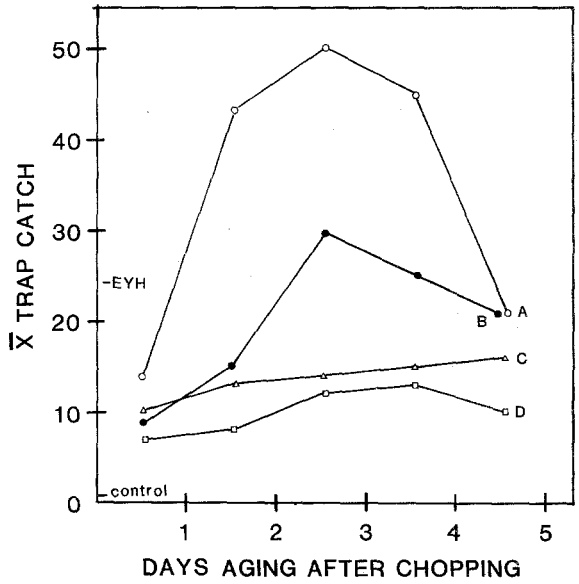

FIG 4. Influence of garlic on the attractiveness of aged onion. Curve A = onion, B = nonhomogeneously mixed onion and garlic, C = homogeneously mixed onion and garlic, and D = garlic. Each of the four materials, pooled across time, was statistically different from all others at $P \leq 0.054$ as determined by paired t tests for grand means.

warmer temperatures in August. This allowed us to document a rapid decline in the attractiveness of aged onion, as well as the rapid rise and peak. Interestingly, the comparatively weakly attractive 4 to 5 day-old onion had a strongly unpleasant odor as perceived by the experimenters. These results indicate that, in chemically identifying this potent attractant for *D. antiqua,* care must be taken when selecting material for analysis. Material that was highly attractive when bioassayed via trapping in the field may pass its prime by the time extracts are made and processed in the laboratory. The challenge in identifying the more potent attractant of *D. antiqua* appears to be isolating the volatile emitted by onions at a particular stage of microbial succession. Figure 4 dictates that bioassay and sample extraction (and stabilization) must be closely linked temporally.

The hypothesis that an onion–garlic mixture would not become as attractive as chopped onion alone was supported by experiment 4. Although the homogeneous admixture (curve C, Figure 4) of onion in garlic was slightly more attractive than garlic alone, this material changed very little over time. We suggest that the antibiotic chemicals in garlic suppressed microbial activity on the onion tissues, and hence, suppressed generation of attractive volatiles. Nonhomogeneously mixed onion and garlic (curve B, Figure 4) shows an intermediate effect of garlic volatiles. Presence of garlic seemed to suppress the responsiveness of *D. antiqua* to onion volatiles but only partially precluded the time-dependent increase in attractiveness.

Experiment 5—Identification of Predominant Microorganism from Optimally Attractive, Decomposing Onion. Bacterial counts from freshly chopped onion vs. optimally attractive decomposing onion were, respectively, 4×10^2 and 4×10^6 viable cells/g fresh wt onion tissue. Six different colony types were observed on isolation plates, but one type accounted for more than 50% of all colonies present. This type was white to cream color, 3–4 mm diam, and round with regular edges (24-hr incubation). From this colony type an isolate was obtained and designated strain JM1.

Cells of JM1 were gram-negative, nonmotile rods, 1×1.5–3.0 μm in size and occurring singly or in short chains. JM1 possessed neither endospores no prosthecae (or other appendages visible by light microscopy). JM1 was facultatively anaerobic, catalase positive, oxidase negative, and reduced NO_3^- to NO_2^-. Organic acids produced from glucose during anaerobic growth included major amounts (>10 μeq/ml) of acetate, but only minor amounts of lactate, succinate, and formate. These data suggested that JM1 was a member of the Enterobacteriaceae. Results of further tests (Table 1) indicated that JM1 was a strain of *Klebsiella pneumoniae.*

Experiment 6—Attractiveness of Ethyl Acetate and Tetramethyl Pyrazine. In this test, none of the synthetics tested singly or in any combination

TABLE 1. BIOCHEMICAL CHARACTERISTICS OF *Klebsiella pneumoniae* STRAIN JM1

Test or substrate	Reaction[a]
TSI[b]	
Slant	A
Base	A
Gas	+
H_2S	−
Indole	−
Methyl red	±
Voges-Proskauer	+
Citrate	+
Urease	+
Arginine dihydrolase	−
Lysine decarboxylase	+
Ornithine decarboxylase	−
Fermentation of	
Glucose	+
Glycerol	+
Adonitol	−
Dulcitol	+
Inositol	+
Sorbitol	+
Polypectate liquefaction	−
Pigmentation	−

[a] A, acid production; +, positive reaction; ±, weak positive reaction; −, negative reaction.
[b] Triple sugar iron agar (Difco).

caught significantly more onion flies than unbaited traps (Table 2), while aged onion again caught more than 50 times more flies than control. In similar tests, where ethyl acetate and tetramethyl pyrazine were added to freshly chopped onion or EYH, no significant increase in trap catch was realized (Harris and Miller, unpublished). These experiments do not support the hypothesis that ethyl acetate and tetramethyl pyrazine are onion fly attractants. However, Harris (1982) has substantiated that ethyl acetate does elevate *D. antiqua* oviposition in response to both propyl and allyl disulfides.

In summary, chopped onions which are exposed to the range of naturally occurring microorganisms in an onion field become highly attractive to onion flies of both sexes, but only at a particular stage of microbial succession. The observation that both male and female onion flies and other insects feed heavily upon this conditioned plant material suggests that it is evoking strong food-finding responses in addition to increased oviposition responses by *D. antiqua* females (Ikeshoji et al., 1980). The

TABLE 2. ONION FLY CATCH IN TRAPS BAITED WITH AGED ONION AND VARIOUS SYNTHETICS, INCLUDING ETHYL ACETATE AND TETRAMETHYL PYRAZINE

Treatment	Mean[a] trap catch (±SD)
1. *n*-Dipropyl disulfide	6.3 ± 1.5 cde
2. Ethanol	5.0 ± 2.9 cde
3. Ethyl acetate	3.8 ± 3.0 de
4. Tetramethyl pyrazine	7.5 ± 4.5 cde
5. acetic acid	4.3 ± 2.2 cde
6. 1 + 2	8.8 ± 6.2 cde
7. 1 + 3	6.5 ± 3.3 cde
8. 1 + 4	7.3 ± 1.0 cde
9. 1 + 5	5.5 ± 1.3 cde
10. 1 + 2 + 3	7.8 ± 4.2 cde
11. 2 + 3	2.5 ± 1.7 e
12. 2 + 3 + 4 + 5	5.3 ± 1.5 cde
13. 1 + 3 + 4 + 5	7.3 ± 4.6 cde
14. 1 + 2 + 4 + 5	11.3 ± 6.1 cde
15. 1 + 2 + 3 + 5	12.0 ± 4.4 cd
16. 1 + 2 + 3 + 4	8.8 ± 3.8 cde
17. 1 + 2 + 3 + 4 + 5	15.3 ± 11.1 c
18. Chopped onion (0–2 days)	71.0 ± 21.7 b[b]
19. Chopped onion (2–4 days)	184.0 ± 55.7 a[b]
20. Unbaited	3.3 ± 1.3 cde

[a] Means followed by a common letter are not statistically different at $P \leq 0.05$ as determined by two-way ANOVA followed by a Student-Newman-Keuls multiple range test on data transformed to log $(x + 0.5)$.
[b] Statistical assignments made with a $(X + 0.5)^{1/2}$ transformation.

microorganism most abundant on optimally conditioned onion is *Klebsiella pneumoniae*. In preliminary experiments (Miller and Weston, unpublished), sterilized onion tissue inoculated with *K. pneumoniae* JM1 became more attractive to onion flies than either uninoculated onion or standard microbiological media inoculated with JM1. We are currently exploring the chemistry of this highly attractive volatile blend generated by the onion–microbe interaction.

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Eucalyptus OILS IN LARVAE OF GUM EMPEROR MOTH, *Antheraea eucalypti*

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Abstract—The ether-soluble portion of the foregut fluid from the larvae of *Antheraea eucalypti* (Saturniidae) was found to contain traces (0.1–0.2%) of isoprenoids. The isoprenoids were those that were major components of the oils from the leaves of the *Eucalyptus* species on which the larvae fed and were also soluble in the foregut fluid. This observation demonstrated that these larvae did not sequester the leaf oils in their foregut or use them for defense. The compositions of the oils, which were steam-distilled from the bodies and feces of the larvae, were identical with those of the oils from the leaves of the host trees. This fact implied that the volatile components of the leaf oils had not been metabolized and had no effect on the growth or feeding of the larvae, at least on the two *Eucalyptus* species examined. In order to explain the coevolution of *A. eucalypti* and *Eucalyptus* species, components of the leaf oils were suggested to serve the function of an ovipositional attractant to the female moths.

Key Words—*Antheraea eucalypti*, Saturniidae, Lepidoptera, larval oral discharge, *Eucalyptus* oils, monoterpenoids, sesquiterpenoids.

INTRODUCTION

This paper examines two aspects of the metabolism of *Eucalyptus* leaf oils by larvae of the gum emperor moth *Antheraea eucalypti*. First, the composition of the ether-soluble portion of the foregut fluid from larvae which had fed on the leaves of *Eucalyptus delegatensis* or *E. cinerea* was determined. Second, the composition of the oil distilled from the bodies and feces of the same larvae was analyzed and compared with that of the oil from leaves of the host trees, in

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order to ascertain whether any leaf oil components had been selectively metabolized.

The gum emperor moth *Antheraea eucalypti* Scott (Saturniidae) (syn. *Caligula eucalypti* Scott) is a large, pale orange-brown moth (female wingspan, 13 cm) which is related to the moon moths of America and India and to the giant silk moth. It is native to Australia but was accidentally introduced to New Zealand about 1930 (Gaskin, 1966).

The caterpillar grows to 12 cm in length. It feeds predominantly on the leaves of *Eucalyptus* species but will also feed, in New Zealand, on leaves of the Californian pepper tree, *Schinus molle* (Anacardiaceae) and the sweet gum, *Liquidambar styraciflua* (Altingiaceae). The larvae of this moth are responsible for considerable defoliation of *Eucalyptus* species in New Zealand (Alma, 1977). Their predilection for *Eucalyptus* leaves suggests that they may have evolved to utilize the aromatic oils which are characteristic of the leaves of *Eucalyptus* species (Penfold and Willis, 1961). The extraordinary size and color of these larvae suggest that a possible use for the oils is to render the caterpillars distasteful to birds.

Morrow et al. (1976) showed that the larvae of sawflies sequester the essential oil from *Eucalyptus* leaves in a diverticular pouch of the foregut and then orally discharge this material in defense. The composition of the oral discharge was identical to that of the oil from the host tree.

The storage of host-plant constituents in a diverticular area of the foregut may be characteristic of sawflies, but the phenomenon of sequestering toxic compounds and using them either systemically or orally for defense is well known for species of other insect orders (Eisner, 1970). Like many caterpillars, the larvae of *Antheraea eucalypti* orally discharge the fluid contents of their foregut when attacked, and the composition of the ether-soluble portion of this fluid is a subject of this paper.

Morrow and Fox (1980) investigated the metabolism of *Eucalyptus* oils in larvae of the chrysomelid beetle *Paropsis atomaria* and in the adult scarabaeid beetle *Anopognathus montanus*. They demonstrated that the proportions of oils in the leaves of different *Eucalyptus* species did not affect the feeding or growth of these insects. In general, most components of the oils were tolerated; they were found to pass through the gut unchanged except for carbonyl compounds which were absorbed and presumably detoxified by conjugation and then excreted. Carbonyl compounds are known to have the greatest germicidal effect on animal cecal microflora, and these compounds are also selectively absorbed in the gut of the koala which feeds exclusively on *Eucalyptus* foliage (Eberhard et al., 1975). The composition of the oils distilled from the bodies and feces of the larvae of *A. eucalypti* is another subject of this paper.

METHODS AND MATERIALS

Larvae Fed on Eucalyptus delegatensis

Extraction of Foregut Fluid. Caterpillars were suspended by their abdomens, an action which induced the larvae to orally discharge the contents of their foregut. The liquid contents (3.44 g) from 14 caterpillars (total weight, 45.1 g) were diluted with aq. NaCl and water, and then extracted with Et_2O (3×) and with EtOAc (3×). The extracts were combined, dried over anhyd. Na_2SO_4, filtered, and concentrated to leave a residue of 4.4 mg (yield, 0.13%).

Extraction of Whole Larvae. The larvae (excluding foregut contents, 41.7 g) were macerated in neutral distilled water (200 ml). Volatile products were then isolated by simultaneous cohobation and extraction into Et_2O (20 ml) over a period of 2 hr in a Likens-Nickerson apparatus. The ethereal solution was dried over anhyd. Na_2SO_4 and evaporation of the solvent left a colorless oil (100.6 mg).

Extraction of Frass. The frass (51.0 g) was extracted as for the whole larvae and afforded a colorless oil in 2.5% yield.

Larvae Fed on Eucalyptus cinerea

Extraction of Foregut Fluid. The foregut contents (10 g) from 11 caterpillars (total weight, 105.5 g) were diluted with water and then extracted continuously with Et_2O for 4.5 hr in a liquid–liquid extractor. The ethereal solution was dried over anhyd. Na_2SO_4, filtered, and concentrated to leave a colorless oil (18 mg, yield 0.18%).

Extraction of Whole Larvae. The larvae (excluding foregut contents, 95.5 g) were macerated and extracted as for the larvae above, to yield a colorless oil (85.5 mg).

Extraction of Essential Oil of Eucalyptus cinerea

Leaves freshly picked in April (19.3 g) were cut into small pieces, and the oil was isolated by simultaneous cohobation in neutral distilled water (500 ml) and extraction into Et_2O (20 ml) over a period of 2 hr in a Likens-Nickerson apparatus. The ethereal solution was dried over anhyd. Na_2SO_4, filtered, and concentrated to afford a colorless oil (310 mg, 1.6%).

Gas Chromatography. Chromatography was performed on a gas chromatograph incorporating a FID, and H_2 was used as the carrier gas with a split ratio of 45:1. Chromatography was performed on a SGE vitreous silica WCOT column (50 m × 0.2 mm ID) with Carbowax 20 M as the stationary phase. N_{eff} = 123,900 plates. The linear gas flow rate was 40 cm/sec. Injector

temperature 220°; detector temperature 275°; initial oven temperature 50°; final oven temperature 200°; oven temperature was held for 5 min, after programing at a rate of 3°/min. Injections of 1 μl of a solution of the oils in ether (10 mg/ml) were used. Components of the oils were identified from previous work (Weston, 1983).

RESULTS AND DISCUSSION

The larvae which had fed on *Eucalyptus delegatensis* were not mature (average weight, 3.2 g), whereas those which had fed on *E. cinerea* were fully grown (average weight, 9.6 g). The proportion of the total amount of oil which was steam-distilled from the larvae was 0.23% for those on *E. delegatensis* and 0.10% for those on *E. cinerea*. The yield of oil from the leaves of the two *Eucalyptus* species was also different, viz., 3.9% for *E. delegatensis* (Weston, 1983) and 1.6% for *E. cinerea*. As a result, the ratio of the amount of oil in the larvae to that in the leaves was the same (0.06) in both cases. This indicated that the amount and composition (see below) of the oil in the leaves had no effect on the feeding or growth of the larvae. The same conclusion was drawn from a study with sawfly larvae (Morrow and Fox, 1980).

The compositions of the oils, which were steam-distilled from the frass and the bodies of larvae which had fed on the leaves of *Eucalyptus delegatensis*, and from the bodies of the larvae which had fed on *E. cinerea*, were identical in every respect with those of the oils from the host trees, except for the inclusion of a number of very minor constituents. Clearly, none of the components of the leaf oils had been metabolized by the larvae.

This result was not unexpected since the digestive system of insect larvae in general is very inefficient and most foliage passes through the gut unchanged (Wigglesworth, 1968). Fox and Macauley (1977) demonstrated that *Eucalyptus* foliage is low in nitrogen (protein) and that serious defoliation of eucalypts was caused by the necessity for insects which fed on this foliage to consume large amounts of leaf material in order to accumulate sufficient nitrogen for growth.

Carbonyl compounds were not constituents of the essential oils of either of the *Eucalyptus* species studied in this work, and the view that these compounds were generally toxic to gut microflora (see above) could not be extended.

The yield and composition of the essential oil from *Eucalyptus* species vary considerably over the many species which have so far been examined (Penfold and Willis, 1961). Several species are noted for the high level of occurrence of a particular component in an essential oil, e.g., cineole in *E. oleosa*, *E. smithii*, and *E. viridis*; citronellal in *E. citriodora*; geranyl acetate in

E. macarthuri; nerolidol in *E. deglupta*; α-pinene in *E. pauciflora*; and α-phellandrene and piperitone in *E. dives* and *E. radiata*. The isoprenoid components of these species have quite different structures and aromas, yet neither of these factors appears to influence directly the feeding of the larvae of *A. eucalypti*, at least on the two *Eucalyptus* species studied here. A generalization, however, cannot be made since the larvae of *A. eucalypti* have been recorded to feed on only 24 of the 60 *Eucalyptus* species so far examined in New Zealand (Alma, 1977) and also on other plant species (see above). Some of the components of *Eucalyptus* oils may, however, act as feeding stimulants for the larvae of *A. eucalypti* and/or have other ecological properties (see below).

The amount of oil which was isolated from the foregut contents of the larvae differed slightly in the two cases but was very low: 0.13% from the larvae on *Eucalyptus delegatensis* and 0.18% from the larvae on *E. cinerea*. These data showed clearly that the leaf oil was not sequestered in the foregut of the larvae, as it was in the sawfly larvae (Morrow et al., 1976), and the low yield of oil from the foregut was simply a reflection of the low solubility of the leaf oil in the foregut fluid (see below).

The composition of the ether extract of the foregut fluid from the larvae of *A. eucalypti* which had fed on the leaves of *Eucalyptus delegatensis* was quite unlike that of the leaf oil. One major product dominated the extract, viz., *trans*-piperitol which constituted 63% of the extract. This proportion contrasted with that of the same compound in a solvent extract of the leaves of the host tree (15%). Other products in the extract of the foregut fluid, with their proportion in the solvent extract of the leaves in parenthesis (Weston, 1983), were *trans-p*-2-menthen-1-ol, 5.6 (11.2); *p*-1-menthen-4-ol, 2.4 (3.4); *cis-p*-2-menthen-1-ol, 9.4 (10.6); α-terpineol, 1.6 (1.0); and 4-phenyl-2-butanone, 1.9 (4.9). These six compounds constituted 84% of the solvent extract of the foregut fluid.

In a recent study of the constituents of the essential oil of *Eucalyptus delegatensis* (Weston, 1983), *trans*-piperitol was shown to be a major component of the leaf oil. Piperitol and the two 2-menthen-1-ol isomers, which are formed from piperitol by rearrangement, were also the major compounds observed in the extract of the foregut fluid from the larvae of *A. eucalypti*. Other major products in the leaf extract which were not soluble in water, e.g., methyl cinnamate, were not detected in the extract of the foregut fluid.

Clearly, the isoprenoids in the foregut fluid were those which were both major components of the oil from the leaves on which the larvae had fed and which were also soluble in the liquid contents of the foregut. To confirm this conclusion, the regurgitate from the larvae of *A. eucalypti* which had fed on

TABLE 1. COMPOSITION OF ESSENTIAL OIL OF *Eucalyptus cinerea*

Component	Percentage in the oil
α-Pinene	2.9
α-Thujene	0.3
α-Phellandrene	1.0
α-Terpinene	0.3
Limonene	1.1
Cineole	34.3
γ-Terpinene	16.2
Cymene	2.9
Terpinolene	1.0
1-*p*-Menthen-4-ol	3.0
α-Terpineol	1.9
Sesquiterpenoid	
1	0.6
2	4.6
3	3.5
4	0.4
5	0.8
α-Eudesmol	9.5
β-Eudesmol	15.6

the leaves of *Eucalyptus cinerea* was extracted, and the composition of this extract was compared with that of the essential oil from the host tree.

The essential oil of *E. cinerea* consisted of three groups of products: monoterpene hydrocarbons (including cineole), monoterpene alcohols, and sesquiterpene alcohols. The composition of the oil is listed in Table 1. Earlier publications recorded a wide variation in oil yield but that cineole was the major component of the oil (Dora de Iglesias et al., 1980); Moreira et al., 1980). These papers did not record the occurrence of sesquiterpenoids which formed a significant proportion (35%) of the essential oil. In the present investigation the major components of the oil were found to be cineole (34%), γ-terpinene (16%), and the sesquiterpenoids α- and β-eudesmol which together constituted 25% of the oil.

The composition of the extract of the foregut fluid from the larvae of *A. eucalypti* which had fed on the leaves of *E. cinerea* was dominated by α- and β-eudesmol which together comprised 63% of the total extract. A similar figure was obtained if cineole and monoterpene hydrocarbons were excluded from the composition of the leaf oil.

It is clear, therefore, that the solubility of the oil components in the foregut fluid, and the proportion in which they occurred in the leaf oil, were

the sole factors which determined their presence in the foregut. These two factors together with the very small amount of ether-soluble material in the foregut fluid indicated that, unlike the sawflies, the larvae of *A. eucalypti* did not use components of the leaf oils for defense. It is possible that the oral discharge from these larvae was merely a reflex reaction upon attack, similar to vomiting which results from trauma in higher animals.

In the introduction, mention was made of the observation that larvae of *A. eucalypti* also feed on the leaves of *Schinus molle*. The leaves of this tree, commonly called the Californian pepper tree, possess an essential oil which has a strong pungent aroma. The composition of this essential oil was recently determined (Bernhard et al., 1983), and the oil was found to contain a range of mono- and sesquiterpene hydrocarbons similar to those found in *Eucalyptus* oils but lacked the monoterpenoid alcohols frequently found in the latter.

It was suggested above that some of the components of a *Eucalyptus* leaf oil may act as feeding stimulants to the larvae of *A. eucalypti*. However, the fact that these larvae will feed on the leaves of other aromatic plants suggested that some of the components of these essential oils might possess the ecologically important role of an ovipositional attractant to the female adult moth (Harborne, 1977). The volatility of the components of *Eucalyptus* oils would enable these compounds to be readily perceived by the adult moths. Despite the absence of experimental support, such a role for the essential oil of *Eucalyptus* leaves may explain the coevolution of *Antheraea eucalypti* and the *Eucalyptus* genus.

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ANTIFEEDANT ACTIVITY OF QUASSINOIDS

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Abstract—The antifeedant activity of 13 quassinoids of different structural types has been studied against the Mexican bean beetle (*Epilachna varivestis Mulsant*) 4th instar larvae and the southern armyworm (*Spodoptera eridania Crawer*) 5th instar larvae. All quassinoids tested displayed significant activity against the Mexican bean beetle and, thus, do not reveal a simple structure-activity relationship. Five quassinoids were active against the southern armyworm. Interestingly, four of these—bruceantin (I), glaucarubinone (VI), isobruceine A (VIII), and simalikalactone D (XI)—possess the required structural features for antineoplastic activity. The noncytotoxic quassin (X) is an exception; it is active against both pests.

Key Words—Antifeedant, Mexican bean beetle, *Epilachna varivestis*, Coleoptera, Coccinellidae, southern armyworm, *Spodoptera eridania*, Lipidoptera, Noctuidae, quassinoids, Simaroubaceae.

INTRODUCTION

Quassinoids are the characteristic bitter principles of the plant family Simaroubaceae (Polonsky, 1973) which is of mostly pantropical distribution. They are degraded triterpenes, and many of them possess diverse and potentially useful biological properties. Thus, much attention continues to be focused on quassinoids because of their potent in vivo antineoplastic activity (Cassady et al., 1980), their ability to inhibit cell transformation induced by the Rous sarcoma virus (Pierré et al., 1980), and the antiamebic (Gillin and Reiner, 1982; Gillin et al., 1982) and the recently observed antimalarial properties (Trager et al., 1981). The quassinoid constituents

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such as bruceantin (I), now undergoing clinical trial by the U.S. National Cancer Institute (Kupchan et al., 1975), have been of special interest.

Since it is highly desirable to develop new methods of pest control (Meinwald, 1978), it seemed interesting to investigate the possible antifeedant effects of the quassinoids. Preliminary antifeedant test (Odjo et al., 1981) were carried out on third stage larvae of *Locusta migratoria migratorioides* R and F (Orthoptera, Acrididae). The quassinoids bruceine A (II), bruceine B (III), chaparrinone (V), glaucarubinone (VI), and simalikalactone D (XI) were found to be insect antifeedants, whereas soulameanone (XIII) (Polonsky et al., 1980), soulameolide (Polonsky et al., 1979), and simarolide (XII) were inactive.

We now report on the antifeedant activity of 13 quassinoids against two pests, *Epilachna varivestis Mulsant* (Mexican bean beetle) and *Spodoptera eridania Crawer* (southern armyworm). The former is monophagous and highly selective, whereas the latter is polyphagous and is a voracious eater.

METHODS AND MATERIALS

The quassinoids tested were isolated in the pure state from natural sources. Only those which did not appear in the review (Polonsky, 1973) have been referenced. Quassin is commercially available, and its total synthesis has been accomplished recently (Grieco et al., 1980). Each compound was dissolved in acetone at the concentrations (in ppm) indicated in Tables 1 and 2.

The feeding bioassays were carried out as follows: A mature bush bean plant, with two leaves approximately the same size, was chosen for each replicate (Figure 1). One leaf top was coated with compound, and one leaf top with solvent alone as a control. These were allowed to dry. In the initial southern armyworm tests, and in all Mexican bean beetle tests, the cut stem was placed in a vial with water and a tissue plug to support the stem. The base of the stem was coated with vaseline to prevent the insects from crawling off. If any compound merited further testing against the southern armyworms, the plant was left rooted in its pot, the leaves coated as before, and the stem covered with vaseline as before. This change in procedure was initiated because the cut stems did not survive the 24-hr test period too well; they tended to wilt and also suffered from the heavier weight of the worms. All plants were then placed inside a cage for the test period to prevent the insects from escaping. Care was taken that no plants or leaves touched, so that the original test subjects stayed on their original plants.

Five replicates were run for each test. For the Mexican bean beetles, two fourth instar larvae were placed on each leaf. For the southern

TABLE 1. ANTIFEEDANT ACTIVITY OF QUASSINOIDS AGAINST MEXICAN BEAN BEETLE

Compound	Conc. (ppm)	R	Eaten (%) C	Eaten (%) X	Result
Bruceantin (I)	500	1–5	50–80	2–5	+
	250	1	90	15	+
		2	80	10	+
		3	100	15	+
		4	50	10	+
		5	20	5	+
	100	1	2	20	−
		2	10	10	−
		3	80	20	+
		4	80	20	+
		5	33	50	−
Bruceine A (II)	500	1	100	15	+
		2	100	20	+
		3	90	2	+
		4	100	50	+
		5	100	20	+
Bruceine B (III)	500	1	95	5	+
		2	80	10	+
		3	90	10	+
		4	95	10	+
		5	95	30	+
Bruceine C (IV)	500	1	90	10	+
		2	90	15	+
		3	95	5	+
		4	75	25	+
		5	70	30	+
Chaparrinone (V)	200	1–5	66–100	5–10	+
	100	1–5	50–80	5–10	+
	50	1–5	50–100	5–20	+
	25	1–5	75–100	5–15	+
	10	1	66	25	+
		2	90	20	+
		3	90	20	+
		4	75	10	+
		5	80	10	+
Glaucarubinone (VI)	500	1	15	15	−
		2–4	10–33	0–5	+
	250	1–5	33–90	5	+
	100	1	10	10	−
		2–4	33–75	2–5	+
	50	1	33	20	±
		2	33	5	+
		3	25	5	+
		4	50	5	+
		5	50	5	+

TABLE 1. Continued

Compound	Conc. (ppm)	R	Eaten (%) C	Eaten (%) X	Result
	25	1	33	5	+
		2	20	5	+
		3	50	5	+
		4	66	5	+
		5	75	15	+
Glaucarubolone (VII)	500	1	10	5	+
		2	25	2	+
		3	10	5	+
		4	80	5	+
		5	33	5	+
Isobruceine A (VIII)	500	1	15	10	±
		2	20	50	−
		3-5	15-50	5	+
	200	1-5	15-100	3-5	+
	100	1	15	10	±
		2	5	10	−
		3-5	10-90	5	+
	50	1-5	50-80	3-15	+
	25	1-5	10-75	5-10	+
	10	1	33	5	+
		2	25	5	+
		3	33	5	+
		4	66	5	+
		5	50	5	+
Picrasin B (IX)	500	1	80	5	+
		2	80	5	+
		3	50	2	+
		4	95	10	+
		5	90	5	+
Quassin (X)	500	1-5	10-66	1-3	+
	250	1-5	50-66	1-5	+
	100	1-5	50-100	5	+
	50	1-5	33-90	2-5	+
	10	1	50	5	+
		2	75	5	+
		3	50	10	+
		4	40	5	+
		5	90	15	+
Simalikalactone D (XI)	200	1	80	5	+
		2	25	5	+
		3	90	5	+
		4	75	5	+
		5	50	5	+
Simarolide (XII)	500	1	20	5	+
		2	50	2	+

TABLE 1. Continued

Compound	Conc. (ppm)	R	Eaten (%) C	Eaten (%) X	Result
		3	50	5	+
		4	50	5	+
		5	50	5	+
Soulameanone (XIII)	500	1	25	25	−
		2	90	66	±
		3	66	66	−
		4	90	80	±
		5	33	25	±

TABLE 2. ACTIVITY OF QUASSINOIDS AGAINST SOUTHERN ARMYWORM

Compound	Conc. (ppm)	R	Eaten (%) C	Eaten (%) X	Result
Bruceantin (I)	500	1	20	15	±
		2	25	15	±
		3	33	15	+
		4	25	15	±
		5	33	15	+
	250	1	25	75	−
		2	50	15	+
		3	20	33	−
		4	20	20	−
		5	66	50	±
Bruceine A (II)	500	1	65	20	+
		2	45	30	±
		3	10	15	−
		4	50	25	+
		5	45	30	±
Bruceine B (III)	500	1	30	50	−
		2	65	45	±
		3	30	75	−
		4	35	45	−
		5	15	40	−
Bruceine C (IV)	500	1	50	50	−
		2	45	70	−
		3	20	10	+
		4	25	45	−
		5	95	99	−
Chaparrinone (V)	500	1	25	15	±
		2	25	10	+
		3	15	15	−

TABLE 2. Continued

Compound	Conc. (ppm)	R	Eaten (%) C	Eaten (%) X	Result
		4	1	10	−
		5	10	3	+
	200	1	25	5	+
		2	15	25	−
		3	33	15	+
		4	25	25	−
		5	10	5	+
Glaucarubinone (VII)	500	1	50	5	+
		2	66	25	+
		3	25	10	+
		4	15	15	−
		5	50	15	+
	250	1	50	33	±
		2	15	15	−
		3	50	15	+
		4	25	25	−
		5	15	10	±
	200	1	10	15	−
		2	15	66	−
		3	10	50	−
		4	10	15	−
		5	10	33	−
Glaucarubolone (VII)	500	1	?	?	?
		2	80	20	+
		3	50	50	−
		4	75	25	+
		5	65	35	±
Isobruceine A (VIII)	500	1	25	10	+
		2	15	5	+
		3	15	5	+
		4	15	5	+
		5	20	10	+
	250	1	5	2	+
		2	15	10	±
		3	10	5	+
		4	15	10	±
		5	15	10	±
	100	1	15	25	−
		2	50	5	+
		3	15	5	+
		4	15	5	+
		5	10	5	+
	50	1	5	5	−
		2	10	10	−
		3	5	5	−

TABLE 2. Continued

Compound	Conc. (ppm)	R	Eaten (%) C	Eaten (%) X	Result
		4	10	10	−
		5	10	5	+
Picrasin B (IX)	500	1	66	50	±
		2	50	20	+
		3	10	20	−
		4	66	?	?
		5	10	10	−
Quassin (X)	500	1	10	5	+
		2	15	2	+
		3	5	10	−
		4	15	5	+
		5	15	5	+
	250	1	15	5	+
		2	20	10	+
		3	15	15	−
		4	50	20	+
		5	10	20	−
	100	1	15	15	−
		2	33	50	−
		3	10	20	−
		4	25	15	±
		5	15	20	−
Simalikalactone D (XI)	200	1	25	0	+
		2	66	2	+
		3	25	2	+
		4	33	2	+
		5	25	2	+
Simarolide (XII)	500	1	All stems		−
		2	chewed		−
		3	throughout		−
		4	but leaves		−
		5	evenly eaten		−
Soulameanone (XIII)	500	1	?	?	?
		2	20	10	+
		3	evenly eaten		−
		4	10	10	−
		5	25	25	−

armyworms, two newly emerged fifth instar larvae were placed on each leaf. The worms were starved for 1–2 hr prior to placements on the leaves, since they do not eat continuously like the beetles. After 24 hr, the percentages eaten from each leaf were estimated visually. Occassionally the leaves that the worms had eaten had to be taped to graph paper and the outline

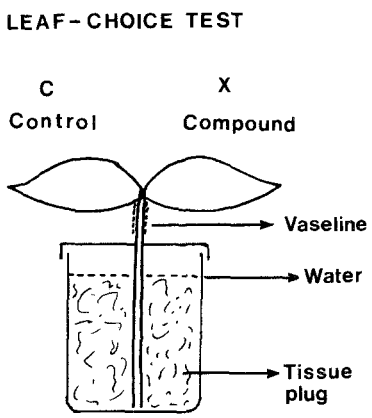


FIG. 1. Feeding bioassay apparatus.

reconstructed, since they ate into the leaf from the edges. Sometimes they ate through the stem, and there was no way to determine which had been the test leaf and which the control. In these cases one could only estimate if both leaves were equally eaten or not. Sometimes the leaves wilted too much to make any kind of determination at all.

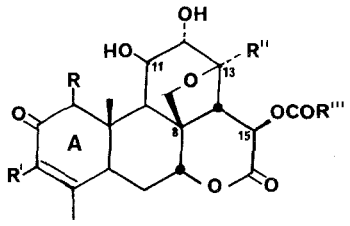
If a compound showed antifeedant activity, it was retested at a lower concentration until there was no more activity. However, some compounds that merited further testing were in limited supply and could not be tested any further.

RESULTS AND DISCUSSION

The results of the antifeedant activity of quassinoids against the Mexican bean beetle and the southern armyworm are presented in Tables 1 and 2, respectively. The quassinoids are listed in alphabetical order, and their formulae are depicted in Schemes 1 and 2. All concentrations are in parts per million (ppm). R is the number of the replicate for each concentration. In the "eaten" column, C and X refer to the percentage of leaf eaten in the control and test experiments, respectively. The results were evaluated as follows: $-$, $X \geq C$; $\pm$, $X < C$ (C not twice as large); and $+$, $X < C$ (C at least twice as large).

In Table 2, a question mark indicates that either the pests ate through the stem or the plant was too wilted to obtain a result.

The structural requirements for the in vivo antileukemic activity of the quassinoids are well established (Kupchan et al., 1976; Wall et al., 1978), viz.: (1) a ring A oxygenated functionality as in (I) to (VIII), (2) an ester group at C-15; (3) an epoxymethano bridge between C-8 and C-11



BRUCEANTIN I R=H; R'=OH; R''=$COOCH_3$; R'''= $CH{=}C(CH_3){-}CH(CH_3)_2$

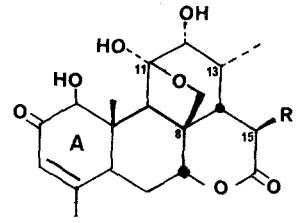
BRUCEINE A II R=H; R'=OH; R''=$COOCH_3$; R'''= $CH_2CH(CH_3)_2$

BRUCEINE B III R=H; R'=OH; R''=$COOCH_3$; R'''= CH_3

BRUCEINE C IV R=H; R'=OH; R''=$COOCH_3$; R'''= $CH{=}C(CH_3){-}C(CH_3)(OH){-}CH_3$

ISO-BRUCEINE A VIII R=OH; R'=H; R''=$COOCH_3$; R'''= $CH_2{-}CH(CH_3)_2$

SIMALIKALACTONE D XI R=OH; R'=H; R''=CH_3; R'''= $CH(CH_3){-}C_2H_5$



CHAPARRINONE V R = H

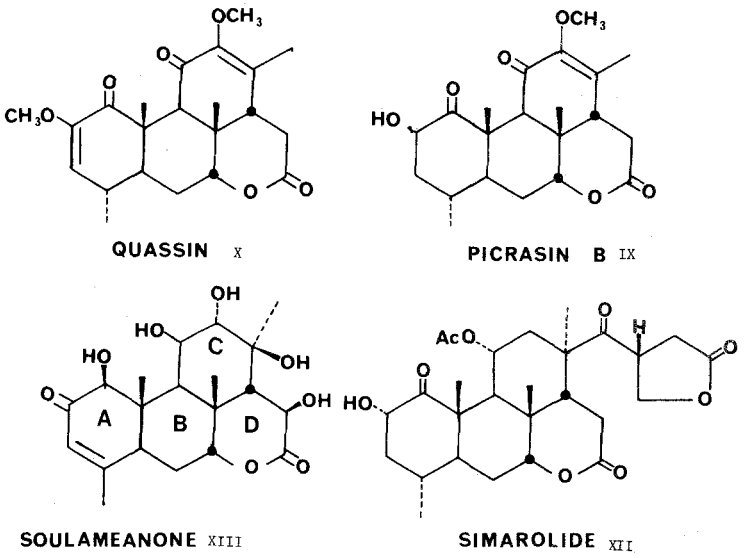
GLAUCARUBINONE VI R = $O{-}C(=O){-}C(OH)(CH_3){-}C_2H_5$

GLAUCARUBOLONE VII R = OH

SCHEME 1.

[quassinoids (V), (VI), (VII)] or between C-8 and C-13 [quassinoids (I), (II), (III), (IV), (VIII), (XI)]. It can also be noted that their antimalarial (Trager et al., 1981) and antiviral (Pierre et al., 1980) activities run roughly parallel to their antineoplastic activity (Cassady et al., 1980). Thus, bruceantin (I), simalikalactone D (XI), and glaucarubinone (VI) seem to be the most active compounds, whereas, for instance, soulameanone (XIII) and simarolide (XII) which have no bridge in ring C are inactive.

In the present invetigation some quassinoids possessing and some



SCHEME 2.

lacking these structural features have been examined. Table 1 shows that all the quassinoids tested at concentrations ranging from 500 to 10 ppm were active against the Mexican bean beetle. Thus, there does not appear to be a simple structure–antifeedant activity relationship among the 13 quassinoids tested. The characteristic bitter taste of quassinoids may perhaps be responsible for the observed activities.

Some species of *Spodoptera* are distributed throughout the world and constitute a major agricultural threat. The antifeedant bioassays against the southern armyworm, *Spodoptera eridania* (Table 2) show that the quassinoids were more active in lower concentrations against the Mexican bean beetles than against the southern armyworm. Simalikalactone D (XI) showed marked activity at 200 ppm (initial work by James Casanova indicated that it was active even at the level of 50 ppm). Bruceantin (I), isobruceine A (VIII) (Polonsky et al., 1975), and glaucarubinone (VI) also showed significant antifeedant activity. It is of interest to note that these quassinoids possess the structural requirements for antineoplastic activity. Chaparrinone (V), which lacks the ester chain at C-15, showed moderate activity against the southern armyworm.

Interestingly the noncytotoxic quassin (X) displays antifeedant properties against the two pests studied. In this connection it can be noted that quassia extractives (which contain quassin) exhibit insecticidal activity against sawflies and aphids (Crosby, 1971) (both contact and stomach poisoning was observed).

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RESPONSES BY KING SNAKES (*Lampropeltis getulus*) TO CHEMICALS FROM COLUBRID AND CROTALINE SNAKES

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Abstract—Four litters of king snakes (*Lampropeltis getulus*), a snake-eating species, were tested for responses to chemicals from colubrid and crotaline snakes. King snakes presented with swabs rubbed against the dorsal skin of living snakes and with swabs treated with methylene chloride extracts of shed snake skins tongue-flicked more to swabs from a northern copperhead (*Agkistrodon contortrix*), a crotaline, than to swabs from some colubrid snakes or to blank swabs. Six out of 10 king snakes in one litter attacked and attempted to ingest swabs treated with snake skin chemicals, implicating these chemicals as feeding stimuli for these ophiophagous snakes. Ingestively naive king snakes presented with plain air and snake odors in an olfactometer tongue-flicked more to snake odors. This study and others suggest that crotaline and colubrid snakes can be distinguished by chemical cues.

Key Words—Colubridae, Crotalinae, snake skin chemicals, king snakes, chemical aposematism.

INTRODUCTION

King snakes (*Lampropeltis getulus*) consume a diversity of vertebrates—mammals, amphibians, birds, lizards, and snakes (Clark, 1949; Hamilton and Pollack, 1956). Numerous crotaline (Crotalinae) and some colubrid (Colubridae) snakes respond to king snakes with fleeing, body posturing, biting, or other defensive behaviors (Bogert, 1941; Chiszar et al., 1978; Marchisin, 1980;

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Weldon, 1982; Weldon and Burghardt, 1979). Chemicals from king snakes elicit these responses. The involvement of chemicals perceived by king snakes from prey snakes or, indeed, from any of the many vertebrates on which king snakes feed is less clear.

Brock and Myers (1979) tested a litter of 11 ingestively naive *L. getulus* for responses to chemicals from 19 stimulus species. Extracts on cotton swabs from an annelid, arthropods, fishes, amphibians, a mammal, several lizards, and the banded water snake (*Nerodia fasciata*) were presented to king snakes, and tongue-flicks (a presumed measure of a snakes' arousal to chemical stimuli) were counted. Brock and Meyers observed no significant differences in tongue-flick rates by *Lampropeltis* to these substances (see also Burghardt, 1970, p. 288), and they concluded that cues other than chemicals are important in the recognition of king snake prey.

Williams and Brisbin (1978) tested 13 adult *L. getulus* to extract swabs from three potential prey species—mouse, chicken, and rat snake (*Elaphe* sp.)— in addition to swabs treated with human hand rinse or physiological saline solutions. These king snakes has been fed only mice for 2–10 years, yet they recognized all prey odors, tongue-flicking more to these stimuli. Although kingsnakes tongue-flicked more to mouse odors, their responses to the mouse, the chicken, and the snake were not significantly different. Thus, whether king snakes discriminate among chemicals from these classes of vertebrate prey is uncertain.

Observations of interactions between *Lampropeltis* and some crotaline snakes have led some investigators to suspect that king snakes avoid these venomous serpents and that crotaline chemicals elicit this response (Cowles, 1938). Bogert (1941), who established that rattlesnakes (*Crotalus* spp.) respond defensively to king snake skin chemicals, also noted king snakes' attempts to escape from rattlesnakes. Other authors have remarked on king snakes' fear of rattlesnakes (Cowles, 1938) or other crotalines, such as copperheads (*Agkistrodon contortrix*) (Meade, 1940) and cottonmouth moccasins (*A. piscivorus*) (Neill, 1947). Neill (1947, p. 205) wrote: "*Lampropeltis getulus getulus* is loath to attack a cottonmouth, under captive conditions. A tame hungry king snake, placed in a cage with a moccasin two-thirds its length, usually becomes frightened, rooting about the cage in a desperate effort to escape." Our laboratory observations of the occasional display by king snakes (*Lampropeltis getulus holbrooki* and *Lampropeltis g. niger*) of sudden withdrawal and flight from northern copperheads (*Agkristrodon contortrix mokasen*) are in agreement with these reports. It is the purpose of this study to examine whether king snakes (1) perceive chemicals from other snake species and (2) discriminate between chemicals from some crotaline and nonvenomous colubrid snakes.

METHODS AND MATERIALS

Swab Presentations. In experiment I, eight black king snakes, *Lampropeltis getulus niger* [snout-vent length (SVL) = 18.0–23.5 cm $\bar{X}$ = 22.9 cm]. hatched from eggs laid by a female captured in Knox County, Tennessee, were tested at three months of age. Snakes were individually housed in 31 × 16 × 8-cm clear plastic boxes with lids. They had free access to water and had been fed a small live mouse every 7–9 days. One snake had occasionally refused food, but all snakes fed at least four days before testing began. None of the snakes was exposed to stimuli from other snake species prior to testing. The temperature of the testing and housing room was 22–24° C.

Snakes were tested once to each of four stimuli (three snakes and a control) in a Latin-square design. Each snake was tested once each day for four consecutive days. Tests were run between 1400 and 1600 hr.

Skin chemicals were collected on cotton swabs from adult females of three snake species sympatric with the king snakes: eastern garter snake, *Thamnophis sirtalis sirtalis* (Colubridae); speckled king snake, *Lampropeltis getulus holbrooki* (Colubridae); and northern copperhead, *Agkistrodon contortrix mokasen* (Crotalinae). A swab was dipped into methylene chloride (CH_2Cl_2) and rubbed against the dorsal surface of a snake while the snake's head and vent were held. Control (blank) swabs were dipped into CH_2Cl_2 without further treatment. Swabs were air-dried at least 7 min before being presented to the snakes. Similar methods of obtaining snake-scented stimuli have been used in other tests of snakes' responses to chemicals (Bogert, 1941; Marchisin, 1980; Weldon, 1982; Weldon and Burghardt, 1979).

Subjects were tested in their home cages after removal of water dishes and paper towel shelters. A sheet of paper was taped around a snake's cage to extend the cage walls vertically and prevent snakes from crawling upwards during testing. Swabs were held in front of a snake's snout (about 2 cm away) for 2 min while all tongue-flicks were hand-counted. No attempt was made to touch the snakes with the swabs, but occasionally a snake would itself touch a swab or the wooden handle of it.

In experiment II, seven speckled king snakes, *Lampropeltis g. holbrooki* (SVL = 21.5–23.0 cm; $\bar{X}$ = 22.1 cm), hatched from eggs laid by a female captured in Shelby County, Tennessee, were tested at three months of age. They were maintained and tested as described for snakes in the first experiment.

Adult females of three species were stimulus snakes: black rat snake, *Elaphe obsoleta obsoleta* (Colubridae); speckled king snake, *Lampropeltis g. holbrooki* (Colubridae); and northern copperhead, *Agkistrodon contortrix mokasen* (Crotalinae). All are sympatric with the king snakes tested.

Shed skins of stimulus snakes were collected within two days of ecdysis. The skins were weighed, wrapped in aluminum foil, and stored in a freezer at −1°C. Within several months, skins were extracted in a Soxhlet apparatus with CH_2Cl_2 for two days each. A viscous yellow residue of skin extract remained after CH_2Cl_2 was removed at reduced pressure (water aspiration). Extractions of the skins of *Elaphe* (3.10 g), *Lampropeltis* (1.42 g), and *Agkistrodon* (3.25 g) yielded 4.2%, 16.2%, and 5.0%, respectively, of the skins' weights in residue.

Solutions of 50 mg skin extract/ml of CH_2Cl_2 were prepared for each species' extract. Cotton swabs were dipped into the solutions and air-dried for 7–10 min. Swabs from each stimulus snake were then wrapped in aluminum foil and stored (−1°C) for several days before being presented to the king snakes. Control swabs were dipped into CH_2Cl_2 and stored in an identical fashion.

In experiment III, ten black king snakes, *Lampropeltis g. niger* (SVL = 19.5–23.5 cm; $\overline{X}$ = 22.7 cm), hatched from a clutch of eggs collected in Knox County, Tennessee, were tested at four months of age. They were fed a small mouse every 5–8 days, and each snake ate one mouse five days before testing began.

A female Central American black and yellow rat snake, *Spilotes pullatus* (Colubridae); a female garter snake, *Thamnophis fulvus* (Colubridae); and a prairie rattlesnake, *Crotalus viridis viridis* (Crotalinae) (sex unknown) were the stimulus snakes. None of these species is sympatric with the king snakes tested. The shed skins of the stimulus snakes were stored and extracted as described for the preceding experiment. Extractions of *Thamnophis* (1.33 g), *Spilotes* (3.16 g), and *Crotalus* (11.50 g) skins yielded 6.2% 5.5%, and 8.9%, respectively, of the skins' weights in residue. Solutions of snake skin extracts were prepared as described above.

In experiment IV, five king snakes from those tested in experiment III were tested four months later to swabs treated with snake skin chemical solutions of *Thamnophis fulvus*; a female banded water snake, *Nerodia fasciata* (Colubridae); and a female timber rattlesnake, *Crotalus horridus horridus* (Crotalinae); only the latter two species are sympatric with the king snakes tested. Solutions of *T. fulvus* skin chemicals were prepared from what remained after the first series of swab preparations. The *Crotalus* (2.56 g) and *Nerodia* (5.50 g) skins yielded 5.1% and 2.4% of their weights on extraction, respectively.

Olfactometer Experiment. The purpose of this experiment was to test king snakes' responses to airborne snake chemicals.

Seven ingestively naive *Lampropeltis g. holbrooki* (SVL = 19.0–24.0 cm; $\overline{X}$ = 21.0 cm), hatched from eggs laid by a female captured in Shelby County,

Tennessee, were tested three weeks after hatching. Other than the postponement of feeding until after testing, snakes were maintained as were those described in the previous experiments.

Snakes were tested once to each of four conditions (three snakes and a control) for four consecutive days in a Latin-square design. The stimulus snakes were a female northern copperhead, *Agkistrodon contortrix mokasen* (SVL = 79 cm); a female speckled king snake, *Lampropeltis g. holbrooki* (SVL = 106 cm); and a male plains garter snake, *Thamnophis radix* (SVL = 59 cm). Tests were run between 1200 and 1700 hr.

The apparatus and methods of odor presentation were similar to those described by Weldon (1982) for tests of *Thamnophis sirtalis* to snake odors. Air flow was generated by a Manostat varistaltic pump. Air passed through Teflon and Tygon tubing to a charcoal air filter and air flowmeter. A tube from the flowmeter connected to a glass bifurcation and stopcock, which directed air through either of two arms leading to two 1.9 liter jars. One arm directed air to a control jar containing cotton soaked in distilled water. The other arm connected to one of three jars containing stimulus snakes. From the control jar and each stimulus snake jar, an air outflow tube directed the airstream into a 14 × 10 × 6-cm plastic container placed inside a soundproof observation chamber (International Acoustics Co.). A test snake was placed into a plastic container, which was fitted with a glass top for overhead viewing of snakes in the chamber. Two tubes from the olfactometer were inserted into holes in the upper rim of the plastic container. One tube delivered air from the control jar; the other conducted air that had passed through one of the stimulus snake jars. Different plastic containers were used for each stimulus condition. Each container was washed with soap and water and dried after each test.

The airstream in the olfactometer was directed from the control jar to a jar containing a stimulus snake by turning the stopcock in the glass bifurcation. During control sessions, where only plain air was presented throughout the trials, the airstream was interrupted momentarily by turning the stopcock to a disconnected arm of a glass bifurcation; this corresponded to a momentary disruption of airflow during tests with stimulus snakes where air was directed from the control jar to a jar containing a stimulus snake. Air was delivered at 400 ml/min.

Test snakes were exposed to plain air for nine minutes during an acclimation period. After 9 min of plain air presentation, baseline data on tongue-flicking and accumlated activity time (scored whenever a snake's head or body moved) were recorded on an Esterline-Angus event recorder for one more minute. After 10 min of plain air presentation, a turn of the stopcock directed air through a jar containing a stimulus snake or (if a control session) a

continuation of plain air. Tongue-flicks and activity were recorded for 5 min after the stopcock was switched. Temperature in the observation chamber was 24° C.

RESULTS

A two-tailed Kruskal-Wallis test on data from experiment I (Table 1) detected overall significant differences in tongue-flick rates ($H = 54$; $P << 0.05$). An STP a posteriori test (Sokal and Rohlf, 1981) detected significantly higher tongue-flick rates to *Agkistrodon* odors than to *Lampropeltis* odors or plain swabs ($P < 0.05$, in both cases). No other significant differences among treatment groups were detected.

A two-tailed Kruskal-Wallis test on the results of experiment II (Table 2) detected overall significant differences in tongue-flicking ($H = 975.5$; $P << 0.05$). An STP test detected significantly greater tongue-flicking by king snakes to the *Agkistrodon* vs. the *Elaphe*, *Lampropeltis*, and control conditions ($P < 0.05$, in both cases). No other significant differences among treatment groups were detected.

It was surprising to find in experiment III that six out of 10 snakes struck at and attempted to ingest swabs laden with snake skin chemicals. In some cases snakes wrapped themselves around the swab handle while grasping the cotton swab tip in the mouth (Figure 1). In the first test with this litter, four attacks were directed at *Thamnophis* swabs, three attacks at *Spilotes* swabs, and one snake, who attacked both of these, once attacked a *Crotalus* swab. No attacks on control swabs occurred.

When attacks on swabs occurred, tongue-flicks could not be counted and trials were terminated. Snakes were placed under running water to induce them to release their grasp on swabs. Snakes were returned to their home cages and tested to another stimulus condition the following day.

During experiment IV, two attacks on *Thamnophis* swabs, and one attack each on the *Nerodia* and *Crotalus* swabs, occurred. Only two of five snakes tested attacked swabs; both individuals had attacked swabs during the previous test. Again, no attacks on control swabs occurred.

TABLE 1. MEAN TONGUE-FLICKS PER MINUTE BY EIGHT *Lampropeltis getulus* FOR 2 MINUTES TO BLANK SWABS AND SWABS FRESHLY RUBBED AGAINST DORSAL SKINS OF STIMULUS SNAKES (±1 SD)

Agkistrodon contortrix	*Lampropeltis getulus*	*Thamnophis sirtalis*	Blank
38.1 ± 16.8	22.2 ± 10.9	29.8 ± 2.6	19.7 ± 3.1

TABLE 2. MEAN TONGUE-FLICKS PER MINUTE BY SEVEN *Lampropeltis getulus* FOR 2 MINUTES TO BLANK SWABS AND SWABS DIPPED IN SOLUTIONS OF SNAKE SKIN CHEMICALS EXTRACTED WITH METHYLENE CHLORIDE (±1 SD)

Agkistrodon contortrix	*Lampropeltis getulus*	*Elaphe obsoleta*	Blank
31.9 ± 13.1	22.3 ± 10.7	23.3 ± 10.8	22.1 ± 11.5

A Kruskal-Wallis test on tongue-flicking data for the first minute of experimental air presentation in the olfactometer experiment fell short of detecting significant overall differences among treatment groups (Figure 2) ($H = 10.4$; $0.20 > P > 0.10$). An STP test detected significantly greater tongue-flicking during this period to each of the stimulus snakes than to plain air, and more tongue-flicking to *Lampropeltis* odors than those from *Thamnophis* ($P \ll 0.05$ for all comparisons).

King snakes were active 93%, 94%, 86%, and 97% of the minute before the stopcock was switched for the *Thamnophis*, *Agkistrodon*, *Lampropeltis*, and control conditions, respectively. Snakes were active 91%, 80%, 95%, and

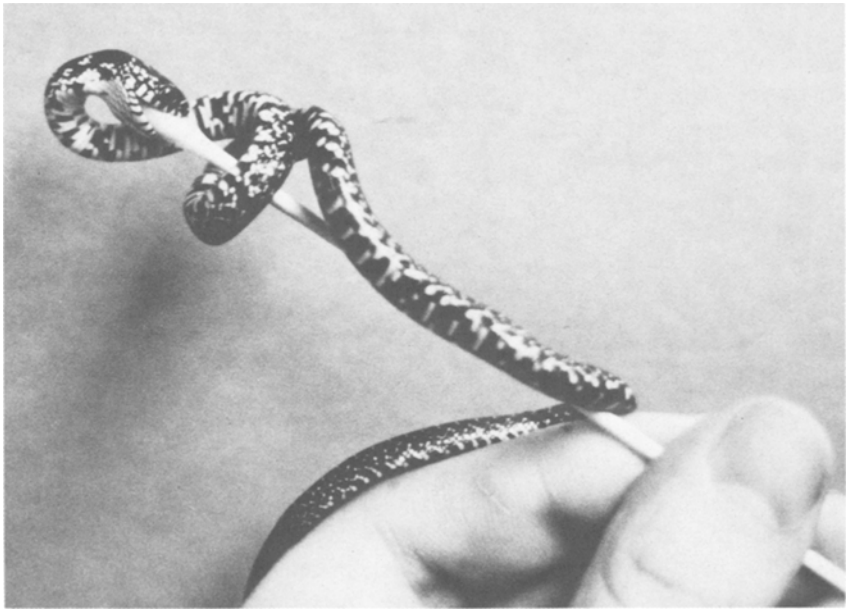

FIG. 1. *Lampropeltis getulus* (23 cm snout-vent length) ingesting a swab dipped into solution of *Thamnophis fulvus* shed skin extract.

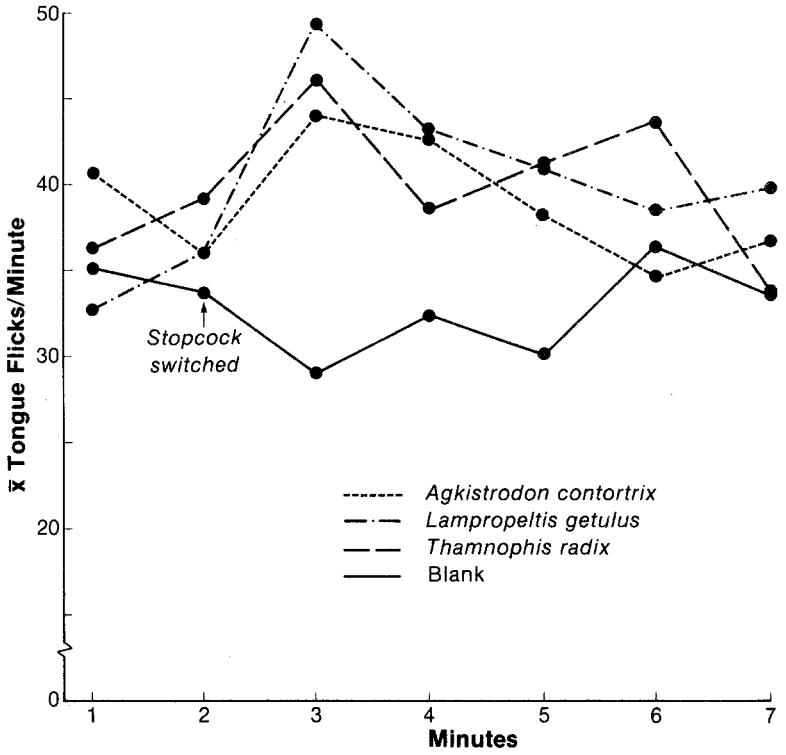

FIG. 2. Tongue-flicking of seven ingestively naive *Lampropeltis getulus* to plain (blank) air and airborne odors of stimulus snakes. Scoring began after 9 min of plain air presentation.

86% of the minute after the stopcock was switched for each of these conditions. No significant differences among these scores (sec/min) were detected. Weldon (1982) also found the accumulated activity time of *Thamnophis sirtalis* presented with snake odors to be less sensitive an indicator of responsiveness than tongue-flicking.

DISCUSSION

These results and those of Williams and Brisbin (1978) indicate that *Lampropeltis getulus* chemically perceive snakes. Further, tests with one king snake litter here indicate that snake skin chemicals elicit predatory attacks. Brock and Meyers (1979) reported that three of 11 king snakes struck at swabs in nine of 220 trials, and one snake seized a control swab and a skink (*Leiolopisma laterale*) -scented swab in its mouth. However, swab attacks by

their king snakes were accompanied by tail vibrations, and they were interpreted to be defensive rather than prey oriented. Since in our study (1) tail vibrations were not observed prior to most attacks, (2) ingestion was attempted after swab tips were grasped, and (3) no attacks on control swabs were observed, we believe that these attacks were prey oriented.

The different methods by which snake chemical samples were obtained by Brock and Meyers, where swabs were dipped into hot water in which snakes had been immersed, and those used here, may account for the discrepant results. Since snake skin chemicals are predominantly lipoidal (Ahern and Downing, 1974; Roberts and Helmkamp, 1982; Roberts and Lillywhite, 1983; Schell and Weldon, in preparation) their water solubility will be low. Our methods undoubtedly provided more concentrated chemical samples.

Individual, interlitter, or subspecific variation in responses to snake chemicals may account for different results in tests with king snakes. These possibilities are underscored by the failure of king snakes from all but one litter tested here to attack swabs, and not all snakes from this litter attacked swabs. In any case, Soxhlet extraction of shed snake skins appears suitable to obtain behaviorally active snake skin chemicals. Ahern and Downing (1974) obtained up to 8% in weight of chloroform–methanol extractable lipids from Florida indigo snake (*Drymarchon corais*) shed skins, and Roberts and Lillywhite (1983) removed 2–12.5% of shed skin weights in lipids from several snake species using different solvent systems. These yields are comparable to those obtained here. By collecting snake shed skin chemicals, samples of uniform concentration may be presented in other tests of the behavioral significance of these substances. Graves and Duvall (1983) recently reported that prairie rattlesnakes (*Crotalus viridis viridis*) respond by mouth gaping to conspecific shed skin chemicals obtained by CH_2Cl_2 extraction, although they did not report extraction yields or the concentration(s) of skin chemical solutions presented to snakes.

Observations of tongue-flicking in the olfactometer confirmed that king snakes perceive airborne snake chemicals. Similar olfactometric tests of a litter of 14 three-week-old, ingestively naive black racers (*Coluber constrictor*), another ophiophagous species, to the same stimuli presented to king snakes gave similar results (Weldon, unpublished). Other snakes respond by tongue-flicking or other behaviors to airborne chemicals from prey (Burghardt, 1977; Burghardt and Abesheenan, 1971; Dunbar, 1979; Halpern and Kubie, 1983) or from other snakes. Cowles and Phelan (1958) observed increased heart rates in rattlesnakes presented with king snake odors in an olfactometer. Weldon (1982) found that garter snakes (*Thamnophis sirtalis*) tongue-flick more to airborne odors from a king snake (*Lampropeltis getulus*) than to those from a rat snake (*Elaphe obsoleta*) or to plain air. The present

results add to the growing list of airborne stimuli known to be perceived by snakes. It is unclear, however, whether king snakes discriminate among snake taxa when presented airborne chemicals.

The results of swab presentations where swabs were not seized (and where tongue-flicks could be counted throughout an entire trial) also indicate that *Lampropeltis* perceives snake skin chemicals. Because king snakes tongue-flicked more to copperhead odors than to other stimuli, we suggest that *Lampropeltis* distinguishes between chemicals from copperheads and some colubrid snakes. The significance of such a discrimination is open to speculation.

Cowles (1938) believed that a fear of rattlesnakes permits king snakes to avoid defensive body blows when attacked. Rattlesnakes and other crotalines also bite king snakes. Although king snakes generally are resistant to crotaline venom (Bonnett and Guttman, 1971, and references therein) occasional deaths and severe wounding from crotaline bites have been reported (Allyn, 1937; Marchisin, 1980). By recognizing crotalines, then, king snakes could avoid body blows or (more likely, we believe) envenomating bites. Our experimental results are ambiguous with respect to crotaline avoidance by king snakes since we did not measure appropriate behaviors.

The idea that crotaline snakes are detected by chemical cues, or that crotaline and colubrid snakes are chemically distinguishable, is suggested from reports of other vertebrates' reactions to snakes. Hennessey and Owings (1979) found that California ground squirrels (*Spermophilus beecheyi douglasi*) respond more defensively by sand kicking and other behaviors to northern Pacific rattlesnakes (*Crotalus viridis oreganus*; Crotalinae) in perforated plastic bags than to those in sealed bags or to Pacific gopher snakes (*Pituophis melanoleucus catenifer*; Colubridae) in sealed or perforated bags. These authors believe that squirrels distinguish between chemicals from these snakes. Anecdotal accounts suggest that American alligators (*Alligator mississippiensis*), occasional snake predators, also distinguish between crotaline and colubrid snakes. McIlhenny (1935, pp. 44–45) states that alligators vigorously shake cottonmouth moccasins (*Agkistrodon piscivorus*) after grasping them, making it difficult for snakes to bite. Nonvenomous colubrid snakes, on the other hand, are grasped and devoured without shaking (see also Neill, 1971, p. 240). Freshly killed snake carcasses without heads and skins were treated as were the living snakes, leading McIlhenny (cited in Klauber, 1972, p. 1110) to suspect that crotaline chemicals elicit head-shaking attacks. These observations call for more rigorous tests of the involvement of chemical cues.

Chemical aposematism may be widespread, but few cases of vertebrate chemicals acting as aposematic signals, and few examples of vertebrate mimicry where aposematic chemicals are involved, are known (Eisner and

Grant, 1981; see also Czaplicki et al., 1975). Rubinoff and Kropach (1970) believe that the venomous yellow bellied sea snake, *Pelamis platurus* (Elapidae), is chemically aposematic because snake pieces, even with skins removed, were refused by several predatory Pacific fishes. We suggest that crotaline snakes are chemically aposematic if, as indicated by some reports, crotaline chemicals cause potential predators to adopt special prey-handling techniques or abstain from attacking. Further observations of interactions between crotalines and their predators and prey, stimulus control studies, and elucidations of snake skin chemicals are needed.

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INTERFERENCE OF SONIC COMMUNICATION AND MATING IN LEAFHOPPER *Amrasca devastans* (DISTANT)[1] BY CERTAIN VOLATILES

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Abstract—Mating between the two sexes in the leafhopper *Amrasca devastans* was inhibited by cineole vapors surrounding host plant leaves bearing the insects. There was a decline in the percentage of pairs mating and mated females fertilized. The vapors were not toxic and did not prevent the insects' arrival on the leaves. The cineole vapors inhibited the mating chiefly by interfering with the surface-mediated sonic communication between the sexes. The inhibition of mating was chemical specific since other volatiles, e.g., citral, were not as effective as cineole. Exposure to cineole vapors resulted in a decline in the sexual response of the females to the sonic signals of the male

Key Words—Volatile mating inhibitor, cineole, sonic communication inhibition, leafhopper, *Amrasca devastans,* Homoptera, Cicadellidae.

INTRODUCTION

In recent years, several reports have shown that appropriate chemicals can interfere with chemical communication in various insects and their consequent behavior (Shorey and McKelvey, 1977; Ritter, 1979; Mitchell, 1981). However, many insect species use mainly sonic communication for attracting mates. Such insects include many species of auchenorrhynchous homopterans (Ossiannilsson, 1949; Claridge and Howse, 1968; Claridge and Reynolds, 1973; Strübing, 1965; Shaw et al., 1974; Ichikawa, 1977; Traue,

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1978) in which the two sexes communicate by means of substrate-borne rather than airborne vibrations. Such communication and mating can be interfered with by certain artificially generated sonic signals as reported by us (Saxena and Kumar, 1980) for the leafhopper *Amrasca devastans* (Distant) which is a serious pest of cotton, okra, eggplant, etc. But, it is not known whether such communication and mating in these homopterans can be interfered with by the vapors of a volatile chemical even though there is no chemical communication between the sexes (Kumar, 1980). We are therefore screening various volatiles for such effects on *A. devastans* in the laboratory. Our observations on the effects of these chemicals are given in this paper.

METHODS AND MATERIALS

The nymphs of *Amrasca devastans* (Distant) were collected from fields of okra (*Abelmoschus esculentus* (Linnaeus) Moench) and reared one each in a vial (5 × 2.5 cm) containing a piece of okra fruit wrapped in a twofold stretched parafilm M_R membrane at 28 ± 1°C under a 13-hr photophase. The emerging adults also were kept singly on the same fruit under the same conditions. Upon attaining the age of 5–7 days when they show maximum mating (Kumar and Saxena, 1978), the females and males were tested for acoustic communication and sexual responses in the presence or absence of cineole vapors, as described below.

For most tests, we used, 30–50 pairs of males and females, arranged in 3–5 replicates of 10 each. However, for recording the number of sound phrases emitted by each individual, 10 pairs of males and females were used. The data on the responses of these insects in the presence or absence of cineole vapors were compared statistically using Student's *t* test (Snedecor and Cochran, 1967).

The effects of the vapors of a test chemical on the acoustic communication and sexual behavior of the leafhopper were studied in a cylindrical chamber (250 ml capacity, 7 cm high) of clear plastic. The chamber had a detachable lid of nylon net (40 mesh/cm) and a fixed nylon net bottom which rested on the top open end of another plastic chamber of the same size. Each chamber was newly constructed, washed thoroughly with the detergent teepol, and dried.

A freshly excised leaf of the host plant cotton (*Gossypium hirsutum* cv. PS-10) was trimmed to form a 5 × 3-cm rectangle with a 2.5-cm petiole which was immersed in water in a stoppered vial (2.5 cm high, 3.5 cm wide). The leaf and the vial were placed in an upper chamber. The required volume of the test chemical in 0.1 ml of liquid paraffin in a glass cup (1 cm high, 1 cm diameter) was placed in the lower chamber so that the chemical's vapors

could diffuse into the test chamber above. The chemicals and their doses, selected on the basis of preliminary trials, were: carvacrol, clove oil, cineole, citral, citronellol, farnesol, geraniol, and linalool. Each of these was tested in a dose of 0.4 ml per test chamber. However, a few found to have a marked effect were subsequently tested at a lower dose of 0.05 ml per test chamber. A control had liquid paraffin in the lower chamber without the chemical. For each test, one or five pairs of males and females were released in the upper chamber for 30 or 60 min. The substrate-borne sounds of the insects on the leaf were picked up by a gramophone cartridge, amplified, and recorded by methods described before (Saxena and Kumar, 1980, 1984). The number of sound phrases emitted by each individual as well as the percentages of insects emitting them and showing different sexual responses also were recorded as described before (Saxena and Kumar, 1984).

The effects of test chemicals were further studied by exposing the insects to their vapors around the host leaves in cylindrical chambers of glass or wire net (40 mesh/cm) having a capacity of 500 ml (10 cm high) or 1000 ml (20 cm high). The required amount of the test chemical in 0.1 ml of liquid paraffin in a glass cup (1 × 1 cm) with open top covered with wire net (40 mesh/cm) having a capacity of 500 ml (10 cm high) or 1000 ml (20 cm high). The required amount of the test chemical in 0.1 ml of liquid paraffin in a glass cup (1 × 1 cm) with open top covered with wire net (40 mesh/cm) was placed at the bottom of the jar. A 5 × 3-cm rectangle of a freshly excised cotton leaf, with its 2.5-cm petiole wrapped in wet cotton wool covered with alumunium foil, was suspended vertically on a string in the center of each chamber, with the base of the petiole almost touching the glass cup top. The vapors of the chemical were allowed 30 min to diffuse through the jar and surround the leaf.

Five pairs of unmated males and females were introduced into the jar but not directly on the leaf. During the tests, the insects sometimes visited the walls, including the top cover of the test chamber. If the cover was of a material like nylon net or muslin cloth, which transmitted the sonic signals of the insects (Kumar, 1980), they would readily mate on it. In order to examine the effects of the vapors of the test chemical surrounding a mating site, i.e., host leaf, it was important that the insects arriving on any other site did not mate so that their responses after their arrival on the leaf could be observed. Therefore, wire net which would not permit sonic communication and mating in the insects was used for the top of the chamber or the entire test chamber. The test jar was kept in the experimental room at 28–30°C under an illumination of 150–160 lux from an overhead fluorescent light (40 W). On the basis of two such tests using 10 pairs of males and females, the percentage of those which mated during successive 60-min intervals over a 4-hr observation period was recorded.

Thereafter, the females were removed from the jar and kept one each in

a separate vial (7.5 cm high, 2.5 cm diameter) containing an unripe okra fruit for feeding and egg-laying. The fruit was replenished on alternate days, and the insects were maintained at 28 ± 1°C under a 13-hr photophase until they were 15 days old, after which egg-laying sharply declines (Saxena and Saxena, 1971). Each okra fruit removed from the vials was retained for 9 days by which time all the fertile eggs laid therein would hatch. This allowed us to determine the percentage of mated females that were fertilized.

The effects of cineole vapors on the leafhopper's arrival, length of stay on the cotton leaf, and the survival of the insects were tested by methods described previously (Saxena and Basit, 1982).

RESULTS

As reported before (Saxena and Kumar, 1980, 1984), when a male and a female are present together on a leaf of their host plant cotton, the male emits its "croaking" sounds. The latter travel through the substrate to the female to render it stationary and stimulate it to emit its "cooing" sounds. These coos also travel through the substrate to the male to stimulate it to: (1) increase the number of its croaks, (2) start "dancing" movements and approach the female, (3) get "arrested" by the side of the female, (4) extend its genitalia towards those of the female, and, finally, (5) copulate. The effects of the test chemical's vapors surrounding the plant leaves on these responses of the leafhoppers were examined.

Effects of Cineole Vapors on Sexual Behavior. The results of our preliminary trials with the vapors of different chemicals on the sexual behavior of the leafhopper are given in Table 1. Since the vapors of cineole inhibited the mating to a maximum degree, this chemical was chosen for detailed study. Carvacrol and clove oil killed the insects within the observation period and thus were not tested further. Of the remaining volatiles, which inhibited mating to almost an equal degree, we took citral as their representative for testing the effect of its vapors on the sexual behavior of the leafhopper.

When single pairs of males and females were released directly on a leaf in the 250-ml control chamber for 60 min, 100% showed the above-mentioned sexual responses leading to mating, as described before (Saxena and Kumar, 1980, 1984). On surrounding the insects and the leaves with the vapors of the test chemicals, the percentage of pairs mating was significantly reduced to 43 ± 3 with citral and to 13 ± 3 with cineole. The remaining test were performed using cineole since it was much more effective than citral in inhibiting mating in the leafhopper. The percentage of individuals of each sex emitting sexually stimulating sounds was not significantly different in

TABLE 1. EFFECTS OF VAPORS OF CERTAIN VOLATILES[a] ON MATING IN LEAFHOPPER *Amrasca devastans*

Test chemical	Source of chemicals[b]	Pairs of males and females mating (Mean % ± SD)[c]
Nil		77 ± 15
Carvacrol	KK	toxic[d]
Clove oil	VMF	toxic[d]
Cineole	GS	13 ± 11.5*[e]
Citral	KK	33 ± 11.5*
Citronellol	KK	37 ± 9.0*
Farnesol	KK	40 ± 0*
Geraniol	KK	43 ± 6.0*
Linalool	KK	43 ± 6.0*
1 SD at $P = 0.05$		19.7

[a]0.4 ml of test chemical in 0.1 of liquid paraffin per test.
[b]GS: Goldensun Manufacturing Co., Bombay, India; KK: K & K Labs Division, ICN Pharmaceuticals, Inc., U.S.A.; VMR: V. Mane Fils, Grasse, France.
[c]For each test, five pairs of males and females were released in the upper compartment of 250-ml cylindrical chamber for 60 min. Two such tests comprised one replicate. Data based on three replicates of 10 pairs each.
[d]All the insects died within the observation period.
[e]Asterisks indicate that the difference in the percent pairs mated in presence of a test volatile and that in the control is significant at $P = 0.05$.

the presence of cineole vapors as in their absence (Figure 1A, B). However, the number of sound phrases emitted by each sex in the presence of the chemical declined sharply to less than one fourth that in the control (Figure 1G, H). Also, the dancing of the males, their arrest by the females, and extension of their genitalia was 23–25% less than that in the absence of cineole (Figure 1C, D, E). Finally, the percentage of pairs which mated was only about one fourth of that in the absence of the chemical (Figure 1F).

The effects of cineole were further studied by exposing the insects to its vapors around the host leaves for durations longer than 60 min in different types of chambers, releasing the insects within the latter anywhere except on the leaves. The insects would then have to arrive on the leaves in response to their visual (color), olfactory, and hygro-stimuli (Saxena and Saxena, 1974) before substrate-borne sonic communication and mating between the sexes could occur. In each type of test chamber, the percentage of pairs mating in the presence of cineole vapors increased with the duration of exposure but remained significantly less than that in the absence of the chemical (Table 2). The interference by cineole of mating in the leafhopper was maximum during the first 60 min and declined with the increase in the time interval. By

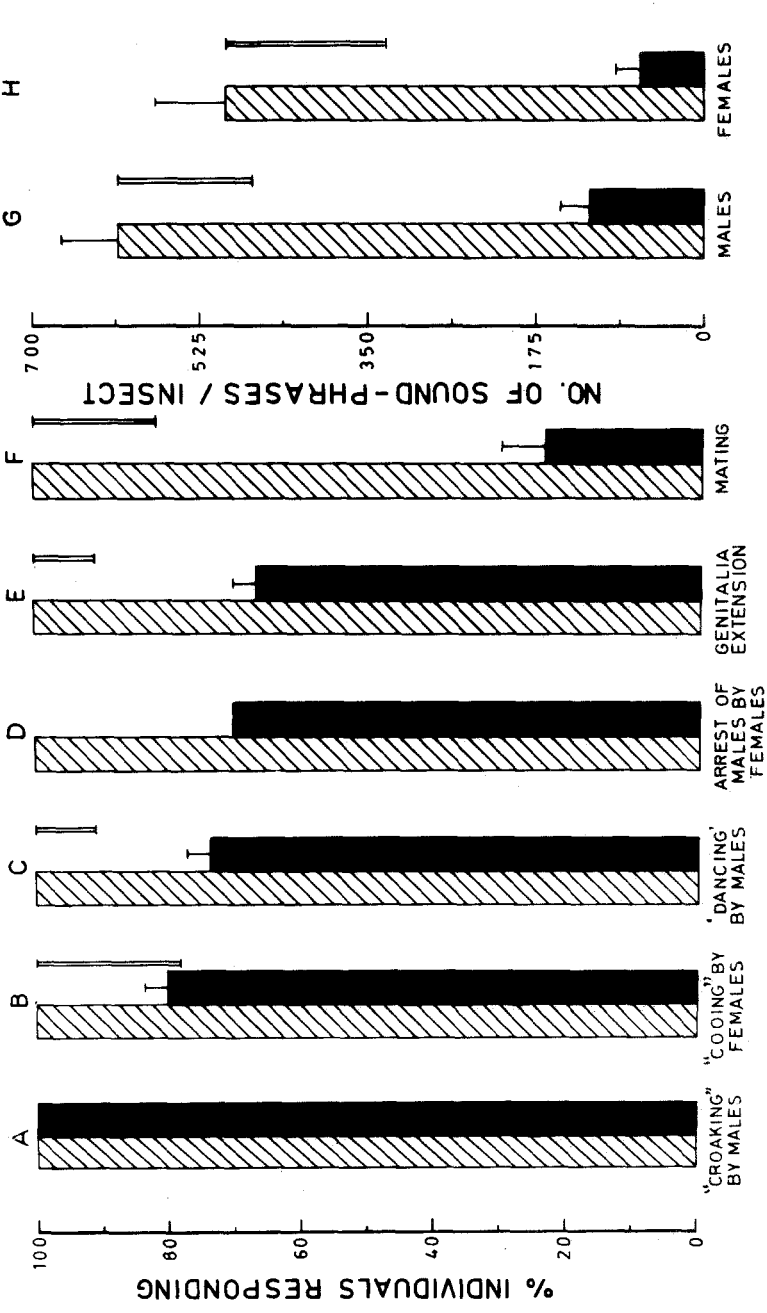

FIG. 1. *Effects of cineole vapors on acoustic communication and mating in Amrasca devastans.* For each experiment, 30 pairs of 5-day-old males and females were tested in three replicates of 10 pairs each for 30 min in the 250-ml test chamber permeated with vapors from 0.05 ml cineole in 0.1 ml liquid paraffin. Solid columns: responses in the presence of cineole; hatched columns: responses in the absence of cineole; single vertical bars: standard errors; double vertical bars: LSD at $P = 0.05$ for the two columns immediately on the left.

TABLE 2. EFFECT OF CINEOLE[a] VAPORS ON MATING IN LEAFHOPPER *Amrasca devastans*

Observation period (min)	Pairs of males and females mating (Mean % ± SD)[b]					
	Glass chamber, 500 ml		Glass chamber, 1000 ml		Wire net chamber, 1000 ml	
	Cineole present	Cineole absent	Cineole present	Cineole absent	Cineole present	Cineole absent
60	26 ± 9.0	62 ± 11.0*[c]	14 ± 9.0	50 ± 20.0*	27 ± 15.0	59 ± 14.0
120	38 ± 13.0	78 ± 15.0*	28 ± 8.5	57 ± 11.5*	39 ± 14.0	71 ± 8.0*
180	54 ± 5.5	92 ± 11.0*	42 ± 13.0	60 ± 10.0*	48 ± 18.0	79 ± 13.0*
240	64 ± 11.0	96 ± 9.0*	50 ± 12.0	70 ± 10.0*	51 ± 18.5	87 ± 9.0*

[a]0.05 ml in 0.1 ml liquid paraffin per chamber.
[b]For each experiment, 50 pairs of 5-day-old males and females in five replicates of 10 pairs each were tested in each type of chamber.
[c]Asterisk indicates significantly different at $P = 0.05$ from that in the presence of cineole.

the end of 4 hr, the percentage of pairs mating in the presence of cineole was reduced by about one third compared to the percentage mating in the absence of the chemical. Even in the wire net chamber of the same size (1000 ml), which allowed the cineole vapors to diffuse out through the side walls, the percentage of pairs mating was reduced by about two fifths as compared with that in the absence of the chemical.

Of the females which mated with the males in the presence of cineole, the percentage of those which laid fertile eggs was significantly less than of those mating in the absence of the chemical vapor (Figure 2). These results suggest that cineole vapors interfere with fertilization.

Effects of Cineole Vapors on Arrival and Length of Stay of Insects on Leaf. As propagation of the sounds of the two sexes occurred through the leaf but not through the glass or wire net wall of the test chambers, it was essential for the insects to arrive and stay on the leaf before they could communicate and mate (Saxena and Kumar, 1980, 1984). These insects have been reported to arrive and stay on the cotton leaf in response to the latter's visual, olefactory, and hygro-stimuli (Saxena and Saxena, 1974). In the presence of cineole vapors, the percentage of insects present on the leaf at the end of 1, 2, 3, and 4 hr was almost the same as in the absence of the chemical (Figure 3). Hence, the decline in mating in the presence of cineole vapors would not be due to the failure of insects to arrive/stay on the leaf and, thereby, to communicate with one another.

Effect of Cineole Vapors on Survival. The survival of insects in the presence or absence of cineole vapors was observed for 6 days after they

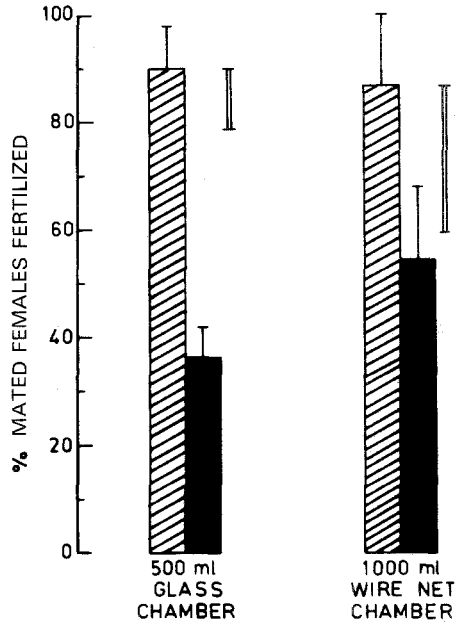

FIG.2. Effects of cineole vapors on the fertilization of mated females by males of *Amrasca devastans* For each experiment, 40 5-day-old females which mated in the presence of 0.05 ml cineole vapors in 0.1 ml liquid paraffin and 30 females mated in the absence of the chemical over a period of 240 min were arranged in replicates of 10 each to calculate the percentages of those laying fertile eggs. Hatched column = control; solid column = treatment Single and double vertical bars: as in Figure 1.

begin oviposition (Saxena and Saxena, 1971). The percentage of insects surviving in the presence of cineole vapors during this period was statistically as high (90% ± 3 SE) as in the absence of the vapors (92% ± 2 SE). Thus, these vapors did not have any acute toxicity to interfere with mating in the leafhopper.

DISCUSSION

There are very few reports on the interaction between sonic and chemical stimuli influencing distance communication between insects for their orientation, mating, or other behavioral responses. The most notable examples include certain species of bark beetles of the genus *Dendroctonus* whose aggregation behavior is inhibited by certain antiaggregative pheromones secreted by the females on stimulation by certain sonic signals from the males (Rudinsky and Michael, 1972). On the other hand, the sonic

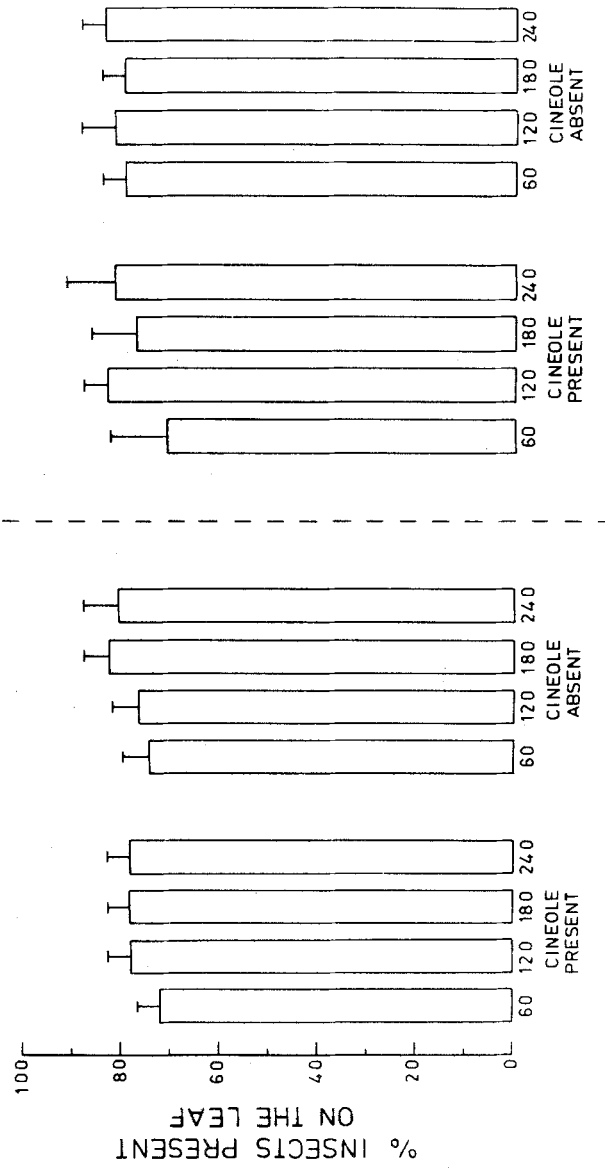

FIG. 3. Effects of cineole vapors on the arrival and stay of *Amrasca devastans* on the host leaves. Fifty pairs of 5-day-old males and females were tested in five replicates of 10 pairs each in the 500-ml glass or 1000-ml wire-net chamber in the absence or presence of 0.05 ml cineole in 0.1 ml liquid paraffin. Single vertical bars: as in Figure 1, the mean responses in the presence of cineole not significantly different at $P = 0.05$ from those in the control for any given time interval as shown by Student's t test.

signals of the males may themselves be evoked by the pheromones of both sexes, those of females eliciting "attractant" chirps and those of males "rivalry" chirps from other males (Rudinsky and Michael, 1974; Rudinsky et al., 1974). Some other important examples are those of certain species of ground crickets of the genera *Allenemobius* and *Pictonemobius* whose males emit sonic signals to attract females in response to chemical stimulation from the latter (Paul, 1976).

The above-mentioned insects are those which emit and use both sonic and chemical signals for distance communication between individuals. However, there is no information on the interaction of these two types of signals in an insect species like *A. devastans,* in which distance communication is mediated by sonic but not by chemical signals (Kumar, 1980). The present work is significant in demonstrating that vapors of a chemical, namely cineole, surrounding the two sexes of this leafhopper on plants/leaves can interfere with their acoustic communication and mating, although this interference declines with the increase in the time interval under the conditions tested. Such an interference by cineole vapors is reflected in: (1) a decline in the proportion of males and females mating, and, (2) a decline in successful matings and, hence, in the proportion of mated females fertilized to lay viable eggs.

Considering the mode of interference by cineole vapors with mating in the leafhopper, the present work shows that the numbers of coos of the female and croaks of the male are reduced in the presence of the chemical, the proportion of each sex emitting these sounds remaining unaffected. Since the mutual acoustic stimulation causes the male and female to increase the emission of their respective sounds (Saxena and Kumar, 1980, 1984), the reduction in the emission of sounds by both the sexes in the presence of the chemical suggests its interference with the acoustic stimulation of the two sexes. Also, the coos of the female stimulate the males to commence their dancing and subsequent sexual responses, leading them to the former and mating (Saxena and Kumar, 1980, 1984). In view of this, the decline in the emission of coos by the females in the presence of cineole vapors would be responsible for the fall in the sexual responses of the males and for the eventual sharp decline in mating, as observed in this work.

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Errata

NEW SEX ATTRACTANTS FOR 35 TORTRICID AND 4 OTHER LEPIDOPTEROUS SPECIES, FOUND BY SYSTEMATIC FIELD SCREENING IN THE NETHERLANDS

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Page 135: in the abstract, *Cydia strobilella* should be *Cydia pactolana.*

Page 136: 3rd paragraph, Kydonieus and Beroza should be Inscoe, 1982. Insect attractants pheromones, and related compounds, pp 201–295, *in* Kydonieus and Beroza (eds) (see references).

Page 137: last paragraph, the following blends should be added: *E*8-12:Ac + *E*8-12:OH (1 : 1), *E*9-12:Ac + *E*9-12:OH (1 : 1), and *Z*10-12:Ac + *Z*10-12:OH (1 : 1).

Page 139: in the 8th and 9th line *E*8-12:Ac should be *E*7-12:Ac, in the 10th and 14th line *E*7-12:Ac should be *E*8-12:Ac. In the 2nd paragraph the sentence . . . Two other *Pammene* species . . . should be replaced by . . . Three other *Pammene* species, *P. obsurana, P. populana,* and *P. splendidulana* were attracted most to Z8-12:Ac + Z8-12:OH (1 : 1) or pure Z8-12:OH. In the bottom line *Cydia strobilella* should be *Cydia pactolana.*

Page 140: the name *Cydia strobilella* should be replaced by *Pammene splendidulana* (Guenée). Of *Eulis ministrana* 126 males were captured in 2 traps from 5/25–6/15.

Page 141: Petrova resinella was captured with *Z*9-12:Ac + *E*9-12:Ac.

Page 143: 1st paragraph, the names *Cydia strobilella* and *Cydia pactolana* should be replaced by *Pammene populana* and *P. splendidulana.* In the 3rd paragraph + *Z*9-12:Ac should be inserted between *E*9-12:Ac and (9 : 1).

Page 144: the following reference should be inserted: Bradley, J.D., Tremewan, W.G. and Smith, A. 1973. British Tortricid Moths. Vol. I: Cochilidae and Tortricidae: Torticinae. The Ray Society, London.

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ROLE OF DIET IN HOST SELECTION OF *Heliothis virescens* BY PARASITOID *Campoletis sonorensis* (HYMENOPTERA: ICHNEUMONIDAE)

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Abstract—Gas chromatographic analysis revealed that chemicals attractive to *Campoletis sonorensis* (Cameron), previously identified in cotton, were not present in wheat germ diet-reared *Heliothis virescens* (F.) larvae. Diet-reared larvae fed cotton obtained the chemicals from cotton, with the consequence of enhanced kairomonal activity of the larvae and their frass to *C. sonorensis*. Parasitoids, presented a choice between cotton, cotton plus hosts, hosts alone, and control in an olfactometer, responded non-randomly, with the greatest number of responses to cotton plus hosts, and three times as many responses to cotton alone as to larvae alone. The role of the plant in the parasitoid–host relationship is discussed.

Key Words—*Campoletis sonorensis*, *Heliothis virescence*, synomone, kairomone, host location, Hymenoptera, Ichneumonidae, Lepidoptera, Noctuidae, parasitoid.

INTRODUCTION

Successful parasitism by insect parasitoids has been divided into five phases: (1) host habitat location, (2) host location, (3) host acceptance, (4) host suitability, and (5) host regulation (Vinson, 1976). Phases 1–3 constitute the host selection process in which chemicals play an important role (Weseloh, 1981; Arthur, 1981). These chemicals have been divided into kairomones (Brown et al., 1970), allomones (Brown, 1968), and synomones (Nordlund and Lewis, 1976) and appear to be key factors determining host location and governing the range of hosts attacked by parasitoids.

One major problem faced by a female parasitoid upon emergence is

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locating a potential host-containing habitat. There is some evidence that plant-produced chemicals play a major role in host habitat location (Vinson, 1981). Read et al. (1970) identified allyl isothiocyanate (mustard oil) as the attractant for *Diaeretiella rapae* (M'Intosh), a parasitoid of *Myzus persicae* (Sulzer) on crucifers. Camors and Payne (1972) found that α-pinene from the host tree attracted *Heydenia unica* Cook & Davis to the site of a southern pine beetle attack, although sex pheromones were also involved. The interaction between host kairomones and plant synomones and the effect of this interaction on parasitoid behavior has been studied in only a few instances (Sauls et al., 1979; Nordlund and Sauls, 1981; Altieri et al., 1981).

Wilson et al. (1974) reported that a kairomone, present throughout the body of the noctuid host, *Heliothis virescens* (F.), elicited antennation and oviposition by the ichneumonid, *Campoletis sonorensis* Viereck. The chemical was associated especially with the cuticle and was also present in frass. Kairomonal activity was greatest in acetone and methanol extracts, but the chemicals involved were not identified.

We showed (Elzen et al., 1983) that *C. sonorensis* is attracted to plants which may serve as food for its noctuid larval hosts. This attraction was due to several volatile sesquiterpenes produced by the plant (Elzen et al., 1984). It was not known what effect feeding by *H. virescens* on the host plant might have on host acceptance by *C. sonorensis*. Our purpose in the present study was to determine if these plant synomones were responsible for the previously reported (Wilson et al., 1974) kairomonal activity of host larvae, the influence of these chemicals on host acceptance, and their fate in the host larvae.

METHODS AND MATERIALS

Adult *H. virescens* moths were held in 2-liter glass containers and supplied with a diet consisting of 5% sucrose and 1% tetracycline hydrochloride. The container was covered with a paper towel and held at 27° C. Eggs deposited on the paper towel were collected every 48 hr, washed in a 15% Chlorox solution, air dried, and placed in 2-liter glass containers. Upon eclosion, *H. virescens* larvae were placed singly in vials containing wheat germ diet (Vanderzandt et al., 1962). *H. virescens* were parasitized by placing three *C. sonorensis* females into a 300-ml styrofoam cup containing 15 third-instar host larvae and diet. Cups were covered with clear plastic and held for 3 hr. Parasitized larvae were placed in plastic vials with diet where, after 12–18 days, the parasitoids emerged. Male and female parasitoids were immediately placed together for mating, separated the following day, and maintained at 26 ± 2°C in a 16:8 light–dark photochamber (Hoelscher and Vinson, 1971) in 600-ml plastic cartons fitted with nylon covers.

The possible existence of previously identified synomones from cotton (*Gossypium hirsutum* L.) in diet-reared *H. virescens* larvae was determined

by removing 200 third-instar larvae from diet and immediately homogenizing them in ethyl ether. Extracts were prepared for gas chromatographic analysis by the procedures outlined in Elzen et al. (1984).

In tests to determine the fate of previously identified cotton chemicals in cotton-fed *H. virescens* larvae, 200 first-instar larvae were removed from wheat germ diet and placed in 50-ml plastic cups containing 10 g of fresh cotton flowers and buds and were allowed to feed for 72 hr. Ethyl ether extracts of whole larvae were prepared as described above and subjected to gas chromatographic analysis.

Another set of *H. virescens* larvae ($N = 256$) which had fed on cotton for 72 hr were removed from the feeding cups and immediately dissected under distilled water in the following manner. Larvae were pinned down and a lengthwise incision was made through the ventral cuticular wall from the head capsule to the anus, the head was cut off and the gut removed intact. The remaining cuticle was scraped with a sharpened spatula to remove adhering tissue. Cuticle and gut were washed separately in two changes of distilled water, dried briefly on a paper towel, extracted as previously described, and analyzed by gas chromatography. Frass (3 g) collected from the feeding cups was extracted and analyzed in the same manner.

The preference of *C. sonorensis* for cotton-fed larvae, obtained after feeding for 72 hr on cotton as described above, was compared to diet-fed larvae of the same instar. Diet-fed larvae were marked with a single 1-mm-diameter spot of white paint on the dorsal, posterior cuticular surface. We confirmed the report of Guillot and Vinson (1972) that small amounts of white paint have no effect on host preference. A 9-cm glass Petri dish, with a Whatman No. 1 filter paper floor, was used as a test chamber. One *C. sonorensis* female was placed in the Petri dish with one cotton-fed and one diet-fed larvae. When either larva was stung by the parasitoid, it was removed and another of its kind introduced. Testing continued until 25 larva, of either type, were stung. The number of cotton-fed and host-fed larvae attacked was recorded in this manner. Tests were repeated three times with a different female used in each test. The replicates were analyzed under a hypothesis of 50:50 parasitoid acceptance of the two types of hosts by chi-square analysis.

Frass from cotton-fed larva and diet-fed larva was tested for attractiveness to *C. sonorensis* females in a 9-cm Petri dish in a bioassay similar to that described previously (Elzen et al., 1983). The two types of frass were pressed onto the filter paper of the petri dish floor 4 mm apart in the approximate center of the dish. A female was introduced and the number of antennal responses to each frass sample was recorded for a period of 3 min. A total of 10 tests were performed with a different female used in each test. Data were analyzed by chi-square analysis with a hypothesized response ratio of 50:50.

The response of *C. sonorensis* when presented plant and larval odor sources was examined using a four-choice olfactometer modified from that

shown in Vet et al. (1983). The four discrete choices were (1) lightly misted refined cotton water pad (control), (2) 0.3 g cotton leaves, (3) 0.3 g cotton leaves and three third-instar *H. virescens* larvae and (4) three third-instar *H. virescens* larvae. All larvae had been fed wheat germ diet as previously described. Twenty parasitoids were placed in the olfactometer for ten min and the number entering the tube, or caught, at each choice was recorded. Six replicates were made. The data were analyzed by a Kolmogorov-Smirnov goodness of fit test (Massey, 1951), with the null hypothesis that given the four choices, parasitoids would distribute evenly.

RESULTS

GC analysis of artificial diet-fed whole-body extracts of *H. virescens* larvae revealed no peaks corresponding to those found in cotton (Table 1). Chemicals matching the retention times found previously in cotton (Elzen et al., 1984) were present in whole body, gut, and frass of cotton-fed *H. virescens* larval extracts. Also, the relative proportion of chemicals was very similar to that found in cotton (Table 1).

When presented a choice between cotton-fed and diet-fed hosts, *C. sonorensis* accepted (stung) a significantly greater proportion of the cotton-fed larve than diet-fed larvae. Similarly, parasitoids responded with significantly more antennal contacts toward cotton frass than diet frass (Table 2).

When *C. sonorensis* were presented a choice between cotton, cotton plus hosts, hosts, and control in an olfactometer, the response to the four choices was significantly differently ($P < 0.05$) from an even distribution (Figure 1). The greatest number of responses was to the choice of cotton plus

TABLE 1. CHEMICALS IDENTIFIED IN COTTON-FED *H. virescens* EXTRACTS

Chemical	Retention time (min)[a]	Relative proportion (%)[b]					
		Cotton standard	Diet fed, WB	Cotton fed			
				WB	CT	GT	FR
β-Caryophyllene	3.6	30.0	0	28.1	T	44.2	31.6
α-Humulene	4.1	7.7	0	8.7	T	12.3	15.2
γ-Bisabolene	4.9	17.4	0	16.7	T	16.8	16.5
β-Caryophyllene oxide + spathulenol	6.5	6.5	0	4.9	0	4.6	5.9
Gossonorol	7.8	1.3	0	1.0	0	0.8	2.3
β-Bisabolol	9.1	37.1	0	40.4	0	21.3	28.5

[a] 5% OV-101 on Chromosorb G, 80–100 mesh, at 160°C; helium flow rate = 20 ml/min.
[b] WB = whole body; CT = cuticle, GT = gut, FR = frass; T = trace.

TABLE 2. RESPONSE BY *C. sonorensi* TO *H. virescens* LARVAE AND FRASS

	Feeding status of *H. virescens*	
	Cotton	Artificial diet
Total No. accepting *H. virescens* larvae (3 replicates)	75[a]	36
Total No. of antennal exams to frass (10 replicates)	50[a]	32

[a]Significantly different from hypothesized 50:50 response ratio ($P < 0.05$) by chi-square analysis.

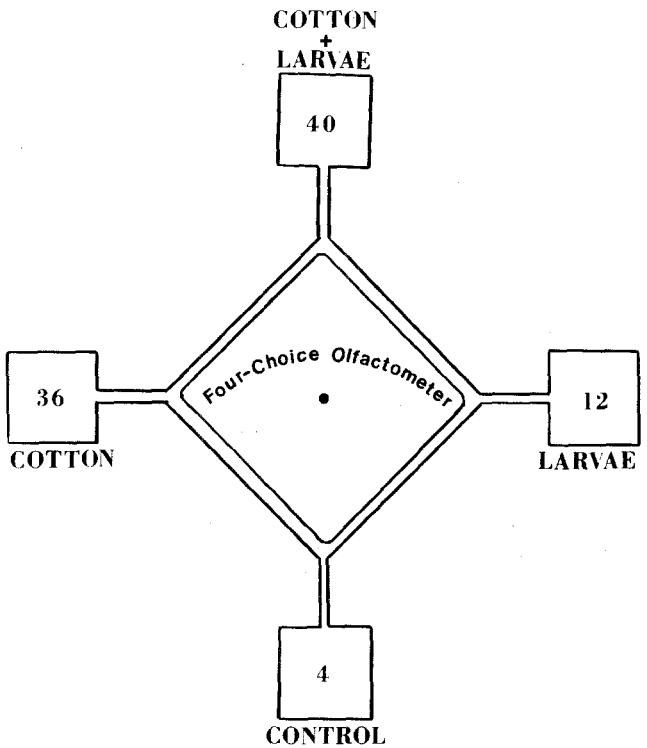

FIG. 1. Four-choice olfactometer design and result of *C. sonorensis* bioassay (six replicates). Four choices significantly different from an even distribution by a Kolmogorov-Smirnov goodness of fit test ($P < 0.05$).

larvae. Further, there were three times as many responses to cotton alone as to larvae alone.

DISCUSSION

Both the cotton frass and the cotton-fed larvae were preferred over the diet frass and diet-fed larvae. This indicates that plant-produced chemicals can affect host preference. Further, the data indicate that the plant plays an important role in attracting *C. sonorensis*, since more females chose a kairomonal source containing cotton over larvae alone or control. We determined that larvae fed cotton obtain the plant synomones and also excrete them in the frass.

Sauls et al. (1979) demonstrated that the diet affects the kairomonal activity for *Microplitis croceipes* Cresson to frass of *H. zea* Boddie larvae and that frass from larvae fed pinto bean diet was significantly less attractive than frass from larvae fed fresh pea cotyledons. Nettles (1979, 1980) found that *Eucelatoria bryani* Sabrosky (Tachinidae) was attracted to the odor of its host's (*H. virescens*) food plants, okra and cotton, and to its hosts fed on okra leaves, but were not attracted to hosts fed artificial diet. Read et al. (1970) found that *D. rapae* preferred to oviposit in aphids feeding on collards containing mustard oil than in aphids feeding on beet, which does not contain mustard oil.

The manipulation of parasitoid behavior to increase their effectiveness in controlling pests may be possible through studies on the role of plant synomones in the plant–host–parasitoid interaction. By isolating and identifying attractive factors from plants for parasitoids, it may be possible to use this knowledge to, for example, increase the level of such chemicals through plant breeding and thereby increase parasitoid effectiveness. The action of parasitoids could also be enhanced by spraying attractive chemicals on suitable or alternate plants that may be food for the parasitoid's hosts. Apart from these beneficial aspects, our study points out the importance of plant secondary metabolic products in the host–parasitoid relationship.

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REVOLVING FRACTION COLLECTOR FOR PREPARATIVE CAPILLARY GAS CHROMATOGRAPHY IN THE 100-μg TO 1-ng RANGE

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Abstract—A gas chromatography fraction collector for use with capillary columns, consisting of an all-glass system with a revolving collector housing six capillary tubes, 100 × 1 mm (internal diameter), has been constructed and tested. The collector is cooled with solid CO_2 and has a recovering efficiency of 50–75% in the 100-μg to 1-ng range for compounds of different polarities and volatilities, exemplified by mono- and sesquiterpenes and long-chain esters. The technique has been used for fractionating blends of semiochemicals prior to microchemical reactions, spectroscopic analyses, and behavioral experiments.

Key Words—Preparative gas chromatography, capillary columns, fused silica columns, fractionation, biologically active odors, volatile compounds, terpenes.

INTRODUCTION

Work with semiochemicals, such as pheromones and allelochemicals, calls for a means of obtaining precise gas chromatographic collection of small amounts of volatile compounds that may be used for biological experiments, such as behavioral tests and olfactive analyses, as well as for various spectroscopic and olfactory analyses. Because the volatile secretions often consist of many components and because they usually occur only in small amounts, capillary, fused silica columns are generally used for the gas chromatographic analysis of these secretions. Techniques for the collection of gas chromatographic fractions have been described by Williams and Vinson (1980),

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Roeraade and Enzell (1979), and Burger and Munro (1983). Our aim was to develop a technique using a revolving fraction collector with a high trapping efficiency in the collection of small amounts of volatile compounds and which would be easy to handle.

METHODS AND MATERIALS

Collector Construction. Figure 1A is a schematic diagram of the various parts of the collector. Capillary D, which leads from the outlet splitter passes through a glass-lined tube (GLT), E (0.7 mm ID, 1.5 mm OD); the capillary tip of D extends 1–2 mm beyond the GLT, which in turn is placed in an outer glass tube, F (2 mm ID, 4 mm OD). The components D, E, and F are held in place in block A, by a nut and ferrule connected to a thread union, C (see Figure 1 B). This assembly passes through the aluminium block A, which is heated electrically by a 200-W heater, G. A Teflon cylinder, H, is placed between the oven wall, B, and the cooling block. The revolving aluminium cooling block consists of two parts, a cylinder, I, 50 mm in length with a diameter of 65 mm, and a housing, N (76 mm ID, 80 mm OD), which serves as a container for solid carbon dioxide with which cylinder I is cooled. Teflon tubes 60 mm in length, 1.6 mm ID, 3 mm OD, are fitted into six 3-mm holes, J, in cylinder I, the Teflon tubes protruding 5 mm beyond each end of the cylinder. In the center of cylinder I is a hole with a Teflon bearing, L (12 mm ID, 20 mm OD). Six steering holes, M, 5 mm deep and 5 mm in diameter are drilled into one end of I. Viton gaskets are used as end seals between cylinder I and housing N. The insulation material around the cooling block is Frigolit (polyurethane) (thickness 5 mm, see Figure 3). The cooling block is movable around a stainless-steel shaft, P, which is 25 cm long and 12 mm in diameter and on which a spring-loaded locking pin, Q, is fixed. The tip of the locking pin, R, is made of Teflon. Figure 2A shows the mounting of the collector before installation, and Figure 2B the component parts in the design. The collector is mounted on an aluminium plate and attached to the wall of the chromatograph, as shown in Figure 3.

Column Preparation. Empty fused silica columns (SGE) in lengths of 25 m and with an internal diameter of 0.35 mm were obtained from Alltech Corp. Columns were pretreated with Superox 4, using the method of Arrendale and Smith (1980) with the following modifications. The column was filled with a solution of 0.2% Superox 4 in methylene chloride, and the static coating method was used. After flushing the column with dry nitrogen, both ends were sealed and the column was deactivated in an oven at 335°C for 1 hr. The column was then rinsed with methylene chloride and filled with a solution of stationary phase (OV-351) in methylene chloride, and the static method was used for applying the phase.

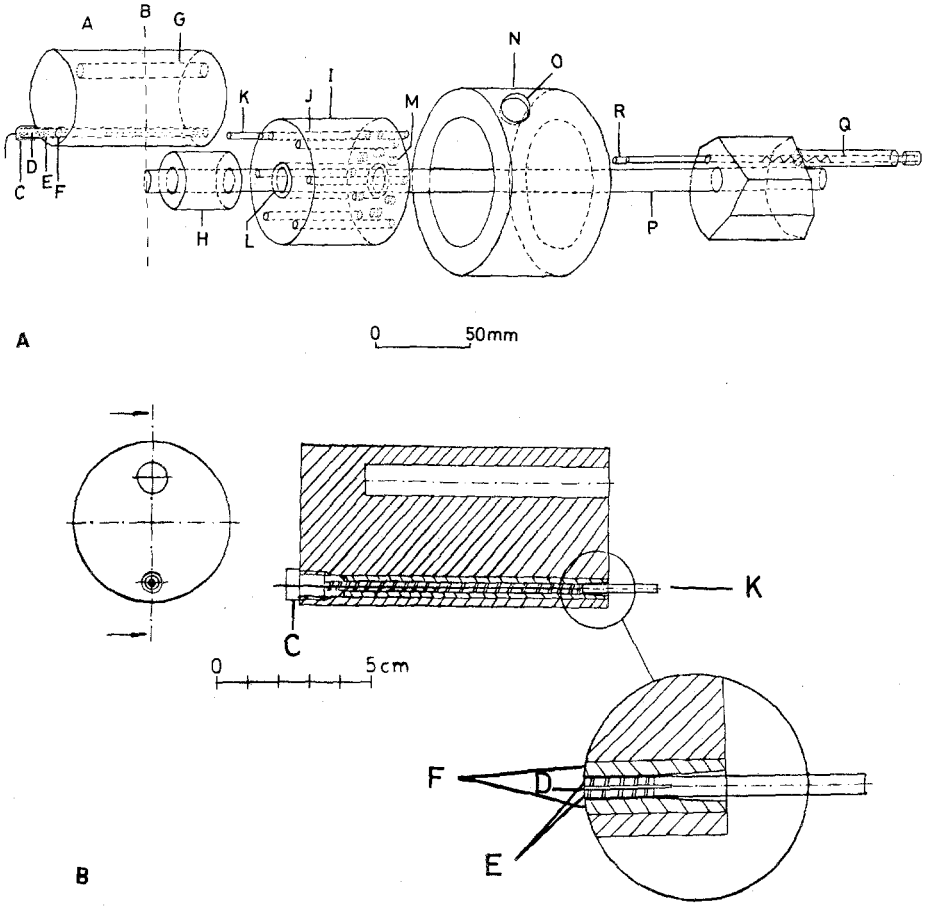

FIG. 1(A). Schematic diagram of the collector. A = complete heating block; B = oven wall; C = connection of capillary in block A. D = capillary from the outlet splitter; E = glass-lined tube (GLT); F = glass tube; G = hole for heater; H = Teflon cylinder; I = cooling cylinder; J = drilled hole with Teflon tube; K = collection capillary; L = Teflon bearing; M = steering hole; N = cooling cylinder housing; O = hole to fill solid carbon dioxide; P = stainless-steel shaft; Q = locking pin; R = Teflon tip. (B) Longitudinal section of the heating block A with an enlarged detail showing the connection to the collection capillary.

Operation. An all-glass splitter is installed in a Carlo Erba 4160 gas chromatograph equipped with a flame ionization detector (FID). The separation column is connected to a zero dead-volume T, SGE GLTT/16, 0.5 mm ID (cf. Williams and Vinson, 1980). The effluent gas to the FID flows through a fused silica capillary (85 cm in length, 0.20 mm ID) and the gas to the collector through a capillary of the same material, 60 cm in length and

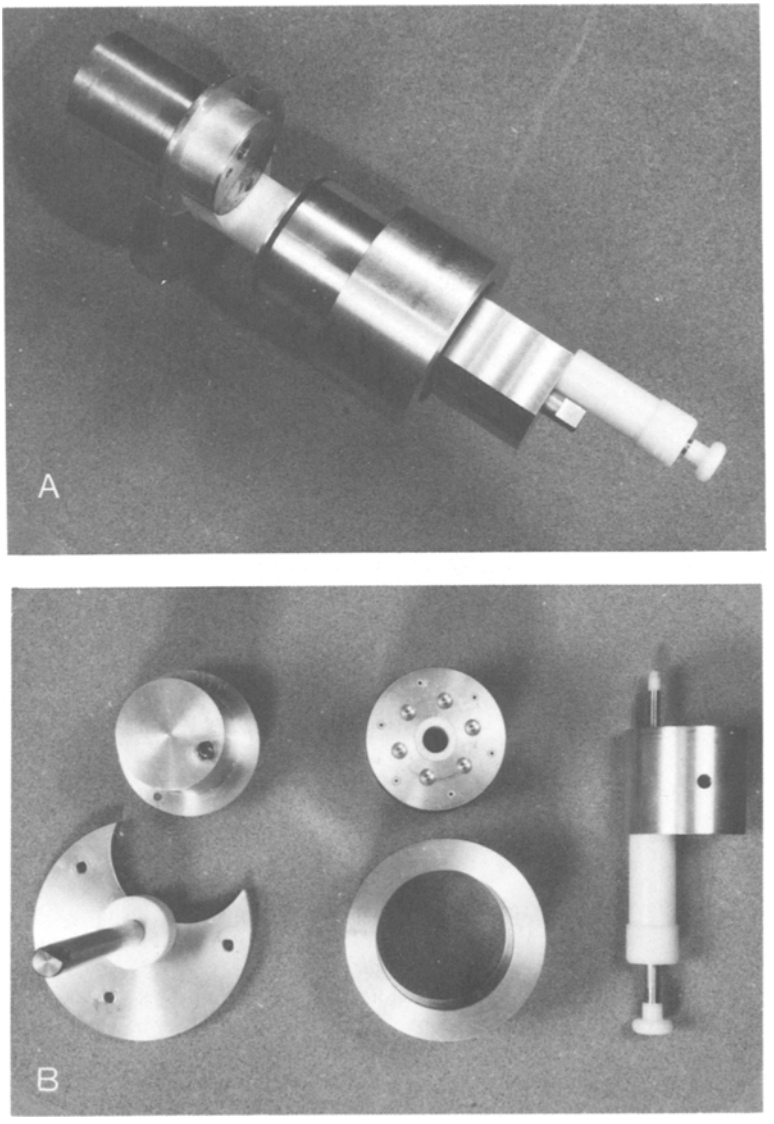

FIG. 2. (A) The mounted collector before installation on the gas chromatograph. (B) Components of the system.

0.35 mm ID. One example of the operation of the fraction collector is the isolation of substances from entrainment of odors from the bark beetle *Pityogenes chalcographus* (Figure 4). In this case, a wall-coated open tubular fused silica capillary column (OV-351, 25 m in length, 0.35 mm ID, film thickness 1.05 μm) was used. Nitrogen was used as carrier gas ($\bar{u}$ = 16 cm/sec) and as

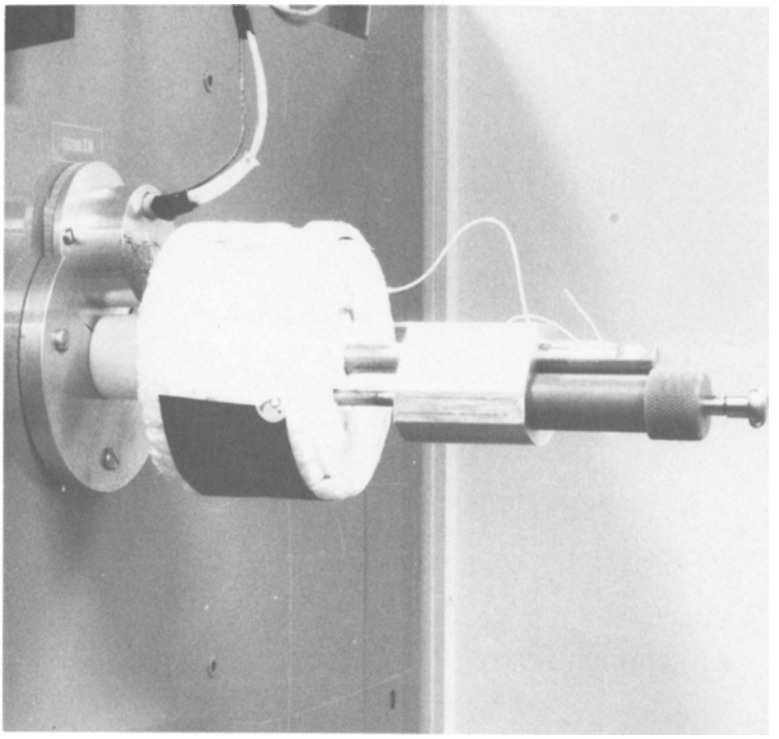

FIG. 3. Photograph of the collector in position on a gas chromatograph (Carlo Erba, model 4160).

makeup gas. The split ratio was 9:1, with 90% of the effluent going to the collector. The detector temperature was set to 230°C. The samples were injected in the splitless mode at an injector temperature of 210°C. The temperature of the heating block was set according to the volatility of the components to be collected, about 20° higher than oven temperature.

To collect components, glass capillaries, K, 100 mm in length and 1 mm ID, 1.5 mm OD, are fitted into the Teflon tubes in cylinder I. If the substances are water-sensitive, condensation of water can be prevented by using a Teflon tube as an extension of the capillary (Figure 3). The space between cylinder I and housing N is filled through the hole Q with pulverized Dry Ice. At the time of collection, the cooling block is put into position with the aid of the spring-loaded locking pin, which is itself pushed into the steering hole in order to obtain a good fit between the capillary and the heating block. The collection capillary is pushed tightly to the GLT-line in the heating block (see Figure 1B). The cooling block is drawn back when one collection capillary is disconnected from the column, switched to the next position, and

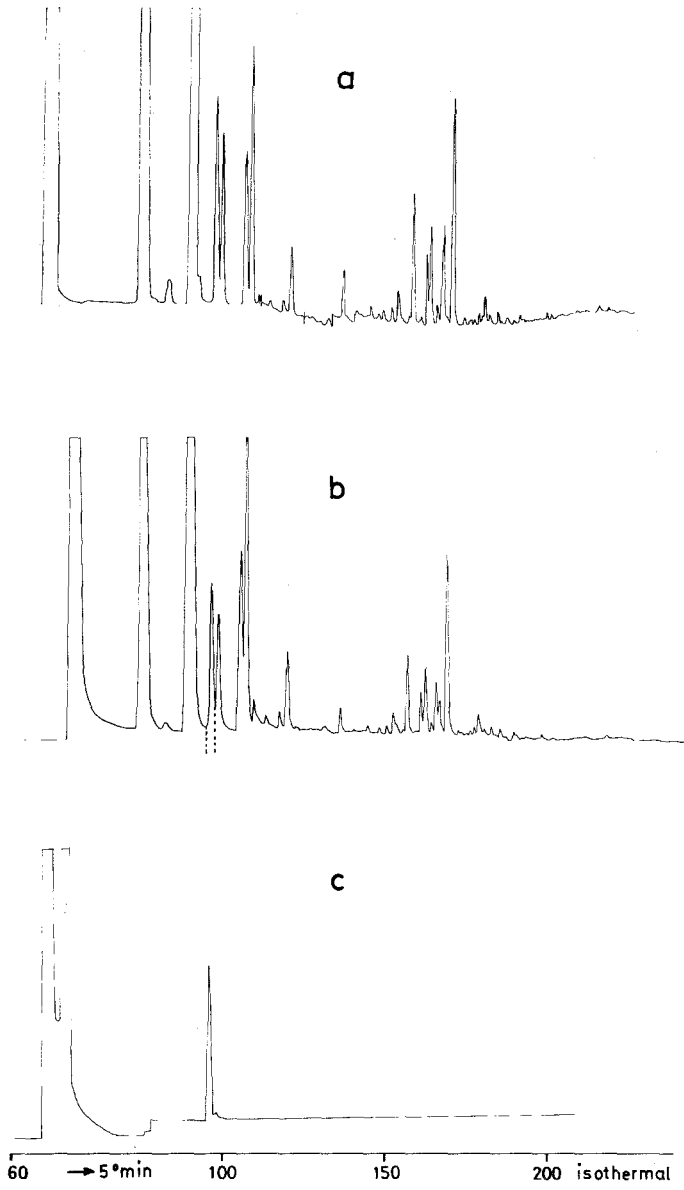

FIG. 4 Gas chromatographic separation of an odor entrainment from a spruce log, containing 40 male *Pityogenes chalcographus*, using splitless injection. Temperature program: isothermal at 60°C for 5 min, and then programed to 200°C at 5°C/min. Column: 25 m × 0.35 mm ID OV-351 on Superox 4 deactivated fused silica. (a) Gas chromatogram without outlet splitter. (b) Gas chromatogram with outlet splitter installed. This is the normal preparative setup. (c) Purity of a collected substance (cf. Figure 4b).

pushed forward again when the next capillary is to be coupled. (Alternatively, the cooling block can be switched one step for each collection without retraction and pushing.) Samples are rinsed from the tubes using several 5-μl portions of the desired solvent.

RESULTS AND DISCUSSION

The outlet splitter has a low dead volume and, because it is an all-glass system, avoids the potential problem of metal-catalyzed decomposition of the sample. The sample in Figure 4a was injected in the splitless mode. As a comparison, Figure 4b shows the same injection with an outlet splitter connected after the column. In this chromatogram, 10 times more material than in Figure 4a passed through the column. The purity of an isolated component is shown in Figure 4c, where a small amount of the following component is also visible as a result of bad resolution. However, higher efficiency of the column is expected to improve the result.

The collector has been used for fractionating samples for bioassay and for the isolation of components for chemical analyses such as hydrogenation, ozonolysis, and for spectroscopic analyses such as NMR. We have found that WCOT columns with OV-351, which is a Carbowax 20 M/nitroterephthalic acid ester, as stationary phase, are suitable for this work, where terpenes with different volatilities and polarities are to be separated. Although bleeding is low, the lifetime of the column is greatly influenced by the purity of the carrier gas. With about 100 μg injected substance, the column is easily overloaded; however, this can be improved by increasing the film thickness. In order to ensure condensation of the collected fractions, efficient cooling of the aluminium cylinder is essential.

In order to determine trapping efficiencies, a number of analyses were made, using substances of different polarities and volatilities. A volume of 1 μl was injected in the splitless mode into an open fused silica column, 25 m in length and 0.35 mm ID, coated with OV-351. Using a 9:1 split ratio, 90% efficiency is the theoretical limit. The results for four compounds are shown in Table 1. The volatile compounds used were monoterpene hydrocarbons

TABLE 1. PERCENTAGE RECOVERY OF VARIOUS COMPOUNDS AFTER COLLECTION USING FRACTION COLLECTOR

Substance	Amount Injected						Temp. of Al block A (°C)
	100 μg	10 μg	1 μg	100 ng	10 ng	1 ng	
α-Pinene	54	58	65	54	56		150
3-Carene	74	68	58	61	54	60	150
Myrtanol	67	60	58	65	57	59	200
Caryophyllene	58	51	69	51	50		200

such as α-pinene and 3-carene. Trapping efficiencies for these compounds were 55–75% in the 100-μg to 1-ng range. Oxygenated terpenes and sesquiterpene hydrocarbons, which are less volatile, were trapped with an efficiency of 50–65% in the 100-μg to 1-ng range. Long-chain esters such as hexadecyl acetate gave trapping efficiencies of 65% in the 100-ng range. Furthermore, verbenol, which is thermolabile, gave a yield of 60%, and there was no decomposition in the heating block.

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POTENTIAL FOR EVOLUTION OF RESISTANCE TO PHEROMONES: Interindividual and Interpopulational Variation in Chemical Communication System of Pink Bollworm Moth[1]

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Abstract—After an extensive examination of the release rates and blend ratios of pheromonal components emitted by field-collected female *Pectinophora gossypiella* (Saunders), we find no evidence of resistance to pheromones applied to cotton fields to disrupt mating. Females from fields with 3–5 years of exposure to disruptant pheromones as well as those from fields with only minimal exposure to disruptant pheromones emitted (*Z,Z*)-7,11-hexadecadienyl acetate at a rate of ca. 0.1 ng/min and (*Z,E*)-7,11-hexadecadienyl acetate at ca. 0.06 ng/min. The ratio of pheromonal components was much less variable than the measured emission rate and was centered about a 61:39 *Z,Z* to *Z,E* ratio. In contrast to the blend ratio emitted by females, the composition of the pheromonal blend used in monitoring populations and disrupting mating is centered about 50:50 *Z,Z* to *Z,E*. In general there was a remarkable consistency in the release rate and blend ratio among populations of females throughout southern California and those from a laboratory colony. It would appear that, although resistance to the *P. gossypiella* pheromone is still a very real possibility when it is used heavily in pest management as a mating disruptant, there are current agricultural practices and conditions which would hinder its development.

Key Words—Resistance, mating disruption, sex pheromone, (*Z,Z*)-7,11-hexadecadienyl acetate, (*Z,E*)-7,11-hexadecadienyl acetate, Lepidoptera, Gelechiidae, pink bollworm, *Pectinophora gossypiella*, cotton, pheromone collection.

[1] *Pectinophora gossypiella* (Saunders), Lepidoptera: Gelechiidae.

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INTRODUCTION

Several years after the identification of the sex pheromone of the pink bollworm moth *Pectinophora gossypiella* (Saunders) as a blend of (*Z*,*Z*)-7,11-hexadecadienyl acetate [(*Z*,*Z*)-7,11-16:Ac] and (*Z*,*E*)-7,11-hexadecadienyl acetate [(*Z*,*E*)-7,11-16:Ac] (Hummel et al., 1973; Bierl et al., 1974), a 1:1 blend of these isomers (gossyplure) became commercially available to cotton growers in the United States for mating disruption (Brooks et al., 1979). The efficacy of mating disruption using gossyplure to control of *P. gossypiella* was established by Gaston et al. (1977) and, on a larger scale, by Brooks et al. (1979). After the Environmental Protection Agency registered the pink bollworm mating disruptant, many growers in Arizona and California began using it for early-season control of this pest. Use of the disruptant grew, and now yearly over 100,000 acres receive at least one application of pheromone (C.C. Doane, personal communication). At this early stage in the use of disruptant pheromones it is important to establish the potential for evolution of resistance to synthetic pheromones.

Since there is a limited understanding of the mechanism(s) by which mating disruption works, it is difficult to anticipate all of the potential means for the development of resistance of pheromones. However, evolution of resistance to pheromones could include selection for an increase in the rate of pheromone emission and/or selection for a shift in the ratio of pheromone components with a concomitant "fine-tuning" of the response of males to the blend released by females. Interindividual variation in release rate or blend ratio may partly determine the potential for resistance to pheromones, since selection would operate on such variation. The development of an efficient system for quantifying the release rate and blend ratio of pheromonal components by Baker et al. (1981) allowed us to measure the interindividual and interpopulational variation in these aspects of chemical communication in *P. gossypiella*, a species that has been subject to commercial control with disruptant pheromones longer than any other species.

METHODS AND MATERIALS

Collection and Handling of Insects. Cotton bolls were collected during August, September, and October, 1982, from selected cotton fields in the three major cotton-producing valleys in southern California: Palo Verde, Imperial, and Coachella valleys. There was at least 100 km separating our sampling sites between these three valleys, and the valleys are separated by deserts and/or mountains. In each valley, fields were selected according to their history of use of mating disruptants. One group of fields had had no exposure or one year of exposure to disruptant pheromones (henceforth

called insecticide-treated fields). The other group had had 3–5 years of treatment with disruptant pheromones (henceforth called pheromone-treated fields). Only in Imperial Valley was it necessary to select insecticide-treated fields with one year of exposure to mating disruptants, since an abatement program requiring the application of disruptant pheromones in 1982 had been enacted by the growers.

Between 1000 and 3000 bolls were collected in each field (the actual number of bolls collected depended on estimates of the infestation level), and then bolls were transported back to Riverside, California, where they were stored in screened cages in a lathhouse. The screened cages consisted of stacks (two to five) of 76 × 122-cm hardware cloth trays (1.25 cm mesh) with 5-cm rims. Last-instar larvae dropped from the bolls onto a fine-mesh nylon screen attached 5 cm below each tray, and the larvae pupated in several layers of cheesecloth. Screening was wrapped around each stack of trays, ensuring isolation of populations from the various fields. Pupae were collected from these cages semiweekly and brought into the laboratory where they were separated according to sex. Female pupae from each field were placed in one-pint paper cartons with nylon screen lids and housed in an environmental cabinet (15:9 light–dark and temperature 25°C). Adult females were transferred daily to one-pint cartons and had access to 8% sugar water.

Laboratory *P. gossypiella* were reared in half-gallon cartons of shredded wheat germ diet described by Adkisson et al. (1960). The procedure for handling the laboratory-reared pupae and adults was identical to that followed for field-collected insects. The laboratory population orignated from insects collected before 1976 from fields in Coachella Valley.

Collection and Quantification of Pheromone. Pheromone was collected from individual female *P. gossypiella* following the procedures first described by Baker et al. (1981) and modified by Haynes et al. (1983). A 2- to 4-day-old female was cold-anesthetized between five and nine hours after the start of the scotophase which is the normal period of calling behavior under the specified laboratory conditions (Haynes, unpublished observations). The female's wings were folded back over her head and she was inserted abdomen-first into a 2.0-mm ID glass tube with a 0.5-mm (diam.) hole at the distal end. This hole was large enough to allow only the ovipositor and associated pheromone gland to emerge when a light pressure was applied to the female's head with a pipe cleaner. The glass tube was then inserted through a Teflon-coated GLC septum into the collector as described by Haynes et al. (1983). Volatiles emitted from the gland's surface were collected for 10 min (at ca. 25°C) onto ca. 10 mg of glass wool. An internal standard [either 3.0 ng of (*Z*)-7-hexadecenyl acetate or 5-hexadecynyl acetate] was added to the glass wool before the inside of the collector was rinsed with ca. 200 μl of CS_2. This volume of CS_2 was then reduced to ca. 6 μl under a nitrogen stream before it was pulled up into a 10-μl Hamilton syringe for injection onto the GLC column.

Analyses were made on a Varian 3700 gas chromatograph equipped with a hydrogen flame detector, a Hewlett-Packard 3380A integrator, a Honeywell Electronik 196 chart recorder, and a Silar 10C packed column (4 g of 10% Silar 10C on acid-washed 100–120 mesh Chromosorb W; glass column 3 m × 4 mm (OD); oven temperature 175°C; N_2 flow rate 30 ml/min). The heights of peaks corresponding to the internal standard and the two pheromone isomers were measured. The amount of each isomer was calculated from a standard curve relating peak heights to mass. These values were then corrected for recovery efficiency by standardizing the measurement relative to the internal standard (recovery efficiency averaged ca. 85%). Recovery efficiency includes loss of compounds at all steps of the work-up, including concentrating the sample under nitrogen. The collection efficiency of the device (adsorption and desorption of pheromone) is ca. 100% (Baker et al., 1981). The lower analytical limit of this system was ca. 0.1 ng (0.01 ng/min); less than 5% of all values fell below this limit (these were discarded from blend ratio data and averaged as 0 ng/min for release rate data).

RESULTS

Pheromone-Treated Vs. Insecticide-Treated Fields. There was no significant difference between the mean emission rate of pheromone by females from fields with different histories of exposure to disruptant pheromone. Females from pheromone-treated fields released (*Z,Z*)-7,11-16:Ac at a rate of 0.095 ± 0.0557 (SD) ng/min ($N = 252$), while those from insecticide-treated fields released 0.102 ± 0.0535 (SD) ng/min ($N = 156$) (Figure 1; no significant difference; $P > 0.05$; two-way analysis of variance). The mean emission rate of (*Z,E*)-7,11-16:Ac was consistently lower than that of (*Z,Z*)-7,11-16:Ac. There was also no significant difference in the emission rate of this *Z,E* isomer in a comparison between females from pheromone-treated fields [0.060 ± 0.0350 (SD) ng/min; $N = 238$] and those from insecticide-treated fields [0.063 ± 0.0338 (SD) ng/min; $N = 145$]. Thus, we could detect no evidence of resistance to pheromones involving a shift in the emission rate of pheromone.

The ratio of the two pheromonal components was not found to be significantly different ($P > 0.05$) in a comparison of females from pheromone-treated and insecticide-treated fields. Females from pheromone-treated fields released 61.7 ± 4.22 (SD) % (*Z,Z*)-7,11-16:Ac ($N = 218$), while females from insecticide-treated fields released 61.9 ± 5.02 % (*Z,Z*)-7,11-16:Ac ($N = 137$) [Figure 2; two-way analysis of variance was run on data transformed by arcsin $\sqrt{p}$, where p is the proportion of (*Z,Z*)-7,11-16:Ac in the two component blend]. Apparently, the use of disruptant pheromones had not resulted in a local evolutionary shift in the blend ratio.

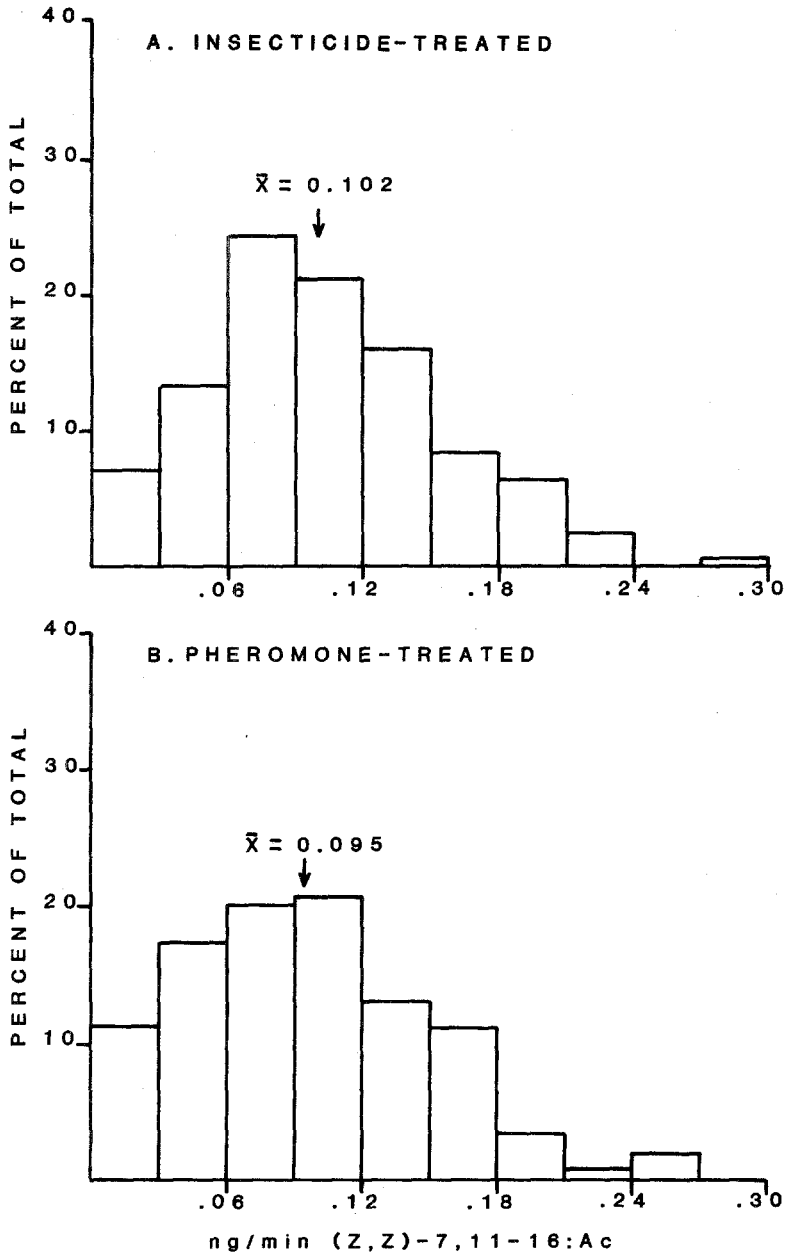

FIG. 1. Variation in emission rates of (*Z*,*Z*)-7,11-16:Ac from female *Pectinophora gossypiella* collected in cotton fields during 1982. (A) Females from insecticide-treated fields (1 year or less of disruptant pheromone treatments) had an average emission rate of 0.102 ± 0.0535 (SD) ng/min ($N = 156$). (B) Females from pheromone-treated fields (3–5 years of disruptant pheromone treatments) released 0.095 ± 0.0557 (SD) ng/min ($N = 252$). There was no significant difference between these samples ($P > 0.05$).

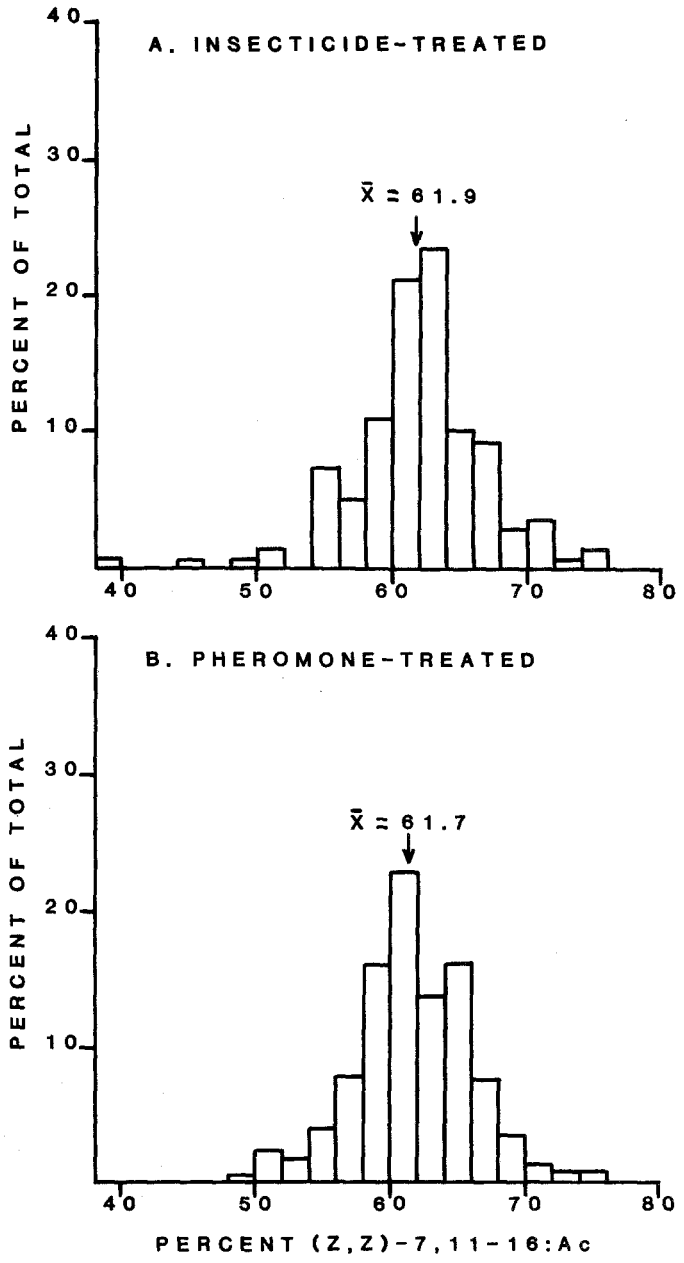

FIG. 2. Variation in the emitted blend ratios from female *Pectinophora gossypiella* collected in cotton fields during 1982. (A) Females from insecticide-treated fields (1 year or less of disruptant pheromone treatments) emitted 61.9 ± 5.02 (SD)% (*Z*,*Z*)-7,11-16:Ac ($N = 137$), while those from pheromone-treated fields (3–5 years of disruptant pheromone treatments) released 61.7 ± 4.22% (*Z*,*Z*)-7,11-16:Ac ($N = 218$). There was no significant difference between these samples ($P > 0.05$).

The similarities of pheromone emission rates and blend ratios by females from fields with different exposures to disruptant pheromones and from a laboratory colony are graphically illustrated in Figure 3. These plots of release rate of (*Z*,*E*)-7,11-16:Ac vs. (*Z*,*Z*)-7,11-16:Ac demonstrate that not only is there very little variation in the ratio of the two isomers, but also that the ratio is independent of release rate. The release rate of the two isomers is relatively more variable, but graphically the distribution of these values is similar in females from pheromone-treated and insecticide-treated fields, as well as in females from our laboratory colony. The similarity of the regression parameters quantifying the relationship between the emission rate of the two isomers demonstrates the consistency of these aspects of chemical communication in these three population samples of females (pheromone-treated fields: $y = 1.53x + 0.0050$, $r = 0.961$, $P < 0.001$; insecticide-treated fields: $y = 1.49x + 0.0092$, $r = 0.941$, $P < 0.001$; laboratory colony, $y = 1.59x - 0.0010$, $r = 0.975$, $P < 0.001$).

Comparison of Populations from Three California Valleys and Laboratory. The consistency of the emission rate of (*Z*,*Z*)-7,11-16:Ac between females from pheromone-treated and insecticide-treated fields was also found in a comparison between populations from the three cotton-producing valleys of southern California and our laboratory colony (Figure 4). There were no significant differences between females from these populations in the release rate of (*Z*,*E*)-7,11-16:Ac [one-way analysis of variance, $P > 0.05$; Palo Verde Valley, 0.062 ± 0.0352 (SD) ($N = 209$); Imperial Valley, 0.062 ± 0.0351 (SD) ($N = 127$); Coachella Valley, 0.056 ± 0.0303 (SD) ($N = 47$); and laboratory, 0.059 ± 0.0298 ng/min (SD) ($N = 51$)]. The percent of (*Z*,*Z*)-7,11-16:Ac in the pheromone blend was significantly different between females from Coachella [60.1 ± 4.17 (SD) ($N = 45$)] and those from Imperial [62.1 ± 5.41 (SD) ($N = 118$)] or Palo Verde Valleys [61.9 ± 3.95 (SD) ($N = 92$)], but there were no other differences (Figure 5; one-way analysis of variance on arcsine $\sqrt{p}$ transformed data; means separated by Duncan's multiple range test). The blend ratio released by females from our laboratory colony [60.7 ± 3.76 (SD)% ($N = 51$)] was not significantly different from that emitted by females from any of the valleys.

DISCUSSION

Selection for an increase in the emission rate of pheromone by females would be perhaps the most straightforward route to resistance in *P. gossypiella*, since it would not necessarily involve a concomitant shift in the males' behavioral threshold. Doane and Brooks (1981), in an experiment designed to test the effect of increasing release rates of pheromone from a hollow-fiber delivery system in pheromone-treated and untreated fields, demonstrated that in pheromone-treated fields such an increase resulted in the cap-

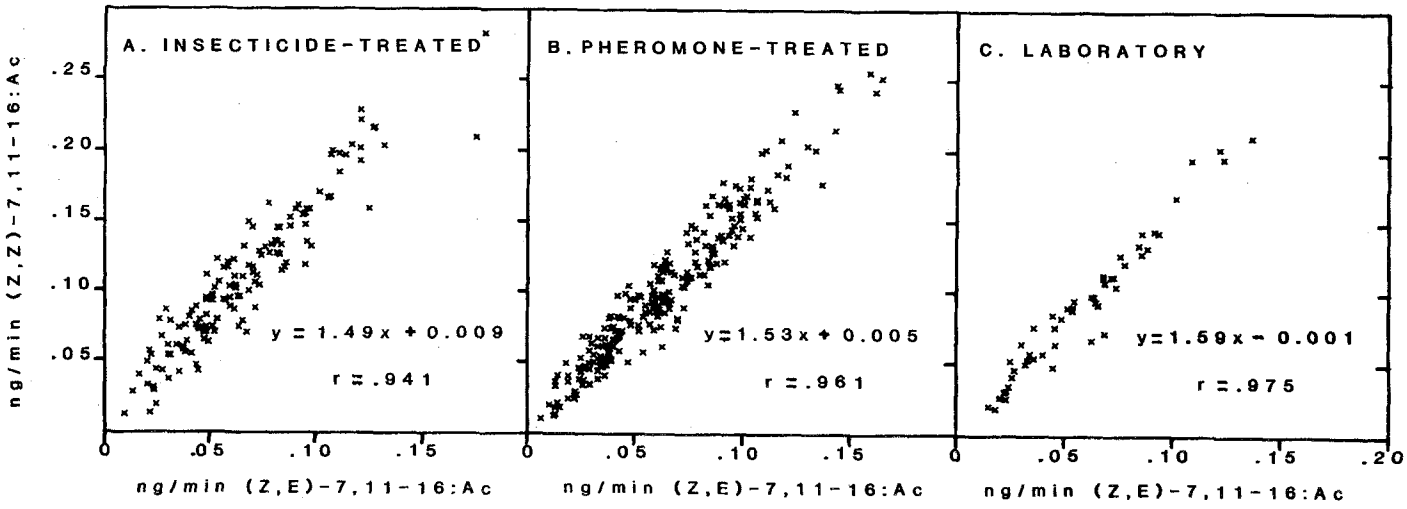

FIG. 3. Emission rate of (*Z*,*E*)-7,11-16:Ac vs. (*Z*,*Z*)-7,11-16:Ac from three samples of female *Pectinophora gossypiella:* (A) insecticide-treated field ($N = 137$), (B) pheromone-treated fields ($N = 218$), and (C) a laboratory colony ($N = 51$). These graphs illustrate the consistency of the emission rate and blend ratio in these three samples of females. The highly significant ($P < 0.001$) correlation coefficients (r) illustrate the tight control over the emitted blend ratio in this species.

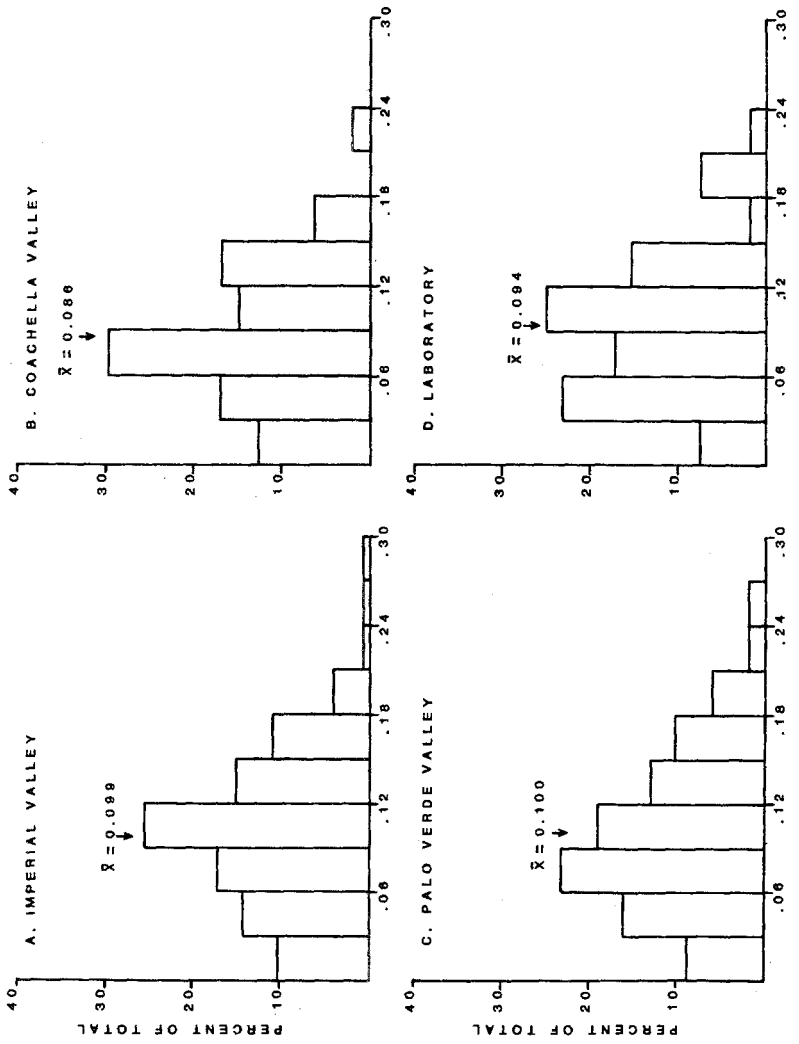

FIG. 4. Variation in emission rates of (*Z*,*Z*)-7,11-16:Ac by female *Pectinophora gossypiella* from the three cotton-growing valleys of southern California and from a laboratory colony. The mean emission rates for these four populations was: (A) Imperial Valley, 0.099 ± 0.0544 (SD), $N = 145$; (B) Coachella Valley, 0.086 ± 0.0498 (SD), $N = 47$; (C) Palo Verde Valley, 0.100 ± 0.0562 (SD), $N = 216$; and (D) laboratory colony, 0.094 ± 0.0498 (SD), $n = 52$. There were no significant differences between any of these means ($P > 0.05$).

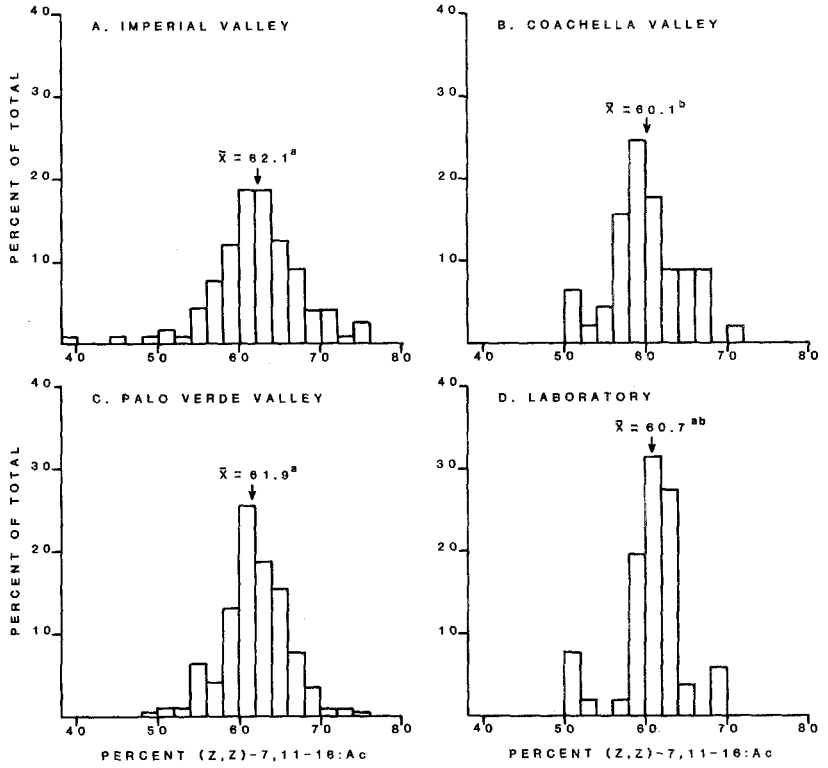

FIG. 5. Variation in the emitted blend ratio from female *Pectinophora gossypiella* from the three cotton-growing valleys of southern California and from a laboratory colony. The mean percent (*Z*,*Z*)-7,11-16:Ac for these four populations was: (A) Imperial Valley, 62.1 ± 5.41 (SD), $N = 118$; (B) Coachella Valley, 60.1 ± 4.17 (SD), $N = 45$; (C) Palo Verde Valley, 61.9 ± 3.95 (SD), $N = 192$; and (D) laboratory colony, 60.7 ± 3.76 (SD), $N = 51$. Means followed by the same letter are not significantly different ($P > 0.05$).

ture of more males in traps. In untreated fields, trap catch did not increase over the entire range of release rates tested, but an upper threshold, above which trap catches would drop off, was not reached. Thus it is clear that males are already capable of responding to a very high emission rate of pheromone and that, within pheromone-treated fields, there would be a selective advantage for females to release more pheromone.

Relatively good analogies to resistance to pheromones are found in examples of reproductive isolation between species. In these cases selection may have favored divergence of communication systems once two related species have come into geographical contact (Dobzhansky, 1970). One hy-

pothesis is that the release rate of pheromone may play a critical role in reproductive isolation between certain species. In *Autographa californica* (Speyer) (the alfalfa looper) and *Trichoplusia ni* (Hübner) (the cabbage looper), there appears to be a difference in how they respond to concentration of the same pheromone component, (*Z*)-7-dodecenyl acetate. *A. californica* preferentially responds to a lower emission rate of pheromone than does *T. ni* (Kaae et al., 1973). It is possible that selection for differences in emission rate and response has minimized reproductive encounters between individuals of these two species. However, recent studies have shown that additional pheromonal components are involved in the communication system of *T. ni* (Bjostad et al., 1980) and additional components are implicated in the pheromonal blend of *A. californica* (Steck et al., 1979), so blend components may play a primary or contributory role to reproductive isolation.

The hypothesis that the blend ratio of pheromonal components may be involved in reproductive isolation of many species of moths suggests that evolutionary shifts in blend ratio may be an effective route to resistance to pheromones. Perhaps the most thorough study concerning the species specificity of pheromonal blends comes from the guild of tortricid moths that feeds on apples in New York (Cardé et al., 1977). In this case, closely related species are reproductively isolated from one another by their blends of pheromonal components. For instance, the pheromone of the fruit-tree leaf roller moth (*Archips argyrospilus*) is a blend of four chemical components: (*Z*)- and (*E*)-11-tetradecenyl acetate, (*Z*)-9-tetradecenyl acetate, and dodecyl acetate (Cardé et al., 1977). A sympatric species, *A. mortuanus*, is maximally attracted to the same four compounds, but only when the compounds are emitted in a different ratio. Roelofs and Brown (1982) cite many examples where the specificity of the sex pheromone blend of closely related tortricid moths involves different combinations or ratios of a restricted number of components. Analogously, the specificity of the communication channel in *P. gossypiella* could be ensured by selection for females that emit a blend ratio different from the disruptant pheromone and a parallel shift in the behavioral specificity of males.

Roelofs et al. (1984) have documented that it is possible to select for a change in the blend ratio of the redbanded leafroller moth, *Argyrotaenia velutinana* (Walker), in two ways. First, offspring of females with high blend ratios of (*E*)-11-tetradecenyl acetate to (*Z*)-11-tetradecenyl acetate tended to have higher than average *E* to *Z* blend ratios. Second, selecting males for mating that were attracted to an abnormally high percentage of (*E*)-11-tetradecenyl acetate generally resulted in female offspring with higher amounts of (*E*)-11-tetradecenyl acetate than their mothers. This pattern suggests genetic coupling or common genes involved in pheromonal perception in males and pheromonal release by females, a phenomenon which has been

found in the pheromone system of another moth, the European corn borer (*Ostrinia nubilalis* (Hübner) (Klun and Maini, 1979). A similar genetic coupling has been documented in acoustical communication of crickets (Hoy and Paul, 1973; Hoy et al., 1977). Involvement of genetic coupling in the control of the emitted pheromone blend and response specificity would make evolution of resistance to disruptant pheromones easier, since selection imposed on one sex could show immediate results in both sexes, and thus resistance would not involve a more complex two-step process.

Selection operates on the existing interindividual phenotypic variation and leads to a change in frequency of genotypes in subsequent generations. Thus, an understanding of the potential for evolution of resistance requires quantification of existing variation. Miller and Roelofs (1980), using glands rinsed with solvent, quantified the interindividual variation in the pheromonal blend of *A. velutinana*, the redbanded leafroller moth. Their report was the first published account of variation in the blend ratio between individual females and between laboratory and field populations. Our comparison between *P. gossypiella* populations from pheromone-treated fields and insecticide-treated fields revealed no difference between these populations of females in either mean emission rate of pheromone or mean ratio of pheromonal components. However, variation in these two aspects of chemical communication was documented. The percent (*Z*,*Z*)-7,11-16:Ac in all populations ranged from 39.4 to 74.7, and the release rate of (*Z*,*Z*)-7,11-16:Ac ranged from 0 to 0.283 ng/min. From our data, we cannot determine the genetic contribution to the measured variation, but such variability would be necessary to rapidly select for resistant phenotypes.

The data presented here suggest an alternative route to resistance to pheromones. Since the blend ratio released by females is centered about a 61:39 ratio of (*Z*,*Z*)- to (*Z*,*E*)-7,11-16:Ac and the blend ratio released by the commercially available disruptant pheromones is approximately 50:50, then resistance may be possible simply by selection for males with a fine-tuned response to the blend released by females. Flint et al. (1979) have documented that populations of males have a relatively broad response spectrum to blends of the *Z*,*Z* and *Z*,*E* isomers. This finding is supported by data collected by Linn (personal communication) and Haynes (unpublished data) in flight tunnels. However, as Cardé et al. (1976) pointed out for another species, populational variation in response could reflect both variation within and between individuals. If response phenotypes exist narrowly centered about the blend released by females, then use of disruptant pheromones of a different blend could rapidly lead to selection for this phenotype. Thus it is important to document the phenotypic variation in the response of males to determine the potential for resistance to disruptant pheromones.

There is some evidence from other species that indicates that small dif-

ferences in the ratio of pheromonal components, such as that between the females' release ratio and the release ratio from disruptant pheromone sources, can be sufficient to aid in reproductive isolation between species. For instance, Roelofs and Brown (1982) cite examples of sympatric leafroller moths that use well-defined blend ratios of (*Z*)-11-tetradecenyl acetate and (*E*)-11-tetradecenyl acetate including 97:3, 91:9, 60:40, 50:50, 33:67, 24:76, 15:85, and 12:88. However, additional components are used by some of these species.

The intensity, continuity, and "homogeneity" of selection pressure are important factors in determining the rate and potential for the development of resistance (Georghiou, 1983). Brooks et al. (1979) showed that gossyplure applied in a disruptant formulation decreased mating in *P. gossypiella* by 97% relative to control fields. The selection pressure on the chemical communication system would seem to be intense. However, cotton growers generally do not apply the disruptant pheromone throughout the growing season. In fact pheromone applications usually end in July in California, leaving two or three generations per year that are not exposed to disruptant pheromones. In addition, the use of disruptant pheromones has not been universally adopted by cotton growers or their pest-control advisors, and as a result, the use of these mating disruptants is patchy in the three cotton-growing valleys of southern California. (One exception was Imperial Valley in 1982, when a program requiring cotton growers to put on at least four applications of gossyplure was in effect.) The patchy nature of pheromone use allows gene flow between untreated and treated fields which could swamp the effect of the locally intense selection pressure. Both long- and short-range dispersal have been documented in this species by several authors (Bariola et al., 1973; Flint and Merkle, 1981; Stern, 1979). However, the relative contribution of this potential gene flow has not been documented. It appears that the disruptant pheromone of the pink bollworm moth is generally being used in a way that makes the evolution of resistance less likely.

The present study has focused on shifts in blend ratios and release rates as potential avenues to resistance to pheromone in *P. gossypiella.* However, there are a number of alternative means to resistance that have not been examined at this point. First, resistance could evolve through selection for males that spend less time in contact with each point source of pheromone, thereby increasing the probability that they will contact females. Second, an increase of flight activity in females between calling bouts could increase the probability of attracting a mate in fields with a high background level of pheromone. Third, there could be selection for females and males that disperse out of the cotton field to mate, with the females returning to oviposit (a behavior which has been observed in the European corn borer on corn by Showers et al., 1976). These hypotheses and a continued investigation of

shifts in emission rate and blend ratio from field-collected females are the central focus of our ongoing investigation into the potential for evolution of resistance to pheromone in the pink bollworm moth.

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IRIDOID GLYCOSIDES AND HOST-PLANT SPECIFICITY IN LARVAE OF THE BUCKEYE BUTTERFLY, *Junonia coenia* (NYMPHALIDAE)

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Abstract—Larvae of the buckeye, *Junonia coenia* (Nymphalidae) feed primarily on plants in four families: Scrophulariaceae, Plantaginaceae, Verbenaceae, and Acanthaceae. These plant families have in common the presence of a group of plant secondary compounds, the iridoid glycosides. Larvae were reared on three plant species and two artificial diets, one with and one without iridoid glycosides. Larvae grew poorly and had low survivorship on the artificial diet without iridoid glycosides, while growth and survival on the artificial diet with iridoid glycosides was comparable to that on plants. Choice tests using artificial diets with and without iridoid glycosides showed that larvae: (1) chose diets with iridoid glycosides (in the form of a crude extract or pure compound) over a diet without; (2) showed no preference between the diet with the crude extract and that with pure iridoid glycoside, and (3) preferred the artificial diet with ground leaves of the host plant, *Plantago lanceolata*, over the diet with pure iridoid glycosides. The artificial diet that larvae had been reared on prior to these tests had no effect on subsequent larval preferences in the choice tests.

Key Words—Iridoid glycoside, *Junonia coenia*, Lepidoptera, Nymphalidae, host-plant specificity, coevolution, insect–plant interaction, *Plantago lanceolata*, buckeye, chemical ecology.

INTRODUCTION

The role of plant secondary chemicals in mediating the interactions of insects and their host plants is well-established (e.g., Burnett et al., 1974;

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Rodman and Chew, 1980; Berenbaum, 1981, 1983; Lincoln et al., 1983; Bowers, 1983). Such compounds may serve as feeding or oviposition stimulants and attractants, deterrents, or toxins (e.g., Schoonhoven, 1972; Bordner et al., 1983; Blum, 1983). One group of secondary compounds, the iridoid glycosides, has been shown to be used as feeding attractants and stimulants for larvae of the chalcedon checkerspot *Euphydryas chalcedona* (Doubleday) (Lepidoptera: Nymphalidae), and probably for other North American *Euphydryas* as well (Bowers, 1983). These compounds are found in several plant families, including those families utilized by the six North American *Euphydryas* species: Scrophulariaceae, Plantaginaceae, Caprifoliaceae, and Oleaceae (Bowers, 1981, 1983).

The larvae of another species of nymphalid butterfly, the buckeye, *Junonia coenia* Hübner, feed on plants in many of the same genera as do the *Euphydras* species, as well as on plants in other families known to contain iridoid glycosides. Larvae have been recorded feeding on plants in the Scrophulariaceae, Plantaginaceae, Verbenaceae, Acanthaceae, and Cornaceae, all of which contain iridoid glycosides (Table 1) (Jensen et al., 1975). These records of host-plant utilization in *J. coenia* suggested that larvae, and presumably ovipositing females, use iridoid glycosides as cues to recognize suitable host-plants. Accordingly, I undertook a series of experiments to assess the potential role of these compounds in host-plant specificity of *J. coenia.* The first experiment was designed to evaluate larval growth and survival on three plant species containing iridoid glycosides and on two artificial diets, one with and one without iridoid glycosides. In a second experiment designed to determine whether iridoid glycosides acted as larval feeding stimulants and attractants, larvae were reared on artificial diet and then given a choice of artificial diets with and without iridoid glycosides, with consumption quantified.

METHODS AND MATERIALS

Caterpillars and Plants. The *J. coenia* larvae used in these experiments were the offspring of three females collected at the Jasper Ridge Biological Preserve of Stanford University, San Mateo County, California, and one female from Sonoma County, California. Larvae were reared in growth chambers with a 14 : 10 light–dark photoperiod and a temperature regime of 25° C day, 20° C night.

The three plant species used for rearing *J. coenia* were *Plantago lanceolata* L. (Plantaginaceae), *Cordylanthus rigidus* (Berth) Jeps., and *C. pilosus* Gray (both Scrophulariaceae). At Jasper Ridge, the primary host plant of *J. coenia* seems to be *P. lanceolata,* and larvae have also been found on *P. erecta* Morris (Brown, personal communication). A freshly emerged

TABLE 1. REPORTED HOSTPLANTS OF *Junonia coenia*, INCLUDING SOURCES OF DATA

Plant family	Food plant	Reference
Scrophulariaceae	*Agalinus purpurea*	Tietz, 1972
	Agalinus maritima	Scott from Hafernik, personal communication
	Aureolaria grandiflora	Kendall, personal communication; Bowers, personal observation
	Antirrhinum sp.	Tietz, 1972; Harris, 1972; Emmel and Emmel, 1973
	Antirrhinum majus	Tietz, 1972; Scott, 1972
	Buchnera floridana	Lenczewski, 1980
	Castilleja purpurea	Kendall, personal communication
	Cymbalaria muralis	Scott, personal communication
	Digitalis sp.	Tietz, 1972
	Diplacus sp.	Shapiro, 1974
	Gerardia sp.	Harris, 1972
	Gerardia harperi	Lenczewski, 1980
	Gerardia purpurea	Scudder, 1889; Tietz, 1972
	Kickxia spuria	Scott, personal communication
	Linaria canadensis	Scudder, 1889; Tietz, 1972
	Linaria vulgaris	Tietz, 1972; Scott, 1975
	Linaria maroccana	Kendall, personal communication
	Maurandya antirrhiniflora	Kendall, personal communication
	Mimulus sp.	Tilden, 1970; Tietz, 1972; Emmel and Emmel, 1973; Shapiro, 1974
	Mimulus prob. *guttatus*	Hafernik, in litt.
	Orthocarpus lacerus	Shields, 1966
	Orthocarpus purpurescens	Scott, personal communication
	Penstemon azureus	Scott, personal communication
	Seymeria cassioides	Scott, personal communication
	Veronica sp.	Scott, 1975
	Veronica anagalis-aquatica	Scott, 1972
	Veronica comosa	Scott, 1972
Plantaginaceae	*Plantago coronopus*	Shapiro, 1974b
	Plantago erecta	Emmel and Emmel, 1973
	Plantago helleri	Kendall, personal communication
	Plantago lanceolata	Emmel and Emmel, 1973; Scott, 1975; Shapiro, 1974b
	Plantago major	Scott from Heitzmann, personal communication
	Plantago rugelli	Scott from Shapiro
	Plantago virginica	Scudder, 1889; Tietz, 1972
Verbenaceae	*Lantana involucrata*	Tietz, 1972
	Lippia (= *Phyla*) sp.	Scott, 1975
	Lippia (= *Phyla*) *lanceolata*	Shapiro, 1974a
	Lippia (= *Phyla*) *nodiflora*	Shapiro, 1974b
	Verbena prostrata	Howe, 1975
Acanthaceae	*Dyschoriste linearis*	Kendall, personal communication
	Ruellia nodiflora	Neck, 1976
	Ruellia runyoni	Kendall, personal communication
Cornaceae	*Acuba* (= *Aucuba*)	Harris, 1972

male collected sitting on *C. pilosus*, bird's beak (personal observation), suggested that this species might also be a potential host plant. *Cordylanthus rigidus* also grows at Jasper Ridge, but *J. coenia* has not been recorded from it. *Junonia coenia* has not been recorded from any other scrophulariaceous plant at Jasper Ridge, although there are many that contain iridoid glycosides, e.g., *Diplacus aurantiacus, Scrophularia californica, Orthocarpus densiflorus, Pedicularis densiflora,* and *Castilleja* sp. (Bowers, 1981, 1983).

The two *Cordylanthus* species were collected at Jasper Ridge and the *P. lanceolata* from the Stanford University campus.

Artificial Diet. The artificial diet was made using the recipe of Bowers (1983). To this basic diet was added either 1g dried *P. lanceolata* leaves (40.0 mg plant/g diet)(designation, AD + P); 0.05g of aucubin and catalpol, the major iridoid glycosides found in *P. lanceolata* (AD + I); 1 ml of the crude extract of *P. lanceolata* from which aucubin and catalpol were crystallized (AD + X); or nothing (AD).

The iridoid glycosides were crystallized from *P. lanceolata* using the charcoal adsorption method of Trim and Hill (1952) (see Bowers, 1983, for details). These white crystals were recrystallized three times, and thin-layer chromatography revealed them to be a mixture of aucubin and catalpol. Although the spots resulting from the TLC were of similar size and intensity, the ratio of aucubin to catalpol was not quantified.

The artificial diets were stored in covered plastic boxes in the refigerator at 3–5°C and larvae recieved pieces about $2 \times 2 \times 10$ mm. Larvae were fed ad libitum and given fresh food every two or three days.

Growth Rate and Survival. This experiment compared larval growth and survival on three plant species and two artificial diets. Egg hatch was synchronized by storing eggs laid over a period of several days in the refrigerator. Four replicates of groups of ten newly hatched larvae were placed on each of five foods and reared for 25 days. The foods used were: (1) *P. lanceolata* leaves, (2) *C. pilosus* leaves, (3) *C. rigidus* leaves, (4) artificial diet containing aucubin and catalpol, and (5) artificial diet with no additives. Larvae were weighed every 5 days for 25 days and the number of larvae surviving at those times noted.

Percent of larvae surviving and mean weight per larva were compared by the Student-Newman-Keuls range test (Sokal and Rohlf, 1969), using the arcsin transformation for percent larvae surviving.

Choice Test. This experiment compared two aspects of the interaction of *J. coenia* and iridoid glycosides. First, larvae were reared from egg hatch to the last instar on one of two diets—the artificial diet with *P. lanceolata* leaves added (AD + P) or the artificial diet with aucubin and catalpol added (AD + I). Then, in the last instar, individual larvae from both rearing

regimes were put into a choice test. The choices were as follows: (1) diet with no additives vs. diet with extract (AD vs. AD + X); (2) diet with no additives vs. diet with aucubin and catalpol (AD vs. AD + I); (3) diet with aucubin and catalpol vs. diet with extract (AD + I vs. AD + X); (4) diet with aucubin and catalpol vs. diet with *P. lanceolata* leaves (AD + I vs. AD + P). Forty larvae from each of the rearing regimes were used, ten in each treatment. All choice tests were conducted in growth chambers with the conditions as previously described. The larvae were given weighed pieces of each diet of approximately equal size which were placed about 1 cm apart in the middle of the dish. The dishes were oriented randomly within the growth chamber. Larvae were allowed to feed for 48 hr. The remaining diet was collected, dried, and weighed. Thus, the effect of rearing history on choice could be studied, as well as the choice that larvae made during the 48-hr test.

Consumption during the test was determined on a dry weight basis (Waldbauer, 1968; Bowers, 1983). The dry weight fed was calculated from the wet weight fed by calculating a wet weight to a dry weight conversion factor for each diet, based on weighing five pieces of each diet wet, drying them at 50°C for 96 hr, and weighing them dry. The mean of these five determinations was used as the conversion factor and was applied to each of the wet weights. The conversion factors for each diet were: (AD + P) = 0.1803; (AD + I) = 0.1774; (AD + X) = 0.1723; AD = 0.1594. Estimates of the amount of diet eaten for each larva included some negative numbers. These negative numbers reflect the small amounts eaten of the artificial diet with no additives, as well as the necessity of using a wet weight to a dry weight conversion method. To correct for this, the amounts eaten were adjusted by adding 6.1 mg to each, because 6.1 was the lowest "negative amount eaten". All statistics and figures reflect these recalculated values.

RESULTS

Growth and Survival. The number of larvae surviving after 25 days was 70% or more on the three plant species and the artificial diet with iridoid glycosides (AD + I) (Table 2). In contrast, larvae fed on artificial diet with no additives (AD) had lower survival, between 10 and 30% (Table 2). The Student-Newman-Keuls range test revealed that there were no significant differences among the four foods with survival of 70% or higher; but survival on these four diets was significantly higher than survival on AD (Table 2) ($P < 0.01$).

The Student-Newman-Keuls range test also showed that growth, as measured by weight gain, was significantly higher on *P. lanceolata* leaves than on any of the other foods (Table 2) ($P < 0.01$). Among the other foods, mean larval weight at day 25 was next highest on *C. pilosus,* followed by AD + I, and *C. rigidus,* and was lowest on AD (Table 2).

TABLE 2. PERFORMANCE OF LARVAE OF *Junonia coenia* ON DIFFERENT FOODS[a]

A. Survival at day 25	Food				
	C.r.	*P.l.*	AD + I	*C.p.*	AD
Mean survival (SD)	90 (8.16)	87.5 (9.57)	80 (8.16)	80 (0)	22.5 (9.57)
B. Weight at day 25	Food				
	P.l.	*C.p.*	AD + I	*C.r.*	AD
Mean weight (SD)	216.36 (46.64)	121.58 (19.23)	92.75 (13.95)	56.34 (18.61)	7.4 (3.12)

[a]Lines connect values that are not significantly different at the 0.01 level by the Student-Newman-Keuls range test (Sokal and Rohlf, 1969). Standard deviations are given in parenthesis. (A) Number out of 10 larvae surviving at day 25. (B) Mean weight of a larva at day 25. Although the weight of larvae reared on *C. rigidus* and AD are not significantly different at the 0.01 level, they are at the 0.05 level, and larvae on AD weigh significantly less than all other larvae. *P.l.* = *P. lanceolata*, *C.p.* = *C. pilosus*, *C.r.* = *C. rigidus*, AD = artificial diet with no additives, AD + I = artificial diet with iridoid glycosides.

Choice Test. Junonia coenia larvae in these choice tests preferred diets containing iridoid glycosides, whether as the pure compounds, the crude plant extract, or as ground up leaves of *P. lanceolata* (Table 3), suggesting that iridoid glycosides play an important role as larval feeding stimulants for *J. coenia* larvae.

As shown by the standard deviations in Table 3, there was a lot of variation in the amounts eaten by individual larvae. In only one instance, however, did two-way analysis of variance reveal significant differences among the replicates: in larvae reared on artificial diet with iridoid glycosides added (AD + I) and given a choice of artificial diet with iridoid glycosides and diet with ground up leaves of *P. lanceolata* (AD + I vs. AD + P) ($P < 0.025$). This variation may have obscured any significant differences between larvae reared on diet with pure iridoid glycosides (AD + I) and diet with plant extract (AD + X), although the means show a small reversal (Table 3).

No detectable differences occurred between the larvae reared on AD + P and AD + I in how they responded to the choices of diets. The concept of

TABLE 3. RESULTS OF CHOICE TESTS QUANTIFYING THE AMOUNTS OF ARTIFICIAL DIETS EATEN BY LAST INSTAR LARVAE OF *Junonia coenia*[a]

	Larvae reared on AD + P		Larvae reared on AD + I	
Choice	Mean (SD)	*P*	Mean (SD)	*P*
AD + I	17.9 (12.0)	N.S.	26.5 (13.4)	N.S.
AD + X	22.5 (17.1)		23.7 (12.6)	
AD	5.5 (4.7)	<0.005	12.8 (10.5)	<0.05
AD + I	16.9 (6.6)		26.1 (13.7)	
AD + I	12.0 (9.7)	<0.01	22.0 (13.2)	<0.025
AD + P	25.8 (9.5)		34.0 (17.8)	
AD	1.8 (.89)	<0.001	2.5 (.7)	<0.01
AD + X	21.3 (11.4)		17.4 (13.3)	

[a]Standard deviations are shown in parentheses. Two-way analyses of variance revealed a significant effect of diet on the amounts eaten by larvae in six of the eight choices.

conditioning (Jermy et al., 1968) suggests that larvae might prefer the diet they had been reared on over others, but such was not the case here. Larvae reared on artificial diet with aucubin and catalpol (AD + I), as well as those reared on diet with *P. lanceolata* leaves (AD + P), significantly preferred the diet with leaves (AD + P) (Table 3).

DISCUSSION

The results of these experiments show that larvae of *Junonia coenia* from northern California use iridoid glycosides as feeding attractants and stimulants. The larvae grew poorly and survivorship was low on an artificial diet without iridoid glycosides, while growth and survival on an artificial diet with iridoid glycosides was not different from that on some potential host-plants. Among the plants, *P. lanceolata* was clearly the best host plant, as might be expected: it seems to be the host-plant most commonly used by *J. coenia* at Jasper Ridge. Larvae feeding on the artificial diet containing aucubin and catalpol, however, showed weight gain and survival comparable to that on two other potential host plants, whereas the artificial diet with no additives clearly did not stimulate feeding in the larvae.

When given a choice between artificial diets with and without iridoid glycosides, larvae preferred diets containing iridoid glycosides, choosing the artificial diets with aucubin and catalpol (AD + I) or the crude extract (AD + X) over that with no additives, regardless of what the larvae had been reared on. Interestingly, larvae preferred the diet containing ground up

leaves of *P. lanceolata* over that containing only iridoid glycosides. This is the choice that had the potential to reflect possible differences in larval behavior due to the diet that larvae had been reared on: artificial diet with ground up leaves of *P. lanceolata* (AD + P) or artificial diet with iridoid glycosides (AD + I). There was no discernable effect of rearing history; larvae clearly preferred the diet containing leaf material of *P. lanceolata,* suggesting that there was a component of the leaf material in addition to the iridoid glycosides that affected food choice. The nature of this additional component is unknown; it may be another iridoid glycoside or some other as yet unidentified compound.

Host-plant records of *J. coenia* suggest that these insects use iridoid glycosides as indicators of appropriate larval host plants (Table 1). Representatives of the five plant families used by *J. coenia* are known to contain iridoid glycosides, although the particular species listed in Table 1 may not have been tested (Kooiman, 1972; Jensen et al., 1975). Records of host-plant genera in the Onagraceae, Compositae, and Crassulaceae (Scudder, 1889; Munroe, 1951; Harris, 1972; Teitz, 1972) are unsubstantiated by more recent observations; thus their status is unclear. It is possible that larvae will accept plants in these genera, although they are not known to contain iridoid glycosides.

Two host-plant records are particularly interesting in view of the use of iridoid glycosides as larval feeding stimulants. The first arises from host-plant records of *Mimulus.* In the past, the genus *Mimulus* included species now classified in the genus *Diplacus* (Thomas, 1961; Munz, 1968). Preliminary examination for iridoid glycosides of some members of the genus *Mimulus,* excluding *Diplacus;* by Kooiman (1972), were negative (*M. cardinalis* Dougl. ex Benth., *M. guttatus* DC; *M. lewisii* Pursh; *M. lutens* L.; *M. moschatus* Dougl.). In contrast, tests on *Diplacus aurantiacus* for iridoid glycosides were positive (Bowers, 1983). Thus some references to *J. coenia* feeding on *Mimulus* (no specific name given) may have referred to *Diplacus;* which does contain iridoid glycosides. However, Hafernik (in litt.) reported finding hundreds of *J. coenia* larvae feeding on *Mimulus,* probably *guttatus.* This suggests that *M. guttatus* may contain iridoid glycosides or that there may be another compound(s) which functions as a larval feeding stimulant for *J. coenia.*

A second unusual host-plant record is of larvae on *Aucuba* (Cornaceae). Harris (1972) reported an outbreak of *J. coenia* larvae on "Acuba" (probably *Aucuba japonica,* the gold-dust plant, a common introduced ornamental) in a local nursery in Georgia. As its generic name suggests, this species contains the iridoid glycoside aucubin (Jenson et al., 1975). This observation further substantiates the importance of iridoid glycosides in host-plant specificity of *J. coenia.*

The genus *Junonia* in North America is now considered to contain three species, *Junonia coenia, Junonia* sp. A, and *Junonia* sp. B (Harvey, 1984; Turner and Parnell, 1983). *Junonia coenia* is distinct from the other two species, which are very similar to each other, although they can be distinguished on the basis of several morphological characteristics (Harvey, 1983). *Junonia* sp. A is reported to feed on *Stachytarpheta jamaicensis* (Verbenaceae), *Ruellia tuberosa,* and *Blechum pyramidatum* (both Acanthaceae) (Harvey, 1984). Although these three plant species have not been tested, another species of *Stachytarpheta, S. mutabilis* was found to contain the iridoid glycoside ipolamiide (Bernays and De Luca, 1981), and the family Verbenaceae is known to contain iridoid glycosides (Jensen et al., 1975) and the Acanthaceae probably does as well (Wieffering, 1966; Bernays and De Luca, 1981). *Junonia* sp. B is reported from black mangrove, *Avicennia germinans* (Avicenniaceae) and *Lippia* (=*Phyla*) *nodiflora* (Verbenaceae) (Harvey, 1984). The Avicenniaceae is considered to be closely related to the Verbenaceae, and therefore may contain iridoid glycosides, although there is no report in the literature of any member of this family being tested for the presence of iridoid glycosides.

Iridoid glycosides play a key role as larval feeding stimulants for *Junonia coenia* and perhaps for the other two species of *Junonia* in North America as well. Larvae of butterflies in the genus *Euphydryas* also use iridoid glycosides as feeding stimulants (Bowers, 1983). Consideration of the genera of host plants used by the six species of *Euphydryas* and *J. coenia* shows that there is quite an overlap, but many notable differences (Bowers, 1983) (Table 1). For example, at Jasper Ridge Biological Preserve, *J. coenia* co-occurs with two species of *Euphydryas: E. editha* and *E. chalcendona.* Larvae of *J. coenia* have been found feeding on *Plantago erecta,* the primary host-plant of *E. editha* in this population (Brown, personal communication), but have not been reported from plants in other genera used by these two *Euphydryas* species, including *Diplacus, Scrophularia, Pedicularis,* and others. *Junonia coenia,* however, will feed on *Scrophularia californica* in the laboratory (personal observation). Thus, both *J. coenia* and the *Euphydryas* species feed on plants containing iridoid glycosides, which function as larval feeding stimulants, and appear to provide the underlying basis for host-plant specialization in these species. However, other factors, such as the discriminatory ability of ovipositing females or microhabitat, may act in ecological time to determine the patterns of host-plant utilization that are observed in nature.

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SEX PHEROMONE COMPONENTS OF FALL CANKERWORM MOTH, *Alsophila pometaria* Synthesis and Field Trapping[1,2]

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Abstract—(*Z,Z,Z,E*)-3,6,9,11-Nonadecatetraene and (*Z,Z,Z,Z*)-3,6,9,11-nonadecatetraene, sex pheromone components of *Alsophila pometaria*, were synthesized by stereoselective Wittig reactions and found to be spectroscopically and chromatographically identical to isolated natural material. Flight-tunnel bioassays and field-trapping experiments confirmed that the two tetraenes together with (*Z,Z,Z*)-3,6,9-nonadecatriene are sex pheromone components. While traps baited with either tetraene individually captured conspecific males in field-trapping experiments, addition of the triene, which captured no males by itself, to either tetraene resulted in synergistic responses.

Key Words—*Alsophila pometaria* (Harris), fall cankerworm, Lepidoptera, Geometridae, sex pheromone, (*Z,Z,Z,E*)-3,6,9,11-nonadecatetraene, (*Z,Z,Z,Z*)-3,6,9,11-nonadecatetraene, (*Z,Z,Z*)-3,6,9-nonadecatriene, synthesis, hydrocarbons.

INTRODUCTION

Recently we reported the identification of three novel unsaturated hydrocarbons, (*Z,Z,Z*)-3,6,9-nonadecatriene [(*Z,Z,Z*)-3,6,9-19:H], (*Z,Z,Z,E*)-3,6,9,11-nonadecatetraene [(*Z,Z,Z,E*)-3,6,9,11-19:H], and (*Z,Z,Z,Z*)-3,6,9,11-nonadecatetraene [(*Z,Z,Z,Z*)-3,6,9,11-19:H], from fall cankerworm, *Alsophila pometaria* (Harris) females (Wong, et al., 1984). The isolated

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compounds elicited strong electroantennographic (EAG) and flight-tunnel behavioral responses from male moths, thereby demonstrating their role as sex pheromone components. Our characterization of these unsaturated hydrocarbons constituted only the second reported example of sex pheromone components from a geometrid moth.

Initially, to confirm the identities of the isolated tetraenes, we synthesized a mixture of (*Z,Z,Z,E*)-3,6,9,11-19:H and the *Z,Z,Z,Z* isomer by the reaction of *n*-octyltriphenylphosphorane with (*Z,Z,Z*)-2,5,8-undecatrienal. Although the desired tetraenes were obtained, the synthesis was unsuitable for the preparation of larger quantities because of the lability of (*Z,Z,Z*)-2,5,8-undecatrienal. Further efforts directed toward the synthesis of the tetraenes have now resulted in the development of a facile, stereoselective route to both compounds.

We report here the synthesis of the two tetraenes and the results of flight-tunnel, EAG, and field-trapping experiments conducted using the synthetic triene and tetraenes during the fall of 1983.

METHODS AND MATERIALS

Synthesis. IR spectra were obtained on neat samples (NaCl disk) with a Perkin-Elmer 257 spectrophotometer. PMR spectra were recorded with a Varian EM-390 (90 MHz) spectrometer or a Bruker WM-360 (360 MHz) instrument on samples in $CDCl_3$ or C_6D_6. CMR spectra were also recorded on the Bruker WM-360 (90.5 MHz) instrument. Chemical shifts are reported in δ units relative to TMS. Chemical ionization (CIMS, isobutane reagent gas) and electron impact (EIMS) mass spectral analyses were obtained using Finnigan model 3300 and 4000 mass spectrometers, respectively, equipped with DB-5 columns (60 m $\times$ 0.32 mm ID). GC analyses were performed on a Hewlett-Packard 5790 gas chromatograph equipped with a 30-m $\times$ 0.32-mm ID fused-silica DB-1 column. HPLC separations were performed on a Spectra-Physics SP8700 liquid chromatograph equipped with a Spectroflow 773 spectrophotometer. Flash column chromatography was performed on Kieselgel 60 (40–63 μm, E. Merck, Darmstadt) as described by Still et al. (1978). All reactions requiring anhydrous conditions were run in flame-dried glassware under a positive pressure of dry nitrogen or argon. Tetrahydrofuran (THF) was distilled from sodium benzophenone ketyl just prior to use. Hexamethylphosphoramide (HMPA) was distilled under reduced pressure from calcium hydride and stored over activated 13x molecular sieves. Boiling points are uncorrected.

3,6-Nonadiyn-1-ol (III). To an ice-cold solution of 1-tetrahydropyranyloxy-3-butyne (I) [23.1 g, 150 mmol prepared from 3-butyn-1-ol (Farchan)

and dihydropyran by the procedure of Parham and Anderson (1948)] in 75 ml of dry THF was added 79 ml (158 mmol) of ethyl magnesium bromide (2 M solution in THF, Aldrich) over 1 hr. The reaction mixture was stirred for an additional 1.5 hr and then treated with cuprous bromide (0.86 g, 3 mmol, Aldrich 98%). After 0.5 hr, 35.7 g (150 mmol) of 1-tosyloxy-2-pentyne [II, prepared from 2-pentyn-1-ol (Farchan) and *p*-toluenesulfonyl chloride by the method of Sendega et al. (1968)] in 50 ml of dry THF was added dropwise over 1 hr to the ice-cold reaction mixture. The reaction was stirred for another 1.5 hr at 23°C and then quenched with 100 ml of saturated aqueous ammonium chloride. The organic layer was separated and the aqueous layer extracted twice with 200 ml of ether. The combined organic phase was washed with water (3 × 150 ml), dried over anhydrous magnesium sulfate, and concentrated with a rotary evaporator to give crude 1-tetrahydropyranyloxy-3,6-nonadiyne. Hydrolysis of the tetrahydropyranyl ether was carried out with 200 ml of 2% *p*-toluenesulfonic acid in methanol at 23°C for 18 hr. The reaction mixture was neutralized with sodium bicarbonate and concentrated to remove the methanol. Water was added to the residue which was then extracted with ether (3 × 100 ml). The ether extracts were dried over magnesium sulfate and concentrated to give the crude alcohol. Distillation afforded 14.7 g of pure III (72.2%); bp 70–72°C (0.3 torr). PMR (90 MHz, $CDCl_3$) δ 3.69 (2H, t, J = 6.5 Hz, —C$\underline{H}_2$—O—), 3.23 (1H, bs, —OH), 3.12 (2H, quintet, J = 2.2 Hz, —C≡C—C$\underline{H}_2$—C≡C—), 2.44 (2H, m, C≡C—C$\underline{H}_2$CH$_2$O—), 2.15 (2H, m, CH$_3$C$\underline{H}_2$C≡C—), 1.10 (3H, t, J = 7.4 Hz, C$\underline{H}_3$—).

(*Z,Z*)-*3,6-Nonadien-1-ol* (*IV*). To 19.0 g (140 mmol) of III in 100 ml of ethanol was added 2.0 g of Lindlar catalyst (Aldrich) and 0.5 ml of quinoline. The reaction mixture was stirred vigorously under an atmosphere of hydrogen at 23°C. After hydrogen uptake ceased (1.5 hr), the reaction mixture was filtered and the ethanol evaporated. The crude diene was dissolved in ether (100 ml), washed successively with 1 N HCl (100 ml), saturated aqueous sodium bicarbonate (2 × 100 ml), and saturated aqueous sodium chloride (2 × 100 ml), and dried over magnesium sulfate. After evaporation of the ether, distillation of the crude product resulted in 17.8 g (90.8%) of pure IV; bp 56–58°C (0.3 torr). PMR (90 MHz, $CDCl_3$) δ 5.42 (4H, m, —C$\underline{H}$=C$\underline{H}$-CH$_2$—C$\underline{H}$=C$\underline{H}$—), 3.62 (2H, t, J = 6.8 Hz, C$\underline{H}_2$—O—), 2.81 (2H, dd, J = 5.4 Hz and 5.4 Hz, —CH=CH—C$\underline{H}_2$—CH=CH—), 2.34 (2H, dt, J = 6.8 Hz, and 6.8 Hz, —CH=CH—C$\underline{H}_2$—CH$_2$—OH), 2.05 (2H, m, CH$_3$C$\underline{H}_2$CH=CH—), 1.30 (1H, bs, —O$\underline{H}$), 0.94 (3H, t, J = 7.5 Hz, C$\underline{H}_3$).

(*Z,Z*)-*1-Bromo-3,6-nonadiene* (*V*). Triphenylphosphine (16.4 g, 62.5 mmol) was added over 15 min to a vigorously stirred, ice-cold solution of IV (7.0 g, 50 mmol) and carbon tetrabromide (18.3 g, 55 mmol) in 200 ml of dry dichloromethane. After 1.5 hr, the dichloromethane was evaporated and

the residue triturated with hexane (4 × 50 ml). Concentration of the hexane extract under reduced pressure afforded the crude bromide which was distilled to give 8.9 g (99%) of V; bp 45–47°C (0.2 torr). PMR (90 MHz, $CDCl_3$) δ 5.35 (4H, m, —C<u>H</u>=C<u>H</u>—CH_2—C<u>H</u>=C<u>H</u>—), 3.30 (2H, t, J = 7.0 Hz, —C<u>H_2</u>Br), 2.85–2.45 (4H, m, —CH=CH—C<u>H_2</u>—CH=CH— and —CH=CH—C<u>H_2</u>CH_2Br), 1.99 (2H, m, CH_3C<u>H_2</u>CH=CH—), 0.88 (3H, t, J = 7.5 Hz, C<u>H_3</u>).

(*Z,Z*)-*3,6-Nonadienyltriphenylphosphonium Bromide* (*VI*). A mixture of V (6.27 g, 30.9 mmol) and triphenyl phosphine (8.5 g, 32.5 mmol) was sealed in a glass tube under argon and immersed in an oil bath held at 75°C for 44 hr. The tube was then opened and the crude product transferred to a round-bottom flask with dichloromethane. The residue left after evaporation of the dichloromethane was washed with ether (4 × 100 ml) to remove excess triphenylphosphine. Evaporation of the ether left 14.3 g (99.5%) of the viscous, hygroscopic salt (VI). PMR (90 MHz, $CDCl_3$) δ 7.75 (15H, m, P—<u>Ph</u>$_3$), 5.38 (4H, m, —C<u>H</u>=C<u>H</u>—CH_2—C<u>H</u>=C<u>H</u>—), 3.87 (2H, m, —$CH_2P^+Ph_3Br^-$), 2.53 (4H, m, —CH=CH—C<u>H_2</u>CH=CH— and —CH=CH—C<u>H_2</u>CH_2—), 1.88 (2H, m, CH_3C<u>H_2</u>CH=CH—), 0.87 (3H, t, J = 7.5 Hz, C<u>H_3</u>).

(*E*)-*2-Decenal* (*VII*). To 2-decyn-1-ol (7.7 g, 50 mmol, Farchan), in 50 ml of ethanol, was added 0.5 g of Lindlar catalyst. After hydrogen uptake ceased (3 hr), the reaction mixture was filtered to remove the catalyst and concentrated to give crude (*Z*)-2-decen-1-ol; PMR (90 MHz, $CDCl_3$) δ 5.52 (2H, m, —C<u>H</u>=C<u>H</u>—), 4.13 (2H, d, J = 5.3 Hz, —C<u>H_2</u>—OH), 2.00 (2H, m, —CH_2—C<u>H_2</u>—CH=CH—), 152, (1H, bs, —O<u>H</u>), 1.22 (10H, bs, CH_3—C<u>H_2</u>)$_5$—), 0.82 (3H, t, J = 6.4 Hz). The crude (*Z*)-2-decen-1-ol (4.68 g., 30 mmol) was added to an ice-cold slurry of pyridinium chlorochromate (9.69 g, 45 mmol) in 75 ml of dry dichloromethane. The dark brown mixture was stirred for 1.5 hr at 0°C followed by 2 hr at 23°C. The precipitate formed by the addition of ether (150 ml) was washed thoroughly with more ether (3 × 50 ml). Filtration of the ether extracts through a 20-cm × 2.5-cm ID column packed with Florisil followed by evaporation of the ether afforded 3.64 g (78.8%) of (*E*)-2-decenal; PMR (90 MHz, $CDCl_3$) δ 9.42 (1H, d, J = 8.4 Hz, —CHO), 6.76 (1H, dt, J = 16 Hz and 6.8 Hz, —C<u>H</u>=CH—CHO), 6.00 (1H, ddt, J = 16 Hz, 8.4 Hz, and 1.5 Hz, —CH=C<u>H</u>—CHO), 2.25 (2H, dt, J = 6.8 Hz, and 6.8 Hz, —CH_2—CH=CH—), 1.22 (10H, bs, CH_3—(C<u>H_2</u>)$_5$—), 0.78 (3H, t, J = 5.9 Hz). GC analysis of the product revealed the presence of 1.8% of (*Z*)-2-decenal.

2-Decynal (*VIII*). To a vigorously stirred suspension of pyridinium chlorochromate in 50 ml of dry dichloromethane was added 2-decyn-1-ol (3.85 g, 26 mmol) at 23°C. Work-up of the crude aldehyde was carried out as described for the preparation of VII. Kugelrohr distillation of the crude

product afforded 2.96 g (77.9%) of pure VIII; oven temperature 120–125° C (0.25 torr). PMR (90 MHz, $CDCl_3$) δ 9.12 (1H, S, —C<u>H</u>O), 2.30 (2H, t, J = 6.5 Hz, —C<u>H</u>$_2$—C=C—), 1.15 (10 H, bs, $CH_3(C\underline{H}_2)_5$—), 0.73 (3H, t, J = 5.8 Hz).

(Z,Z,Z,E)-3,6,9,11-Nonadecatetraene (IX). To an anhydrous solution of VI (2.69 g, 5.79 mmol), in a mixture of HMPA (15 ml) and THF (25 ml) at −45° C, was added 1.06 g (5.79 mmol) of sodium bis(trimethylsilyl)amide. The reaction mixture, which turned to a red-brown color upon addition of the base, was stirred for 30 min at −45° C and then treated with 0.89 g (5.79 mmol) of (*E*)-2-decenal (VII). After 3 hr, the reaction mixture, which had slowly warmed to 0° C, was diluted with water (30 ml) and extracted with hexane (3 × 20 ml). The hexane extract was washed with water (3 × 30 ml), dried over magnesium sulfate, and concentrated to give crude IX. Elution of the crude product through silica gel (15-cm × 2.5-cm ID column) with hexane afforded 1.0 g (66.4%) of the tetraene IX; PMR (360 MHz, C_6D_6) δ 6.47 (1H, dddd, J = 15.0, 11.0, 2.6, and 1.4 Hz, —C(H)=C(H)—C(H)=C(H)—), 6.09 (1H, dd, J = 11.0 and 11.0 Hz, —C(H)=C(H)—C(H)=C(H)—), 5.65 (1H, dt, J = 15.0 and 7.3 Hz, —C(H)=C(H)—C(H)=C(H)—), 5.45 (2H, m, —C(H)=C(H)—), 5.40 (2H, m, —C(H)=C(H)—), 5.34 (1H, m, —C(H)=C(H)—C(H)=C(H)—), 2.97 (2H, dd, J = 6.0 and 6.0 Hz, —C(H)=C(H)—C<u>H</u>$_2$—C(H)=C(H)—), 2.81 (2H, dd, J = 5.2 and 5.2 Hz, —C(H)=C(H)—C<u>H</u>$_2$—C(H)=C(H)—), 2.08–1.96 (4H, m, $CH_3C\underline{H}_2$—CH=CH— and —C(H)=C(H)—C<u>H</u>$_2$—CH$_2$), 1.23 (10H, bs, $CH_3(C\underline{H}_2)_5$—), 0.91 (3H, t, J = 7.6 Hz, $C\underline{H}_3CH_2$—CH=CH), 0.89 (3H, t, J = 7.0 Hz, —$(CH_2)_5C\underline{H}_3$). IR 3040 cm^{-1} (m, C—H stretch of alkenes), 2980 cm^{-1}, 2950 cm^{-1}, 2880 cm^{-1} (s, C—H stretch of alkanes) 1650 cm^{-1} (w, C—C stretch of alkene), 970 cm^{-1} (m, C—H bending of *E* double bond). CMR (90 MHz, $CDCl_3$) δ 135.4, 132.0, 129.0, 128.6, 127.9, 127.4, 127.1, 125.4, 32.9, 31.8, 29.4, 29.21, 29.17, 26.1, 25.6, 22.6,

20.6, 14.2, 14.0. EIMS (% relative abundance) 260 (0.4), 231 (0.2), 217 (0.1), 191 (0.1), 180 (2.1), 178 (2.8), 161 (1.2), 147 (1.0), 133 (1.7), 119 (4.6), 108 (61.1), 93 (25.3), 91 (21.9), 79 (100), 67 (23.8), 55 (16.9).

GC analysis revealed that 92.0% of the product was the desired (*Z,Z,Z,E*)-3,6,9,11-19:H. The remainder of the product was composed of the *Z,Z,E,E* isomer (3.0%), the *Z,Z,Z,Z* isomer (1.0%), and two unknown isomers (4.0%). Purification by HPLC on a Partisil M9 10/50 SCX column, loaded with silver ion (Merritt and Bronson, 1977) and eluted with 0.1% dimethoxyethane in hexane, gave material which was 97.2% pure (0.5% *Z,Z,Z,Z* isomer, 1.7% *Z,Z,E,E* isomer, and 0.6% unknown isomer). Analysis: Calc. for $C_{19}H_{32}$: 260.46. C, 87.62; H, 12.38. Found: C, 87.76; H, 12.29.

(*Z,Z,Z*)-3,6,9-Nonadecatrien-11-yne (X). Preparation of the ylide of VI (4.8g, 10.3 mmol) was carried out as described for the preparation of IX. To the red-brown solution of the ylide, held at −45° C, was added 1.57 g (10.3 mmol) of VIII. The reaction mixture was warmed to −10° C over a 1-hr period and worked up following the procedure described for the work-up of IX. Elution of the crude product through silica gel with hexane afforded 2.28 g (85.7% yield) of a light yellow liquid. GC analysis revealed the presence of the *Z,Z,E* isomer (8.1%) [PMR(90 MHz, $CDCl_3$) δ 6.0 (1H, dt, J = 15.8 Hz and 6.4 Hz, $-C(H)=C-C=C-$), 5.58-5.14 (5H, m, $-CH=CH-$), 2.76 [(4H, m, $-CH=CH-CH_2-CH=CH-$) 2.33-1.77 (4H, m,] $CH_3CH_2CH=CH-$ and $-C=C-CH_2-$), 1.22 (10H, bs, $CH_3(CH_2)_5-$), 0.90, and 0.82 (6H, overlapping t, CH_3-)] and two other unknown isomers (4.6%). Flash chromatography on silica gel (pentane elution) afforded material of 93.9% purity which still contained the *Z,Z,E* isomer (1.5%) and the two unknown isomers (4.6%); PMR (360 MHz, C_6D_6) δ 5.65 (1H, dt, J = 10.5 and 7.3 Hz, $-C(H)=C-C\equiv C-$), 5.55 (1H, m, $-C=C(H)-C\equiv C-$), 5.45 (2H, m, $-C(H_6)=C(H_7)-$), 5.41 (2H, m, $-C(H_3)=C(H_4)-$), 3.23 (2H, dd, J = 6.1 and 6.1 Hz, $-C(H)=C(H_7)-CH_2-C(H_9)=C(H)-$), 2.88 (2H, dd, J = 4.9 and 4.9 Hz, $-C(H)=C(H_4)-CH_2-C(H_6)=C(H)-$), 2.21 (2H, dt, J = 2.0 and 6.9 Hz, $-C\equiv C-CH_2-$), 2.02 (2H, dq, J = 5.6 and 7.5 Hz, $CH_3CH_2C(H)=C(H)-$), 1.44 (2H, m, $-C\equiv C-CH_2-CH_2-$), 1.18 (8H, bs, $CH_3(CH_2)_4-$), 0.91 (3H, t, J = 7.5 Hz, $CH_3CH_2CH=CH-$), 0.86 (3H, t, J = 7.1 Hz,

$CH_3(CH_2)_6$—). IR 3040 cm^{-1} (m, C—H stretch of alkenes), 2980 cm^{-1}, 2950 cm^{-1}, 2880 cm^{-1} (s, C—H stretch of alkanes), 2220 cm^{-1} (w, C—C stretch of disubstituted alkyne), 1650 cm^{-1} (w, C—C stretch of alkene). CMR (90 MHz, $CDCl_3$) δ 139.9, 132.0, 129.4, 127.1, 126.6, 109.8, 95.2, 77.2, 31.8, 28.93, 28.91, 28.81, 28.51, 25.7, 22.6, 20.6, 19.6, 14.2, 14.0. CIMS (% relative abundance) 287 (M + 29, 8.6), 259 (M + 1, 71.6), 243 (4.4), 231 (3.5), 217 (13.0), 203 (20.6), 189 (30.1), 175 (52.7), 163 (23.1), 149 (63.7), 135 (38.9), 119 (76.1) 109 (85.3), 95 (100), 91 (76.9), 83 (46.5), 79 (52.1), 69 (66.4).

(Z,Z,Z,Z)-3,6,9,11-Nonadecatetraene (XI). To a solution of the trienyne (X; 516 mg, 2 mmol) in 10 ml of dry pentane (0° C) was added 4.2 ml of 0.5 M dicyclohexylborane in pentane (Brown et al., 1977). After 2 hr at 0° C, the reaction mixture was diluted with 15 ml of THF and treated with 0.6 ml of glacial acetic acid for 3 hr at 50° C. The mixture was then made basic with 4 ml of 5.0 N aqueous sodium hydroxide and treated with 0.86 ml of 30% hydrogen peroxide for 0.5 hr. The hexane extract (3 × 30 ml) was washed with water (3 × 30 ml), dried over $MgSO_4$, and evaporated to leave an oily residue. Elution of the residue through silica gel (hexane) gave 292 mg (56.2%) of the desired (*Z,Z,Z,Z*)-3,6,9,11-19:H (89% isomeric purity). This material contained (*Z,Z,Z,E*)-3,6,9,11-19:H (1.1%) and three major unknown impurities (8.3%). Purification by HPLC on the silver-ion-loaded Partisil M9 10/50 SCX column (eluted with 0.1% dimethoxyethane in hexane) yielded material which was greater than 99.0% pure; PMR (360 MHz, C_6D_6) δ 6.40 (2H, overlapping d, J = 9.0 and 9.0 Hz, —C=C—C=C—), 5.48 (2H, m, H $\underline{H}_{10}$ $\underline{H}_{11}$ H

—C=C—C=C—), 5.44 (2H, m, —C=C—), 5.40 (2H, m, $\underline{H}_9$ H H $\underline{H}_{12}$ $\underline{H}_6$ $\underline{H}_7$

—C≡C—), 2.96 (2H, dd, J = 6.0 and 6.0 Hz, —C=C—C$\underline{H}_2$—C=C—), $\underline{H}_3$ $\underline{H}_4$ H H_7 H_9 H

2.80 (2H, dd, J = 5.4 and 5.0 Hz, —C=C—C$\underline{H}_2$—C=C—), 2.14 (2H, dt, H H_4 H_6 H

J = 7.4 and 7.0 Hz, C=C—C$\underline{H}_2$—CH_2—), 2.0 (2H, dq, J = 5.5 and 7.5 Hz, H H

$CH_3C\underline{H}_2C$=C—), 1.23 (10H, bs, $CH_3(C\underline{H}_2)_5$—), 0.90 (3H, t, J = 7.5 Hz, H H

$C\underline{H}_3CH_2C$=C—), 0.88 (3H, t, J = 7.0 Hz, $C\underline{H}_3CH_2CH_2$—). IR 3020 cm^{-1} H H

(m, C—H stretch of alkenes), 2980 cm^{-1}, 2950 cm^{-1}, 2880, cm^{-1} (s, C—H stretch of alkanes). CMR (90 MHz, $CDCl_3$) δ 132.8, 132.1, 129.4, 128.8, 127.7, 127.1, 123.9, 123.3, 31.8, 29.65, 29.26, 29.17, 27.6, 25.90, 25.60, 22.6, 20.6, 14.21, 14.03. EIMS (% relative abundance) 260 (0.3), 231 (0.2), 217 (0.2), 206 (0.2), 191 (0.1), 180 (1.5) 178 (0.8), 161 (1.2), 147 (1.1), 133 (1.8), 119 (5.0), 108 (67.5), 93 (26.3), 91 (24.1), 79 (100), 67 (25.6), 55 (18.2). Analysis: Calc. for $C_{19}H_{32}$: 260.46. C, 87.62; H, 12.38. Found: C, 87.66; H, 12.32.

(Z,Z,Z)-3,6,9-Nonadecatriene (XII). The preparation of this triene has been described by Underhill et al. (1983) and by Becker et al. (1983). More recently, we have synthesized XII by the coupling of lithium dimethylcuprate with (*Z*,*Z*,*Z*)-1-tosyloxy-9,12,15-octadecatriene (Conner et al., 1980).

Flight-Tunnel Tests. The flight-tunnel and experimental methods used have been reported (Underhill et al., 1983; Palaniswamy et al., 1983). Field collected males were held in an environmental chamber (15° C, 16:8 light-dark photoperiod) for at least 24 hr before experimentation. Adult moths were also obtained by collecting late-instar larvae from the field. The larvae were fed elm leaves and allowed to pupate in soil-filled containers. Sexed pupae were stored separately at 22° C until mid-October when they were placed outdoors in screen cages to emerge. Illumination within the tunnel was maintained at an intensity of 3.0 lux and all assays were done between 0.5 and 2.5 hr into scotophase. Moths were exposed to a treatment only once during a day and then returned to the environmental chamber for 24 hr before they were used again.

Field Trapping. Field tests were conducted with Pherocon ICP® traps (Zoecon Corp., Palo Alto, California) or Hara Traps (Hara Products Ltd., Swift Current, Saskatchewan), which are similar to the cone-orifice model 3 trap described by Steck and Bailey (1978). Traps were baited with chemical lures impregnated in rubber septa (A. H. Thomas, No. 8753-D22) or virgin females held within wire screen cages. Female baited traps were inspected daily and new females introduced in place of dead ones. Field trapping was carried out near Saskatoon, Saskatchewan in areas containing American and/or Siberian elm trees known to have been attacked by fall cankerworm larvae during June 1983. Two procedures were adopted for trap placement. When the line of traps was within 5 m of trees, traps were spaced 2 m apart with the ends facing perpendicular to the trap line. Traps within lines located at least 20 m from trees were spaced 12 m apart with the ends facing perpendicular to the trap line. In replicated tests, traps were set out in randomized block designs. To assess disorientation of males to monitoring traps, eight stakes 1.5 m high were placed in a square 3 m apart to form a test plot 36 m^2. A rubber septum attached near the top of each stake was used to release the potential disruptant in the test plot. An additional stake (1.5 m high) centered in the test plot carried a Pherocon 1CP monitoring trap baited with (*Z*,*Z*,*Z*,*E*)-

3,6,9,11-19:H (60 μg), (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-19:H (15 μg), and (*Z*,*Z*,*Z*)-3,6,9-19:H (25 μg). The extent of male orientation disruption was assessed by comparing the number of males captured in disruptant plots and in control sites which contained identically baited traps. All disruptant plots and control sites were separated by at least 150 m.

Statistical Analysis. Trap capture data were transformed $(X + 0.5)^{1/2}$ and subjected to analysis of variance. The means were compared by Duncan's new multiple-range test.

RESULTS AND DISCUSSION

The key steps in the syntheses of (*Z*,*Z*,*Z*,*E*)- and (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-19:H are the stereoselective Wittig reactions of two α,β-unsaturated aldehydes (VII and VIII) with the phosphorane of a common intermediate (VI). Formation of the (*Z*,*Z*)-3,6-nonadienyl carbon skeleton was achieved by a copper catalyzed Grignard coupling of the terminal alkyne (I) with the tosylate (II) (Verkruijsse and Hasselaar, 1979). Catalytic semihydrogenation of diyne III with Lindlar catalyst, poisoned with quinoline, afforded (*Z*,*Z*)-3,6-nonadien-1-ol (IV) in 99.6% purity after distillation. This preparation of IV gave a much better yield (66% from I) than a previous synthesis (33%) carried out by a slightly different route (Kajiwara et al., 1975). IV has recently been identified as a component of the sex pheromone for the Caribbean and Mexican fruit flies (Battiste et al., 1983). The alcohol (IV) was brominated with carbon tetrabromide and triphenylphosphine to give an 88% yield of V after distillation (Hooz and Gilani, 1968). Conversion of bromide V to the triphenylphosphonium salt occurred slowly and quantitatively in a sealed tube at 75°C. Although the reaction time could be decreased by using higher reaction temperature, thermal isomerization of the product made this approach undesirable. Similarly, attempted quaternization with triphenylphosphine in refluxing acetonitrile led to virtually complete isomerization of the product after only 3 hr. The hygroscopic phosphonium salt (VI) was dried under high vacuum and stored under dry argon until required.

The stereoselectivity of Wittig reactions between the phosphorane of VI and the aldehydes VII and VIII are dependent upon solvents, reaction temperature, and the base used for ylide generation. The use of *n*-butyllithium in THF–HMPA resulted in poor stereoselectivity (<85% isomeric purity) while the use of sodium hydride in dimethylformamide did not lead to any desired product. Optimum yields and isomeric purities were obtained for both IX and X with the use of sodium bis(trimethylsilyl)amide (Bestmann et al., 1976) in a mixture of THF and HMPA at −45°C. A reaction temperature lower than −45°C was unfeasible since VI crystallized below −45°C from the THF-HMPA mixture. On the basis of the quantities of IX and the

Z,Z,E,E isomer (isolated by reverse-phase HPLC and characterized by 400-MHz PMR spectroscopy) found in the product, the stereoselectivity of the Wittig reaction for the preparation of IX is 96.9*Z*:3.1*E*. Under virtually identical reaction conditions, a lower stereoselectivity (91.5 *Z*:8.5 *E*) was observed in the preparation of the trienyne X. This difference in stereoselectivity is probably a consequence of the greater electrophilicity of the carbonyl carbon in 2-decynal (VIII) compared to (*E*)-2-decenal (VII). As a result of this difference, betaine formation is more reversible with VIII, thus leading to a slightly increased proportion of the thermodynamically favored (*Z,Z,E*)-3,6,9-nonadecatrien-11-yne (Gosney and Rowley, 1979). Recently the sex pheromone of the processionary moth, (*Z*)-13-hexadecen-11-yn-1-yl acetate, was synthesized via a stereoselective Wittig reaction by Camps et al. (1983). These workers also discovered that the use of sodium bis(trimethylsilyl)amide at low temperature (−70° C) afforded the best stereoselectivity (95*Z*:5*E*).

The reduction of X to (*Z,Z,Z,Z*)-3,6,9,11-19:H was best effected with dicyclohexylborane in pentane at 0° C. The choice of solvent is critical in this reaction, since no reduction occurred in THF even at reflux temperature. Presumably the conformation of the molecule in polar solvents protects the triple bond from the bulky dicyclohexylborane. Although the product mixture contained some (*Z,Z,E,Z*)-3,6,9,11-19:H (1.1%, GC retention time identical to authentic material isolated by reverse-phase HPLC and characterized by 400-MHz PMR spectroscopy from an earlier preparation) along with three other uncharacterized isomeric impurities (8.3%), none of the *Z,Z,Z,E* isomer could be detected. Thus the dicyclohexylborane reduction must be highly stereospecific, since the isomeric impurities were probably derived from impurities present in the starting material (X). Attempted reductions of X with Lindlar catalyst and P2-nickel failed completely due to extensive isomerization of the product.

The stereoselective synthesis of (*Z,Z,Z,E*)-3,6,9,11-19:H was achieved in five steps from I with an overall yield of 40%. Six steps, with a 28% overall yield, were required for the synthesis of (*Z,Z,Z,Z*)-3,6,9,11-19:H. Both tetraene hydrocarbons could be purified to 97% purity or better by HPLC on a silver-ion-loaded strong cation-exchange column. The synthetic compounds were spectroscopically and chromatographically identical to the compounds isolated from female fall cankerworm moths. EAG responses of 2–3 mV were elicited by the two tetraenes separately applied to filter paper disks at a dose of 0.01 μg. A much higher dose of the triene (1.0 μg) was required to elicit a similar response.

Prior to extensive field tests of the synthetic compounds, the efficacy of HPLC-purified tetraenes [(*Z,Z,Z,E*)-3,6,9,11-19:H (97.2%) and (*Z,Z,Z,Z*)-3,6,9,11-19:H (>99%)] were compared to that of unpurified material (92% and 89% respectively). Initially we tested a three-component mixture

containing (*Z,Z,Z,E*)-3,6,9,11-19:H, (*Z,Z,Z,Z*)-3,6,9,11-19:H, and (*Z,Z,Z*)-3,6,9-19:H in the ratio of 60:15:25. This ratio was chosen because it approximated the ratio found in the dichloromethane extract of ovipositors (Wong et al., 1984). The results of this test (Table 1) showed that captures in traps baited separately with purified and unpurified compounds were not significantly different at the doses and ratio tested. Since the impurities present in unpurified (*Z,Z,Z,E*)-3,6,9,11-19:H and (*Z,Z,Z,Z*)-3,6,9,11-19:H neither inhibited nor potentiated the response of male moths, we used the unpurified compounds for further field tests. The absence of a dose–response relationship between the traps baited with total doses of 100 and 500 μg may be due to a maximum release rate for our single septum formulation at the 100-μg dose. The release of long-chain hydrocarbons from rubber septa is very slow, less than 1 μg/day for (*Z,Z,Z*)-3,6,9-20:H (unpublished results), and as a result we do not know if release of our baits is proportional to the applied dose. Heath et al. (1983) have also reported similar observations for the velvetbean caterpillar moths with (*Z,Z,Z*)-3,6,9-20:H and (*Z,Z,Z*)-3,6,9-21:H in half-septa formulations.

Our initial assessment of the flight tunnel behavioral responses of male moths to compounds isolated from female ovipositors revealed that the two tetraenes individually elicited wing fanning, plume-oriented flight, and landing on the source (Wong et al., 1984). To further assess the role of the tetraenes, traps baited with the synthetic tetraenes at various doses were set out. Both tetraenes captured male fall cankerworms specifically with threshold doses of approximately 30 μg (Table 2). However, the *Z,Z,Z,E* isomer exhibited greater potency by capturing more than twice the number caught by the *Z,Z,Z,Z* isomer at the 100-μg dose. In a separate experiment, various combinations of the two tetraenes were compared to the single components (Table 3). None of the binary mixtures captured significantly more moths than the *Z,Z,Z,E* isomer alone.

TABLE 1. CAPTURE OF *A. pometaria* MALES IN TRAPS BAITED WITH SYNTHETIC COMPOUNDS (*Z,Z,Z,E*)-3,6,9,11-19:H, (*Z,Z,Z,Z*)-3,6,9,11-19:H, AND (*Z,Z,Z*)-3,6,9-19:H OF DIFFERENT PURITIES

Lure composition (μg, purity)			Total males captured[a]
(*Z,Z,Z,E*)-3,6,9,11-19:H	(*Z,Z,Z,Z*)-3,6,9,11-19:H	(*Z,Z,Z*)-3,6,9-19:H	
60 (97%)	15 (>99%)	25 (>98%)	73 a
300 (97%)	75 (>99%)	125 (>98%)	66 a
300 (92%)	75 (>89%)	125 (>98%)	75 a

[a]3× replicated: Pherocon ICP traps, September 28–October 6, 1983. Values followed by the same letter are not significantly different ($P = 0.05$).

TABLE 2. CAPTURE OF *A. pometaria* MALES IN TRAPS BAITED WITH SYNTHETIC (*Z,Z,Z,E*)-3,6,9,11-19:H OR (*Z,Z,Z,Z*)-3,6,9,11-19:H AT VARIOUS DOSES

Lure composition (μg)	Total males captured[a]
(*Z,Z,Z,E*)-3,6,9,11-19:H	
100	73 a
30	46 ab
10	11 c
3	9 c
1	3 c
(*Z,Z,Z,Z*)-3,6,9,11-19:H	
100	35 b
30	28 b
10	2 c
3	3 c
1	1 c

[a]3× replicated: Pherocon 1CP traps, October 12–14, 1983. Values followed by the same letter are not significantly different ($P = 0.05$).

The role of (*Z,Z,Z*)-3,6,9-19:H, which captures no fall cankerworm males by itself, was explored in two experiments. In one experiment, Hara traps were baited with various combinations of (*Z,Z,Z,E*)-3,6,9,11-19:H, (*Z,Z,Z,Z*)-3,6,9,11-19:H, and (*Z,Z,Z*)-3,6,9-19:H. The results (Table 4) show that addition of the triene to (*Z,Z,Z,E*)-3,6,9,11-19:H clearly increases the trap captures compared to traps baited only with the two tetraenes, individually or together. While the same potentiation was not observed for the combination of the triene and the (*Z,Z,Z,Z*)-tetraene in this experi-

TABLE 3. CAPTURE OF *A. pometaria* MALES IN TRAPS BAITED WITH VARIOUS RATIOS OF (*Z,Z,Z,E*)-3,6,9,11-19:H AND (*Z,Z,Z,Z*)-3,6,9,11-19:H

Lure composition (μg)		
(*Z,Z,Z,E*)-3,6,9,11-19:H	(*Z,Z,Z,Z*)-3,6,9,11-19:H	Total males captured[a]
100	0	180 a
90	10	193 a
80	20	186 a
50	50	157 ab
25	75	139 ab
10	90	134 ab
0	100	109 b

[a]4× replicated: Pherocon 1CP traps, October 8–10, 1983. Values followed by the same letter are not significantly different ($P = 0.05$).

TABLE 4. CAPTURE OF *A. pometaria* MALES IN HARA TRAPS BAITED WITH VARIOUS COMBINATIONS OF (*Z,Z,Z,E*)-3,6,9,11-19:H, (*Z,Z,Z,Z*)-3,6,9,11-19:H, AND (*Z,Z,Z*)-3,6,9-19:H

Lure composition (μg)			Total males captured[a]
(*Z,Z,Z,E*)-3,6,9,11-19:H	(*Z,Z,Z,Z*)-3,6,9,11-19:H	(*Z,Z,Z*)-3,6,9-19:H	
60	15	25	21 ab
60	0	25	31 a
0	15	25	12 bc
60	15	0	3 c
60	0	0	3 c
0	15	0	2 c

[a]4× replicated: Hara traps, October 12–24, 1983. Values followed by the same letter are not significantly different ($P = 0.05$).

ment, this may be due to the subthreshold dose of tetraene that was used. The three component mixture was not better than the binary mixture of triene and (*Z,Z,Z,E*)-tetraene. To eliminate the uncertainty caused by the use of low doses of synthetic compounds, a second experiment was performed. Pherocon 1CP traps were baited with various ratios of the tetraenes and triene at higher doses. The results of this experiment (Table 5) clearly show that captures of male moths by both tetraenes are significantly increased by addition of the triene. Again, no improvement in trap captures was observed for any of the three-component baits compared to the triene-tetraene binary mixtures.

TABLE 5. CAPTURE OF *A. pometaria* MALES IN PHEROCON 1CP TRAPS BAITED WITH VARIOUS COMBINATIONS OF (*Z,Z,Z,E*)-3,6,9,11-19:H, (*Z,Z,Z,Z*)-3,6,9,11-19:H, AND (*Z,Z,Z*)-3,6,9-19:H

Lure composition (μg)			Total males captured[a]
(*Z,Z,Z,E*)-3,6,9,11-19:H	(*Z,Z,Z,Z*)-3,6,9,11-19:H	(*Z,Z,Z*)-3,6,9-19:H	
120	30	50	109 a
50	150	25	97 ab
0	150	25	92 ab
50	0	25	80 ab
50	150	0	55 bc
50	0	0	35 c
0	150	0	38 c

[a]6× replicated: Pherocon 1CP traps, October 19–22, 1983. Values followed by the same letter are not statistically different ($P = 0.05$).

In another experiment our synthetic compounds were compared to virgin females. Pherocon 1CP traps baited with (*Z*,*Z*,*Z*,*E*)-3,6,9,11-19:H (60 μg), (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-19:H (15 μg), and (*Z*,*Z*,*Z*)-3,6,9-19:H (25 μg) were set out at the same time as traps baited with virgin females which had emerged over the previous five days. Treatments, replicated three times, consisted of traps which contained three females of the same age. Males were captured over a 15-day period (October 10–24, 1983) in both chemical and female-baited traps. On five days during the experiment (October 16–20), no males were captured in any of the traps. For those days on which males were captured, the mean number of males captured/trap/day was 2.99 ± 3.58 ($N = 133$) and 10.4 ± 11.1 ($N = 30$) for the female- and chemical-baited traps, respectively. The number of males captured by female-baited traps never exceeded the captures by chemical-baited traps. Male captures in traps baited with females varied greatly within replicates and from day to day, and no correlation was observed between age of females and number of males captured. However, a correlation was observed between mean daily temperature and captures of fall cankerworm. On the days during which the mean temperature was less than 6°C, no males were captured. This result suggested either a lack of flight activity or a threshold temperature for response to pheromone.

Although our initial report (Wong et al., 1984) suggested that *n*-nonadecane did not function as a sex pheromone component for the fall cankerworm, we set out traps baited with the three-component mixture (IX, 60 μg; XI, 15 μg; XII, 25 μg) and compared them to traps baited with the same mixture plus 25 μg of *n*-nonadecane (3× replicated). No significant difference ($P = 0.05$) was observed in the male captures, thus corroborating our previous observations.

To assess the potential for mating disruption of the fall cankerworm, we set out a preliminary trial for the disruption of male orientation to synthetic pheromone baits as described in Methods and Materials. During the 2 days prior to the placement of the disruptant septa, the test sites and control sites were monitored with chemical-baited traps. The number of males captured in the test sites (13) and the control sites (15) were similar. Results of the experiment (Table 6) ranged between 80% and 100% disruption of male orientation. Although lacking in a number of important details, this preliminary test demonstrates that male orientation to a pheromone source can be disrupted and suggests the potential for pheromone-mediated disruption of mating.

During the course of our studies problems were encountered in obtaining replicated trap captures. These problems were traced mainly to trap placement and trap design. The fall cankerworm is intimately associated with its host tree, and consequently population density is highest near host trees (Schneider, 1980). Thus a trap may capture more males than its replicate

TABLE 6. CAPTURE OF *A. pometaria* MALES IN PHEROCON 1CP TRAPS IN UNTREATED PLOTS AND PLOTS TREATED WITH (*Z*,*Z*,*Z*,*E*)-3,6,9,11-19:H + (*Z*,*Z*,*Z*,*Z*)-3,6,9,11-19:H + (*Z*,*Z*,*Z*)-3,6,9-19:H

Date	Total males captured[a]		Disruption (%)[b]
	Disruptant plots	Control plots	
Oct. 4	2	10	80
Oct. 5	2	19	89
Oct. 6	0	4	100
Oct. 7	9	65	86

[a]Total number of males caught in 3× replicated treatments.
[b]% Disruption = [(Number caught in control plots) − (Number caught in disruptant plots)/ Number captured in control plots)] × 100.

if it is located closer to a host tree. Placing the traps further from trees (>20 m) reduces the problem caused by nonuniform local populations of fall cankerworms, but makes trap captures highly dependent on wind direction. The trap placement procedures we have described yielded reproducible results.

Neither of the two types of traps employed were completely satisfactory. At the commencement of the flight period, the presence of fall cankerworm males was noted only in the Pherocon 1CP traps. However, these sticky traps became functionally saturated after capturing approximately 40 males, and at peak emergence (October 7–10), most became saturated within 24 hr. The Hara traps captured approximately 5% of the number captured in the Pherocon traps and were favored under conditions of high population. Males approached both types of traps with similar efficiencies in flight-tunnel tests; however, most of those which landed on the cone-shaped screen of the Hara trap failed to enter through the trap orifice and flew away after a short period.

The results obtained from flight-tunnel bioassays of the synthetic compounds corroborated the results obtained in our field-trapping experiments. When tested individually, both tetraenes elicited a complete sequence of behavioral response from activation to landing, while the triene failed to elicit any observable response. The results are in complete agreement with our earlier results obtained with compounds isolated from female moths (Wong et al., 1984). In a choice test comparing the two tetraenes (Table 7, entry 1) male moths landed predominantly on the septum impregnated with the *Z*,*Z*,*Z*,*E* isomer, thus supporting our hypothesis that the *Z*,*Z*,*Z*,*E* isomer is more potent than the *Z*,*Z*,*Z*,*Z* isomer. The synergistic effect of (*Z*,*Z*,*Z*)-3,6,9-19:H is also clearly shown in the flight-tunnel choice tests (Table 7, entries 2 and 3). Male moths showed a preference for the septum containing

TABLE 7. LANDING RESPONSE OF *A. pometaria* MALES TO SYNTHETIC PHEROMONE COMPONENTS IN FLIGHT TUNNEL CHOICE TESTS

Entry	Composition of lures (μg)[a]		No. of moths initiating flight[b]	Landing (%)	
	A	B		A	B
1	(*Z,Z,Z,E*)-3,6,9,11-19:H (30)	(*Z,Z,Z,Z*)-3,6,9,11-19:H (30)	10	70	20
2	(*Z,Z,Z,E*)-3,6,9,11-19:H (30) + (*Z,Z,Z*)-3,6,9-19:H (30)	(*Z,Z,Z,E*)-3,6,9,11-19:H (30)	11	73	27
3	(*Z,Z,Z,E*)-3,6,9,11-19:H (10) + (*Z,Z,Z,Z*)-3,6,9,11-19:H (30) + (*Z,Z,Z*)-3,6,9-19:H (5)	(*Z,Z,Z,E*)-3,6,9,11-19:H (10) + (*Z,Z,Z,Z*)-3,6,9,11-19:H (30)	25	68	24

[a]Lures A and B were positioned 10 cm apart at the upwind end of the flight tunnel.
[b]Refers to forward or upwind progress of those moths which, when released from their holding cages, orient in and follow the plume for at least 10 cm from the release point.

the triene plus tetraene when offered as a choice against the tetraene alone. The choice tests failed to show synergism between the two tetraenes (results not shown) and thus supported our field-test results (Tables 3–5).

All of the evidence that we have gathered indicates that (*Z,Z,Z,E*)-3,6,9,11-19:H, (*Z,Z,Z,Z*)-3,6,9,11-19:H, and (*Z,Z,Z*)-3,6,9-19:H are sex pheromone compounds for the fall cankerworm. These female-produced compounds elicit upwind flight and mating behavior from male moths. The specific role of the triene appears to be in maintaining upwind flight near the source and in landing, based on our previous flight tunnel observations (Wong et al., 1984). Increased captures of male moths in field traps containing tetraene and triene strengthens this hypothesis. Our results, however, have not allowed us to distinguish different behavioral responses for the two tetraenes. The success we achieved on the preliminary trial disruption of male orientation is encouraging and, as a result, we plan to continue work toward the goal of control of the fall cankerworm by mating disruption with synthetic pheromones.

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RELATIVE KAIROMONAL ACTIVITIES OF 2-ACYLCYCLOHEXANE-1,3-DIONES IN ELICITING OVIPOSITION BEHAVIOR FROM PARASITE *Nemeritis canescens* (GRAV.)

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Abstract—The relative activities of sixteen 2-acylcyclohexane-1,3-diones from the larval mandibular glands of *Ephestia* (=*Anagasta*) *kuehniella* Zeller in causing the parasite *Nemeritis* (=*Venturia*) *canescens* (Grav.) to make oviposition movements are reported.

Key Words—Kairomone, 2-acylcyclohexane-1,3-diones, oviposition *Ephestia kuehniella* Zeller [syn. *Anagasta kuehniella* (Zeller)], Lepidoptera, Pyralidae, *Nemeritis canenscens* (Grav.) [syn. *Venturia canescens* (Grav.)], Hymenoptera, Ichneumonidae.

INTRODUCTION

A series of studies (for example, Lewis et al., 1975a,b; Gross et al., 1975) demonstrates the possibility of manipulating the behavior of parasites with kairomones to increase their effectiveness. Utilization of kairomones in pest control, particularly in conjunction with inundative releases of entomophagous insects, appears promising (see Gross, 1981, for a review) and is receiving inceasing attention.

Previous work (Mudd and Corbet, 1982) showed that a series of novel 2-acylcyclohexane-1,3-diones from the mandibular gland secretion of *Ephestia* (=*Anagasta*) *kuehniella* Zeller larvae elicited oviposition responses from the larval parasite *Nemeritis* (=*Venturia*) *canescens* (Grav.). Using larger amounts of the secretion and with improved separation techniques, eight new naturally occurring 2-acylcyclohexane-1,3-diones (compounds

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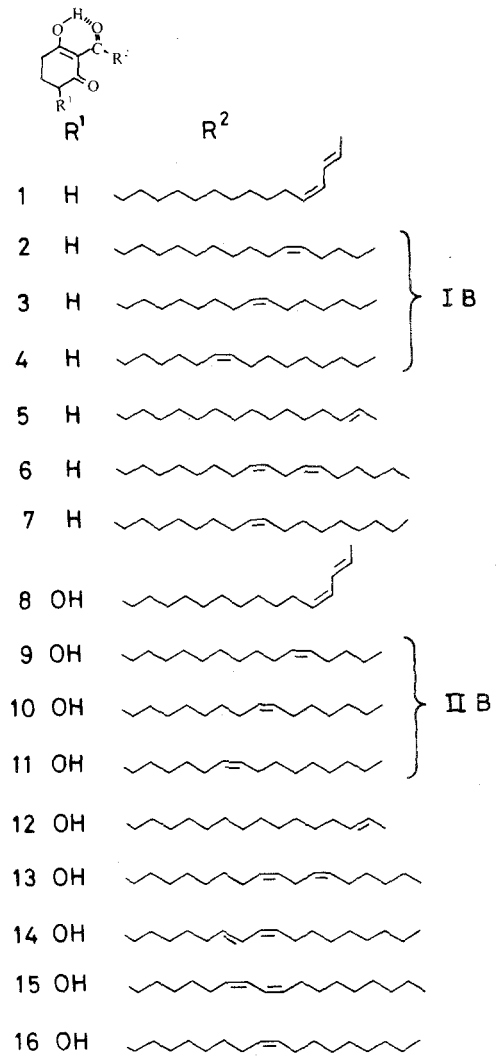

FIG. 1. Compound identified from mandibular gland secretions.

2,3,4,5,6,13,14, and 15, Figure 1) were identified (Mudd, 1983). We now report on the relative activities of these compounds in eliciting oviposition movements from *Nemeritis*.

METHODS AND MATERIALS

The isolation and identification of the components (Figure 1) have been reported (Mudd, 1981, 1983). Three pairs of components (5 and 6, 12 and

13, 14 and 15, Figure 1) which chromatographed as single peaks in high-pressure liquid chromatography (HPLC) on silica and reverse-phase ODS3 columns were separated by HPLC on an ion-exchange column loaded with silver ions (Chromopack, Nucleosil 10 SA Ag^+, 25 cm × 4.6 mm). Fractions IB and IIB chromatographed as single components on all HPLC columns under a wide variety of conditions and were therefore tested as such.

The bioassay procedure was similar to that described earlier (Mudd and Corbet, 1981). Each of the 12 components (10 μg) was placed, on a glass cover slip, in a muslin-covered arena (1 cm high and 15 cm diam.) containing 12 parasites, and the number of oviposition movements made by the parasites in a 5-min period was recorded. In this experiment, the Latin-square design used 12 replicate groups of insects forming the rows and 12 time periods forming the columns to enable time-dependent changes in responsiveness to be eliminated. As before, the insect groups were tested in the same order during each time period so that each group had 60 min to recover between each of the 12 tests to which it was exposed. Each treatment was preceded once by each other treatment to eliminate any carry-over effects from exposure of the parasites to the previous compound, but none were detected.

The higher standard errors of the differences of the means (SED) in this bioassay (Table 1) compared with earlier work are attributable to variations in response within insect groups reflecting a less homogeneous insect population. For example, a slightly wider age range among the insects tested had to be accepted to obtain the much larger number of parasites required for this experiment, and a smaller proportion responded positively. Whether or not particular active individuals performed within the test period greatly

TABLE 1. RELATIVE ACTIVITY OF COMPOUNDS FROM MANDIBULAR SECRETIONS

Component[a]	Mean of $\log_{10}$ (number of movements + 1)	Activity rating
1	1.550	High
IB	0.584	Low
5	0.963	Medium
6	0.535	Low
7	0.738	Medium
8	1.503	High
IIB	0.548	Low
12	0.477	Low
13	1.086	Medium
14	1.820	High
15	1.570	High
16	1.676	High
SED	0.220	

[a]See Figure 1.

influenced the result. Nevertheless, as before, differences were clearly apparent in the parasites' oviposition behavior towards the various kairomonal components.

RESULTS AND DISCUSSION

The relative activities of the components can be divided into three distinct groups having high, medium, or low activity as indicated in Table 1. Previous work (Mudd and Corbet, 1982) showed that conjugated unsaturation in the side chain was important for high activity and that the hydroxyl group at C-4 also contributed to the activity. These findings are supported by the present results since all but one of the compounds with high activity have conjugated unsaturation in the side chain. The difference between the hydroxylated and corresponding unhydroxylated compounds is less marked but only in one instance is the hydroxylated compound (12) less active than its unhydroxylated counterpart (5). The unresolved mixtures, fractions IB and IIB were, as expected from earlier work, of low activity. The higher activity of the conjugated dienes (1, 8, 14, and 15) was confirmed and also the relatively high activity of compound 16, 4-hydroxy-2-oleoylcyclohexane-1,3-dione.

Experiments to explore the potential use of these compounds in pest control require larger amounts of material than can be conveniently isolated from natural sources. Previous work showed that the activity of the natural secretion could be equaled or exceeded by the same amounts (10 μg) of the more active individual components so that single components should be effective for these experiments. The present work indicates the most appropriate active components for synthesis and use in such experiments to be compounds 1, 8, 14, 15, or 16, but the final choice depends on a variety of factors such as stability, ease and, therefore, cost of synthesis.

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POSSIBLE CHEMICAL BASIS FOR HISTOCOMPATIBILITY-RELATED MATING PREFERENCE IN MICE

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Abstract—High-resolution chromatographic profiles of urinary volatiles were quantitatively recorded and statistically evaluated for the female mice genetically differing in a small region of the major histocompatibility complex on the 17th chromosome. Both immature and estrogenized animals were evaluated. While there seem to be no specific volatile products of the histocompatibility genes, statistically significant differences were readily observed with the immature females of different haplotypes, involving the general range of secondary volatile metabolites. Their possible role in olfactory communication is discussed.

Key Words—Female mice, mating preference, major histocompatibility complex, olfactory communication, capillary gas chromatography, *Mus musculus*, urine volatiles.

INTRODUCTION

Chemical communication plays a very important role in the reproductive physiology and behavior of the common house mouse, *Mus musculus*. Primer pheromones (for a review, see Whitten and Champlin, 1972; Bronson, 1979) as well as various behavioral traits, such as aggression and protection from aggression (Mugford and Nowell, 1970), territorial marking (Jones and Nowell, 1973), and male–female attraction (Scott and Pfaff, 1970; Bellamy and Davies, 1971) have been investigated extensively.

There is considerable evidence that various forms of chemical communi-

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cation in the mouse are genetically influenced. Thus, certain strains appear to impart enhanced sensitivity towards pregnancy blockage in the females, while different males are endowed with lesser or greater ability to produce this effect (Hoppe, 1975). Chapman and Whitten studied the genetics of the pregnancy-blocking pheromone and found it to be a recessive trait, apparently controlled by a single gene for both males and females (Chapman and Whitten, 1969). The genetic component of male mouse pheromonal facilitation of PMSG- or HCG-induced ovulation has also been investigated (Zarrow et al., 1971), revealing that the C57BL strain males are weak producers of the pheromone responsible for this effect. In addition, the releaser pheromone responsible for intermale aggression is known to have a genetic dependency. Recent research regarding the genetics of this pheromone production by the male may be related to the observed genetic variations of testosterone levels (Bartke, 1974).

It has also been suggested that the major histocompatibility gene complex (H-2) exerts influence over mate selection under laboratory conditions. Males of a given H-2 genotype showed either a preference for or against females of their own type, depending on the haplotypes investigated (Yamazaki et al., 1976). Mating preference tests were performed by presenting single males with a choice between two estrous females, one of the same H-2 type and the other of a different H-2 type. When no H-2 influence over preference was observed, males chose either females 50% of the time. In trials with B10 ($H\text{-}2^b$) and B10.A ($H\text{-}2^a$) congenic strains, males of both H-2 haplotypes chose against their own females in 60% of the cases. With a different strain, BALB ($H\text{-}2^d$) and BALB ($H\text{-}2^b$), homozygous $H\text{-}2^d$ males did not demonstrate a preference in the first test but did exhibit consistency of choice in subsequent trials: for example, $H\text{-}2^d$ animals in 68% of subsequent matings. While it is not yet clear whether females actively participate in choosing their mate, a conclusion was reached (Yamazaki et al., 1976) that males discriminate against females of certain H-2. Thus, both the information transmission (by the females) and reception and recognition (by the males) appear to be under some influence of the major histocompatibility complex.

Further experiments with a Y-maze arrangement, using groups of male mice as odor sources and trained males or females as test subjects, indicated (Yamazaki et al., 1979) that both males and females were able to discriminate different H-2 odor sources; the experimental arrangement prevented tactile and visual signals, with olfaction being the only source of information. The following tests using recombinant strains (Andrews and Boyse, 1978; Yamaguchi et al., 1978) suggested that several sites in H-2 could be responsible for production of the olfactants involved in recognition through the Y-maze trials. While it is unlikely that the genes directly control production of specific olfactants, some qualitative and/or quantitative differences in the "mouse odor display" could be caused by H-2 through the secondary metab-

olism. Mouse urine is a highly effective source (Yamazaki et al., 1979) of this H-2-based olfactory discrimination.

While the volatile fraction of mouse urine is exceedingly complex, its individual components can effectively be separated by capillary gas chromatography (GC) and identified through gas chromatography/mass spectrometry (GC/MS). In the initial investigations of this laboratory, quantitative comparisons of chromatograms obtained from the urine samples of different mouse strains readily revealed that the differences in background strain were far more obvious than differences due to H-2 (Jorgenson, 1979).

It is expected that any differences in olfactory cues based on H-2 (which represent only a small section of the total genome) are likely to be more subtle than the influence of a background strain (for which many variations in the genome may exist). The very existence of this H-2-related olfactory discrimination appears to indicate that (1) mice have a remarkable capability to distinguish small quantitative differences within a complex profile of odorants, and (2) H-2 may exert an exceptionally powerful influence over secondary metabolism.

In this report, capillary chromatograms obtained from the urines of different H-2 females were statistically compared to provide chemical evidence in support of earlier biological experiments concerning the existence of H-2 olfactory discrimination. In addition, a possible relationship of urinary volatile profiles to serological sensitivities is postulated.

METHODS AND MATERIALS

General Procedures. Although male mice were employed as odor sources in the Y-maze experiments (Yamazaki et al., 1979), females were used in this study because of the more straightforward design of the earlier mating preference work in which males discriminated between females of different H-2 type (Yamazaki et al., 1976). In addition, it was hypothesized that higher levels of estrogen in estrous females might interact with H-2-regulated metabolism and accentuate differences in volatile secondary metabolites. This hypothesis was shown to be unjustified by our observations, but serves as a further explanation for our use of females in this study.

Urine samples were collected from five immature (3–4 weeks old) and estrogen-treated mature females of the C57BL/10 strain; the animals were raised in the Department of Immunogenetics, Max Planck Institute, Tübingen, West Germany. All animals were housed in polycarbonate boxes (29.2 × 18.4 cm, 12.7 cm height, Hazelton Systems, Inc., P.O. Box 700, Aberdeen, Maryland) provided with hardwood chip bedding material (P.J. Murphy Forest Products Corp., Rochelle Park, New Jersey), food (Purina Mouse Chow, St. Louis, Missouri), and water ad libitum. The congenic lines inves-

tigated were B10 ($H\text{-}2^b$), B10.A ($H\text{-}2^a$), B10.BR ($H\text{-}2^k$), and B10.RIII ($H\text{-}2^r$). Urine samples were collected in metabolism cages, and the collected urine was immediately frozen over Dry Ice. Total collected urines were thawed, filtered through glass wool, and stored at −5°C before analysis.

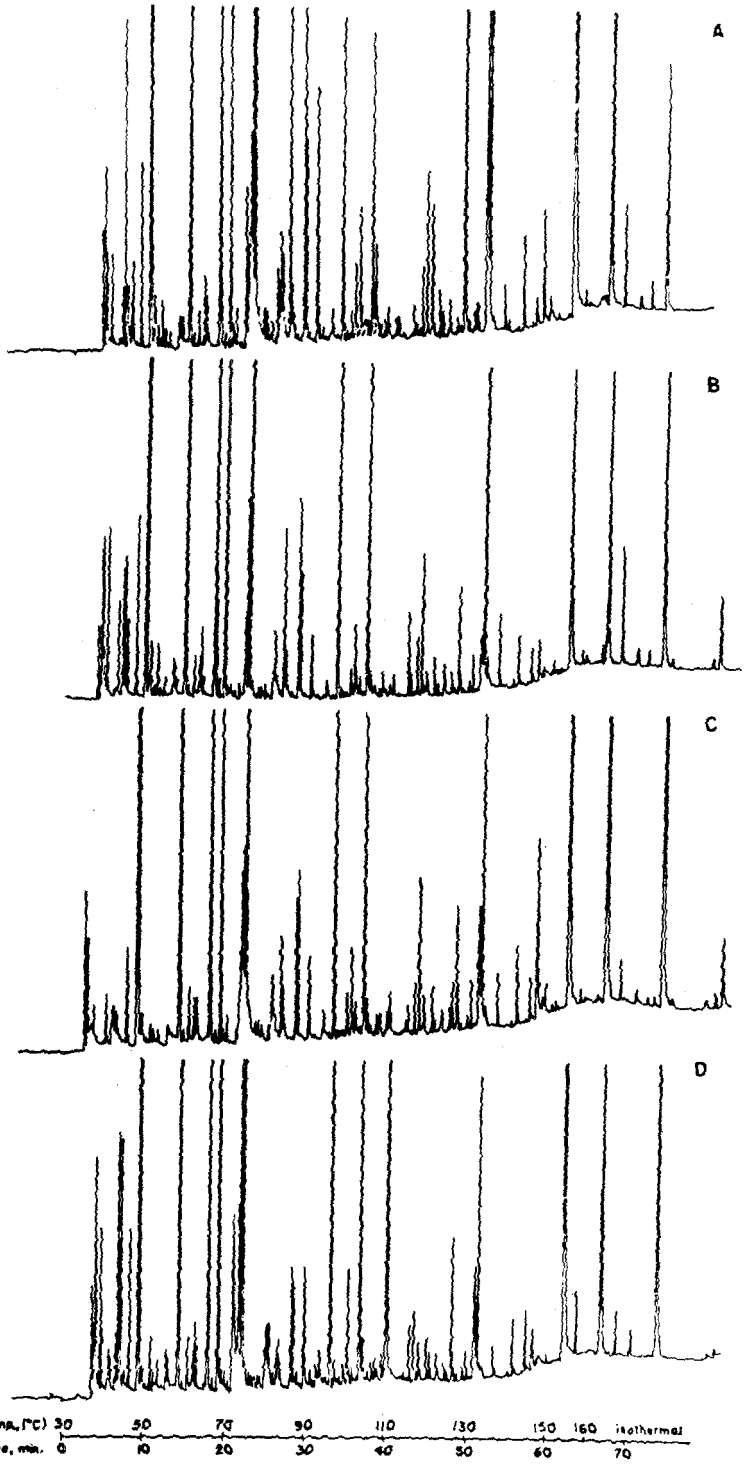
Urinary volatiles were sampled by sparging a 1-ml aliquot of urine with purified helium and trapping the volatiles onto a precolumn packed with a porous material (Tenax GC, Applied Science Laboratories, State College, Pennsylvania). The general procedure was described earlier (Novotny et al., 1974). By inserting the precolumn into the gas chromatograph (Sigma 3, Perkin-Elmer Corporation, Norwalk, Connecticut) and initiating carrier-gas flow, volatile samples were thermally desorbed onto the analytical column (70 m × 0.25 mm ID, glass capillary column coated with UCON-50HB 2000); the front section of this column was cooled with liquid nitrogen to avoid an initial band spreading. The column temperature was programed from 30°C to 160°C at 2°C/min. Figure 1 shows representative chromatograms of the mouse urinary volatiles.

Haplotype comparisons were performed for both immature and estrogen-treated mature animals. The estrogen treatment involved silastic tubing implants (Dow Corning No. 602-305, 5 mm long × 2 mm ID) packed with pure 17β-estradiol, and sealed with Dow Corning type A medical adhesive (Maruniak et al., 1975). In a few days, these implants produced a continuous state of estrus as verified by vaginal swabs. Urine from immature females was collected several days after receiving the animals, in order to allow for acclimation to their surroundings. In all cases, urine collections were performed in 24- or 48-hr periods to minimize diurnal fluctuations.

Statistical Design. As a cursory examination of Figure 1 suggests, differences in the volatile profiles of congenic mice are not readily discernible. Thus, a statistical means of comparison was sought in which the chromatographic parameters (retention times and integrated peak areas) could be used to evaluate a single statistic. Similarity or dissimilarity of any two chromatograms could be judged as based on such a "comparison score."

An approach used in this work involved calculating correlation coefficients (Pearson's r) for corresponding peak areas of chromatograms being compared, while using log/log Cartesian coordinates. The logarithmic plots are required to restrict the range of data points and eliminate bias in indeterminant error favoring the larger peaks. A residual analysis (Neter et al., 1978) of a nonlogarithmic comparison demonstrated a nonuniform distribution of indeterminant error, thereby negating the application of linear regression to nontransformed data. Conversely, log/log comparisons demonstrated a uniform distribution of residual error.

FIG. 1. Chromatograms from (A) B10, (B) B10.A, (C) B10.BR, and (D) B10.RIII immature females.


A
B
C
D
Temp., °C) 30 50 70 90 110 130 150 160 isothermal
Time, min. 0 10 20 30 40 50 60 70

The statistical significance of correlation coefficients may be evaluated by calculating 95% confidence intervals. This is not a straightforward task, because correlation coefficients have a discontinuous range ($-1.0000 < r < 1.0000$); distributions of sample correlation coefficients are skewed with the moments of skew dependent on the magnitude of r (Showhart, 1931). The z-transformation, described by Fisher (Fisher, 1948), was used to evaluate the 95% confidence intervals.

RESULTS

Since the mating preference experiments (Yamazaki et al., 1976) initially employed estrous females, immature and estrogen-pellet-implanted animals were investigated first. The results of this comparison are shown in Table 1. While this experiment demonstrated differences in correlation coefficients for the strains investigated as immature females, the correlations all improved (approached +1.000) with estrogen treatment. A more detailed examination of immature females thus appeared worthwhile.

An intrinsic problem with the first set of experiments was the lack of a control for comparing correlation coefficients. Obviously, the analytical reproducibility itself would be a source of variation that is capable of masking meaningful comparisons. While analyzing data from replicate chromatograms of the same sample, it became apparent that correlation coefficients of better than 0.90 were not consistently obtained. The source of error was attributed to certain "unreliable" mixture components (most likely those which suffer from an irreversible adsorption in the GC system), a problem typically encountered in multicomponent trace organic analysis. While some information could be lost in such a procedure, these "unreliable" peaks were eliminated for all comparisons and the correlations recalculated. This elimination process was repeated until replicate chromatograms showed a high degree of

TABLE 1. PRELIMINARY COMPARISONS OF FEMALE CONGENIC STRAINS (IMMATURE AND MATURE-ESTROGENIZED CORRELATION)

Comparison	Correlation coefficients, r	
	Immature	Mature-Estrogenized
B10 vs. B10.A	0.7109	0.8735
B10 vs. B10.BR	0.7201	0.8783
B10 vs. B10.RIII	0.6735	0.8412
B10.A vs. B10.BR	0.8161	0.8490
B10.A vs. B10.RIII	0.8033	0.8537
B10.BR vs. B10.RIII	0.7537	0.8977

TABLE 2. COMPARISON OF C5BL/10-CONGENIC H-2 IMMATURE FEMALES

Comparison group	H-2 haplotype	r	95% Confidence interval
B10 vs. B10	b/b	0.9581	0.9658–0.9487
B10.A vs. B10.A	a/a	0.9394	0.9505–0.9259
B10.BR vs. B10.BR	k/k	0.9446	0.9547–0.9323
B10.RIII vs. B10.RIII	r/r	0.9426	0.9531–0.9298
B10 vs. B10.A	b/a	0.8069	0.8402–0.7676
B10 vs. B10.BR	b/k	0.8074	0.8406–0.7681
B10 vs. B10.RIII	b/r	0.8174	0.8490–0.7799
B10.A vs. B10.BR	a/k	0.9095	0.9258–0.8898
B10.A vs. B10.RIII	a/r	0.9016	0.9193–0.8803
B10.BR vs. B10.RIII	k/r	0.9179	0.9328–0.8999

correlation (+0.90). Correlation coefficients were calculated for all possible combinations of chromatograms, and the resulting numbers were used to calculate mean correlation coefficients for each comparison of H-2 types. These mean correlation values are reported in Table 2 together with 95% confidence intervals as determined by Fisher's z-transformation.

A possible effect of "individual variation" was investigated by comparing two separate but genetically identical populations of mice. Ten C57BL/10Sn females were arbitrarily divided into two groups of five mice each. The results of chromatographic analysis and correlation calculations are shown in Table 3; these provide yet another control.

DISCUSSION

Comparison of Correlation Coefficients. The information in Table 1 is included here in order to demonstrate why only immature females were investigated in later experiments. In all comparisons, estrogenization decreased differences between chromatograms, i.e., increased correlation coefficients. We have shown that estrogenization of mature female mice dramatically in-

TABLE 3. COMPARISON OF GENETICALLY IDENTICAL POPULATIONS OF IMMATURE FEMALES (C57BL/10 Sn)

Comparison groups	r	95% Confidence interval
A vs. A	0.9427	0.9532–0.9299
B vs. B	0.9302	0.9430–0.9147
A vs. B	0.9256	0.9392–0.9091

creases the concentration of a number of urinary volatiles (Schwende et al., 1984). Such increases apparently act to mask the influence of H-2 type as determined by our statistical model. Odor perception and individual recognition by the mouse is probably not affected in the same way, although one may postulate that odor information is interpreted on a hierarchical scale of significance to the organism. The significance of H-2 type would certainly rank lower than information regarding species, sex, and endocrine status. Our statistical model is apparently affected by this hierarchical ordering in that the significance of communicating a state of estrus negates our ability to discriminate H-2 types. Therefore, only female urine free from estrous information could be reliably evaluated.

The correlation coefficients calculated for immature females with the benefit of the deletion of unreliable peak data are contained in Table 2. Replicate chromatograms are all very similar, as evidenced by the high (>0.90) correlation coefficients, and are equivalent to the values determined for two genetically identical populations of mice shown in Table 3. Low correlations are obtained only when genetically different groups are compared. Moreover, all comparisons with the B10 congenic strain demonstrate the lowest correlations, implying that the H-2^b haplotype has the least in common with the other types investigated. Interestingly, this dissimilarity of the B10 line is also generally reflected in serological antigen sensitivities determined by H-2 alloantigens. Of the H-2 antigens reported in a standard H-2 chart (Klein, 1975), the H-2^b haplotype has the least in common with the other types used in our study. The serological comparison of B10 and B10.A appears to be intermediate. A list of identical H-2 antigens for the congenic lines compared in our study is shown in Table 4. The distribution of measures of similarity by serology and by urinary volatile profiles (correlation coefficients) is shown in Figure 2. Except for the case of B10 vs. B10.A, serological sensitivities and urinary correlation coefficients appear to exhibit similar trends.

TABLE 4. COMPARISON OF H-2 ANTIGENIC REACTIVITIES

Comparisons			
Strains	H-2 haplotypes	Identical antigens	Number of identical antigens
B10 vs. B10.BR	b/k	5	1
B10 vs. B10.RIII	b/r	5, 6	2
B10 vs. B10.A	b/a	5, 6, 27, 28, 29, 35, 36	7
B10.A vs. B10.BR	a/k	1, 3, 5, 8, 11, 23, 24, 25, 45, 47, 49, 52	12
B10.A vs. B10.RIII	a/r	1, 3, 5, 6, 8, 11, 25, 45, 47, 49, 52	11
B10.BR vs. B10.RIII	k/r	1, 3, 5, 8, 11, 25, 45, 47, 49, 52	10

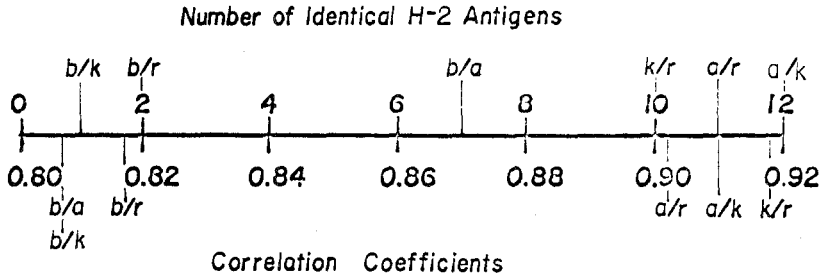


FIG. 2. Relationship of serological typing and urinary volatile correlation coefficients.

Isolation of Components Responsible for Poor Correlations. In addition to correlation coefficients, comparisons of chromatographic profiles by a computer yield "scattergrams." Examples of such plots are shown in Figure 3. It is apparent from the B10–B10.A comparison that a number of points lie far from the line determined by linear correlation analysis. The chromatographic peaks obviously responsible for such points can be identified through GC-MS; identifications are listed in Table 5.

One can delete information derived from the deviating peaks and recalculate correlation coefficients. If a lack of correlation were primarily caused by these peaks, the newly calculated values should be insignificantly different from each other. This is not the case for B10–B10.A, B10–B10.BR, and B10–B10 RIII comparison. Wide deviations from linearity are still preserved, and it is nearly impossible to isolate the individual components responsible for the remaining differences. This tends to support the proposition that the perceivable odor differences between H-2 congenics are due to variations in the pattern of general secondary metabolites rather than differences in the production of specific components.

Patterns of secondary metabolites can be influenced by a number of environmental and genetic influences. Urinary volatile components are typical products of secondary metabolism. On the other hand, many other nonimmunological H-2-linked traits have already been discovered (Klein, 1978; Klein et al., 1982).

The alteration of H-2-mating influence and Y-maze discrimination by using progeny of F_1 crosses (Yamazaki et al., 1978) supports the concept of a nonspecific influence of H-2 on olfactory cues, since non-H-2-related differences altered the mating preference and Y-maze discrimination effects. Specific components under direct genetic control would certainly be expressed in heterozygotes from F_1 progeny at the same level as the homozygous parental stock. The alteration of H-2 recognition effects after passing through an F_1 heterozygous cross is perhaps best explained by non-H-2-related genetic drift, or maternal influence, both of which are likely to alter secondary metabolism pathways in nonspecific ways.

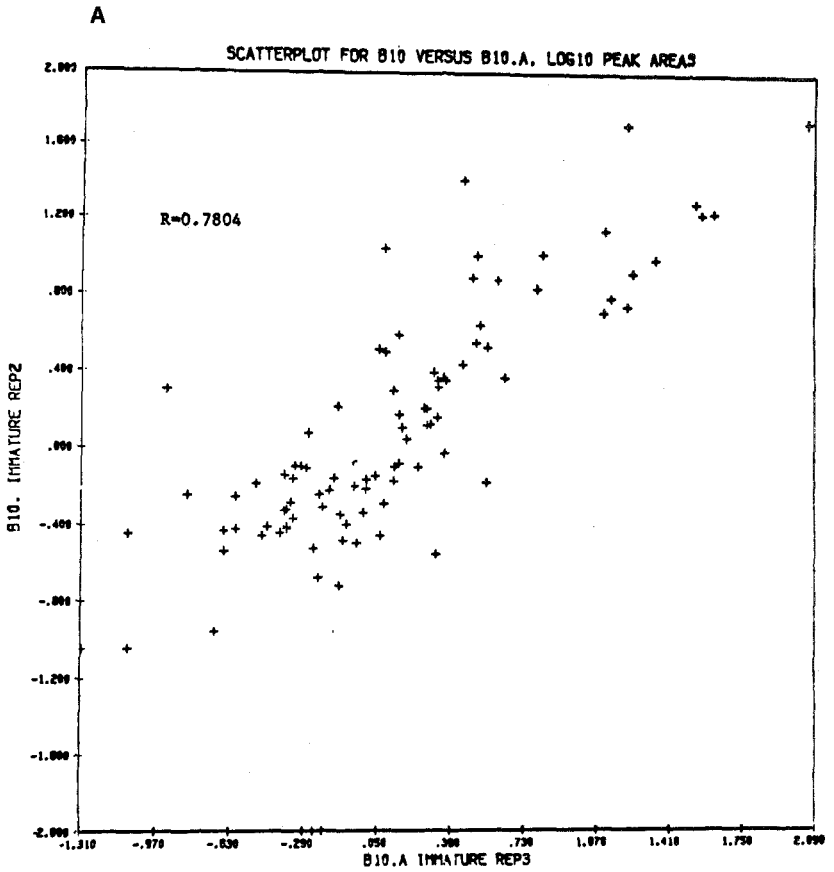


FIG. 3. Scatterplots of B10 vs. B10.A (A) and B10 vs. B10 (B).

CONCLUSIONS

It was reasoned (Yamazaki, 1979) that heterozygosity at the H-2 locus should confer an advantage to the individual by providing for a wider range of immunological defenses. In wild mouse populations, excessive heterozygosity for certain antigens was observed (Nadeau et al., 1981). Although other explanations may account for these observations, the potential influence of H-2 olfactory recognition cannot be discounted. Mating preference and Y-maze tests clearly demonstrated that mice can distinguish one another on the basis of their H-2 types and that the recognition signals are airborne. Our observations reported here indicate that differences in H-2 type affect the pattern of volatile secondary metabolites found in female urine. Although a number of specific volatile components account for a great deal of the differences observed, statistically significant variations among most of the other

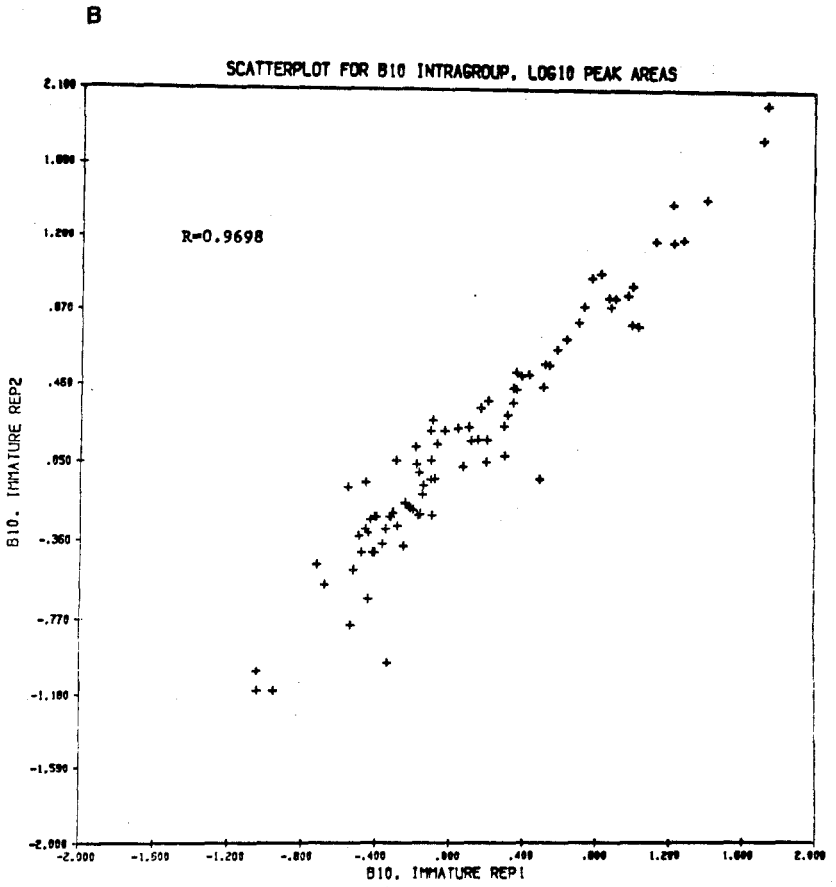
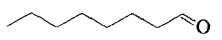
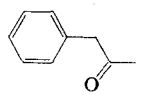
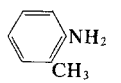
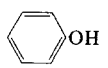


FIG. 3. Continued

TABLE 5. ISOLATED COMPONENTS RESPONSIBLE FOR STRAIN DIFFERENCES

Octanal	
Phenylacetone	
Ortho-toluidine	
Phenol	

components exist. Thus, our results indicate that the pattern of volatile secondary metabolites rather than the presence of specific components in the urine may serve to communicate H-2 information to males in mating preference tests and to males and females in Y-maze discrimination tests. In wild populations, the expression of H-2 type via its influence over secondary metabolites in the urine of females would be modified by other variable factors controlling secondary metabolism. The possible role of H-2 recognition in wild populations as a means of maintaining H-2 heterozygosity remains a matter of speculation.

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REPELLENT EFFECT OF VOLATILE FATTY ACIDS OF FRASS ON LARVAE OF GERMAN COCKROACH, *Blattella germanica* (L.) (DICTYOPTERA: BLATTELLIDAE)

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Abstract—Two- to 12-day-old larvae of *Blattella germanica* are repelled by certain volatile fatty acids present in the frass. Propionic, isovaleric, and valeric acids are strongly repellent, isobutyric acid is slightly repellent, and acetic and butyric acids are without effect. These results are discussed in relation to control of population density.

Key Words—*Blattella germanica*, Dictyoptera, Blattellidae, aggregation, volatile fatty acids.

INTRODUCTION

Aggregation of larvae of *Blattella germanica* on filter paper "conditioned" or contaminated by the same species has been demonstrated by Ishii (1967). He showed that the factor(s) was present in the gut and was soluble in ether and methanol. A similar factor was found in three *Periplaneta* species and the attraction was cross-specific (Ishii, 1970).

Watler (1979) demonstrated similar aggregation in larvae of *Acheta domesticus* (L.). McFarlane et al. (1983) showed that volatile fatty acids were present in the excreta of *A. domesticus* and that propionic acid, when applied to filter paper, aggregated the larvae.

In a survey of eight species of omnivorous and phytophagous insects, including *B. germanica*, McFarlane and Alli (1984) found volatile fatty acids in the frass of all of them. Inasmuch as the behavior of *A. domesticus* larvae was affected by propionic acid, the possibility exists that the behavior of other species is also affected by the acids. In this article the effects of the vola-

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tile fatty acids present in the excreta on the aggregating behavior of larvae of *B. germanica* are examined.

METHODS AND MATERIALS

Blattella germanica (*L.*). A culture of the German cockroach was obtained from Mr. G.J. Hilchie, Department of Entomology, University of Alberta, Canada. It was reared originally on Ralston Purina rabbit chow, and later on Ralston Purina Dog chow, at 30 ± 2°C and 55 ± 5% relative humidity with a photoperiod regime of 14:10 light–dark.

In experiments to test the attractancy or repellency of the volatile fatty acids, the method of McFarlane et al. (1983) was adopted. The larvae were 2–12 days old; newly hatched larvae and older larvae tended not to settle under the conditions of the experiment. Two 14 × 3.8-cm strips of Whatman No. 1 filter paper folded twice (to form a W) were placed on edge and spaced evenly in a culture dish (diam. 19.2 cm). Individual fatty acids were tested by making serial dilutions from 1.0×10^{-1}% to 1.0×10^{-7}% and treating a filter paper strip with 0.68 ml of the solution. This amount of solution completely wetted the paper and permitted uniform distribution of each acid. The control paper was wetted with 0.68 ml of distilled water. Twenty larvae were introduced into the culture dish, and the dish was immediately covered with fine-mesh Tergal which was secured with a rubber band. Each culture dish was then covered with a small cardboard box which maintained darkness for the test. If this was not done, the larvae would not settle on the papers. In fact, in nearly all trials, the great majority of the larvae came to rest on the papers, and these were counted. Larvae not on the papers were not considered in the analysis of results, as the normal response to the experimental situation was to come to rest on a paper; wandering larvae could therefore not be said to have been repelled. The few trials in which less than half of the larvae came to rest on the papers were also not considered, for the same reason. The duration of each experiment was 7 hr. Larvae were counted 1.5 hr after the experiment was set up, i.e., after the papers had dried, and thereafter at 0.5-hr intervals. The insects for the most part remained at rest during the brief observation period.

Preliminary experiments were conducted with two strips of filter paper wetted with distilled water only to see whether the orientation of the strips in the culture dish influenced the distribution of larvae on the papers.

In testing the volatile fatty acids, each concentration of each fatty acid was tested at least six times on six different groups of larvae. As only one experiment was carried out each day, repeated use of some larvae allowed for a recovery period of 17 hr. This was deemed to be a sufficient recovery period, in view of the fact that the dog chow on which the cockroaches were

TABLE 1. ORIENTATION OF THE GERMAN COCKROACH IN TWO-CHOICE TRIALS WITH UNTREATED FILTER PAPER

Location	Total no. of trials	No. of trials with greater number on paper directed to					No. of insects on paper directed to			
		Left	Right	Top	Bottom	Even	Left	Right	Top	Bottom
Examined at 0.5-hr intervals										
Laboratory 1										
Bench 1	36			9	26				5.3 ± 6.8[a]	13.1 ± 6.8
Bench 1	36	31	5				14.9 ± 5.4	3.2 ± 4.5[a]		
Bench 2	42	29	13				12.2 ± 6.5	5.6 ± 6.2[a]		
Bench 2	37			33	4				15.8 ± 4.8	2.6 ± 3.9[a]
Laboratory 2	35	8	25			2	5.4 ± 5.2[a]	12.9 ± 5.6		
Examined at end of 7-hr period										
Laboratory 1										
Bench 1	32	20	10			2	8.8 ± 5.2	6.3 ± 5.0[b]		

[a]Significantly less than opposite paper at $P = 0.01$.
[b]Significantly less than opposite paper at $P = 0.05$.

maintained contained all of the volatile fatty acids (McFarlane and Alli, 1984); this reduces the risk that larvae might be trained to any particular volatile fatty acid.

RESULTS

Experiments with Untreated Filter Paper. All two-choice experiments with untreated (but wetted) filter paper showed nonrandom aggregation of the insects (Table 1). The experiments were done with different orientations of the papers, on different laboratory benches, and in different laboratories. A geomagnetic effect seemed not to be involved because the preferred paper was variously approximately north, south, east and west.

To test if light was contributing to the orientation, the insects were left covered for the entire 7-hr period and counted only at the end of that period. Table 1 shows that there was a reduced, but still significant, difference between numbers on both papers at the 5% level, and that the total number responding was reduced compared with those observed at 0.5-hr intervals. The orientation seemed therefore to be at least in part due to light.

Experiments with Volatile Fatty Acids. In view of the bias in these experiments, the experimental design was as follows. The left and right orientations of bench 1, laboratory 1 were used, where the bias was to the left. In half the trials, the treated paper was on the left, in the other half on the right. Instead of comparing treated with control, the treated on the left were compared with the control on the left, and the treated on the right were compared with the control on the right. The results are presented in Table 2.

Propionic, isovaleric, and valeric acids were highly repellent, and iso-

TABLE 2. EFFECT OF VOLATILE FATTY ACIDS ON AGGREGATION OF *Blattella germanica* WHEN TESTED SEPARATELY AT CONCENTRATIONS RANGING FROM 1.0×10^{-1} TO 1.0×10^{-7}%

Volatile fatty acid	Number on left paper				Number on right paper			
	Trials	Treated	Trials	Control	Trials	Treated	Trials	Control
Acetic	21	14.4 ± 5.4	21	16.8 ± 3.7	21	2.4 ± 3.4	21	4.7 ± 5.6
Propionic	17	8.8 ± 6.9[a]	19	16.2 ± 5.1	19	2.4 ± 4.9[a]	17	9.0 ± 6.0
Isobutyric	27	9.7 ± 7.2	22	13.7 ± 7.5	22	5.1 ± 7.4[b]	27	9.5 ± 7.1
Butyric	32	6.6 ± 6.5	25	7.7 ± 7.6	25	10.8 ± 7.7	32	11.1 ± 6.6
Isovaleric	33	7.0 ± 6.4[a]	35	12.5 ± 6.6	35	6.4 ± 6.5[a]	33	12.4 ± 6.4
Valeric	21	5.0 ± 7.2[a]	21	12.9 ± 7.7	21	6.5 ± 7.8[a]	21	14.3 ± 7.1

[a]Treated significantly less than control at $P = 0.01$
[b]Treated significantly less than control at $P = 0.05$.

TABLE 3. RESPONSES OF *Blattella germanica* AT VARIOUS CONCENTRATIONS OF ACETIC ACID

Concentration (%)	Number on left paper				Number on right paper			
	Trials	Treated	Trials	Control	Trials	Treated	Trials	Control
10^{-1}	3	9.6 ± 10.0	3	19.0 ± 1.0	3	1.0 ± 1.0	3	9.6 ± 10.0
10^{-2}	3	16.0 ± 2.6	3	16.6 ± 5.8	3	3.3 ± 5.8	3	3.0 ± 3.0
10^{-3}	3	17.6 ± 1.5	3	18.6 ± 1.2	3	0.3 ± 0.6	3	1.6 ± 1.2
10^{-4}	3	13.6 ± 3.1	3	15.0 ± 4.4	3	3.0 ± 3.0	3	4.7 ± 3.2
10^{-5}	3	16.6 ± 2.3	3	16.0 ± 3.0	3	3.7 ± 2.5	3	2.7 ± 3.1
10^{-6}	3	12.0 ± 9.2	3	18.7 ± 2.3	3	0.3 ± 0.6	3	7.7 ± 9.3
10^{-7}	3	15.0 ± 2.6	3	13.6 ± 5.1	3	5.0 ± 6.1	3	3.3 ± 4.2

butyric acid tended to be repellent. Acetic and butyric acids were without effect. The concentration of fatty acid used was not important in these experiments. This is apparent from Table 3, which shows the influence of concentration of an ineffective acid, namely acetic acid, and from Table 4, which shows the influence of concentration of a repellent acid, namely valeric acid.

When a mixture of the volatile fatty acids, in the amounts and proportions in which they are found in 0.3 g of frass when fed dog chow (McFarlane and Alli, 1984), were presented, larvae in all instances chose the untreated paper, i.e., were repelled.

DISCUSSION

The nonrandom orientation shown in experiments with untreated (but wetted) filter paper strips is not understood. Orientation to light seems to be

TABLE 4. RESPONSE OF *Blattella germanica* AT VARIOUS CONCENTRATIONS OF VALERIC ACID

Concentration (%)	Number on left paper				Number on right paper			
	Trials	Treated	Trials	Control	Trials	Treated	Trials	Control
10^{-1}	3	7.0 ± 11.3	3	18.0 ± 2.6	3	0.3 ± 0.6	3	11.7 ± 10.1
10^{-2}	3	0.7 ± 1.2	3	5.3 ± 9.2	3	14.3 ± 9.0	3	19.0 ± 1.0
10^{-3}	3	1.3 ± 2.3	3	13.0 ± 11.3	3	6.3 ± 11.0	3	18.7 ± 2.3
10^{-4}	3	9.7 ± 6.0	3	12.7 ± 4.5	3	6.3 ± 5.1	3	8.3 ± 4.0
10^{-5}	3	2.3 ± 2.5	3	12.7 ± 11.0	3	7.0 ± 11.3	3	16.7 ± 2.5
10^{-6}	3	0.3 ± 0.6	3	11.0 ± 7.2	3	9.0 ± 7.2	3	19.3 ± 1.2
10^{-7}	3	13.3 ± 10.7	3	17.7 ± 4.0	3	2.3 ± 3.2	3	6.3 ± 10.1

a contributing factor, but this does not seem to be the complete explanation, as the bias persisted, although reduced, when the larvae were in darkness throughout the experimental period. Geomagnetic orientation cannot be completely ruled out, as the magnetic field varies in laboratories with steel furniture and numerous power lines. On the other hand, air currents or chemical gradients seem unlikely influences, inasmuch as the experimental dishes were covered. A completely shielded laboratory would seem to be the best way of avoiding this kind of bias.

In any case, it is apparent from these results that volatile fatty acids do not play a role in aggregation of *B. germanica.* In fact, most of them are repellent, and this repellency must be overcome, or masked by other compounds, when *B. germanica* is attracted to "conditioned" paper. Yet they may play a role in controlling population density, if the build-up of excreta in a dense population disperses them.

In this connection, Sexton and Hess (1968) demonstrated the presence of an attractant (presumably nonvolatile) and a volatile dispersant on wooden blocks "conditioned" by the house cricket, *Acheta domesticus.* They interpreted the presence of these substances in terms of the control of population density in this species. The volatile fatty acids in *B. germanica* may thus be fulfilling a similar function.

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BEHAVIORAL RESPONSES OF ELM BARK BEETLES TO BAITED AND UNBAITED ELMS KILLED BY CACODYLIC ACID

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Abstract—Diseased elms, treated with various doses of cacodylic acid in northwest England, became attractive to elm bark beetles (Coleoptera: Scolytidae). This attraction seemed to be independent of pheromone baits. However attractive the trees became, they were unsuitable to the beetles as breeding sites since significantly more beetles visited the trees than were stimulated to penetrate and attempt to breed. It seems as if colonization of trap trees by the bark saprophyte *Phomopsis oblonga* following cacodylic acid treatment made the trees unsuitable to beetles for breeding.

Key Words—Cacodylic acid, pheromone, *Phomopsis oblonga*, Dutch Elm disease, elm bark beetles, *Scolytus scolytus*, *Scolytus multistriatus*, Coleoptera, Scolytidae.

INTRODUCTION

The trap-tree technique of controlling the elm bark beetle vectors of the Dutch elm disease (DED) (O'Callaghan et al., 1980) has met with success in the United States, where it was developed (Lanier, 1981, 1982). Attempts made to adapt the technique to the situation in northwest England have failed to reproduce the success, in terms of the numbers of beetles attacking trap trees, of the American trials (O'Callaghan and Fairhurst, 1983). Major ecological and climatic differences between the northeastern United States and northwest England have been assumed to be responsible for the lack of success in Britain. In England, two of the vectors of the disease are *Scolytus*

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scolytus (F) and *S. multistriatus* (Marsh), the large and smaller European Elm bark beetles respectively (Gibbs et al., 1977), and there are seven common species/varieties of elm (Jobling and Mitchell, 1974). Differences in climate between the United States and northern England mean that the timing of cacodylic acid (CAC) treatments to coincide with beetle flight periods is significantly more difficult in Britain (O'Callaghan and Fairhurst, 1983; Fairhurst and King, 1983). Such coincidence is necessary for optimum "trap effect" and attack density (Lanier, personal communication).

A major factor that affects beetle colonization of trap trees in northwest England is the presence of the bark saprophyte, *Phomopsis oblonga* (Desm) Trav. (O'Callaghan and Fairhurst, 1983). This fungus can make the inner bark of elm unsuitable for beetle breeding (Webber, 1981) and can exclude beetle attacks from particular trees or parts of trees (O'Callaghan, 1982). Furthermore, following treatment with CAC, trees are rapidly colonized by *P. oblonga* (O'Callaghan and Fairhurst, 1983), thus making them inhospitable to bark beetles. Therefore, difficulties in synchronizing treatment and beetle flights lead to time lags that result in *P. oblonga* colonization and a reduction in the trap effect of the treated trees.

As previous work suggests that CAC can promote the growth of *P. oblonga* in vitro (Edwards, 1981), it is not surprising that rapid colonization of inner bark by this fungus follows treatment with CAC. We decided to explore the relationship between CAC treatment of elms and subsequent *P. oblonga*/beetle colonization. Specifically, we studied the effects of various doses of CAC on *P. oblonga* colonization and on attraction of elm bark beetles when treatment and beetle flights were synchronized. We also investigated the efficacy of some pheromone components as lures on the CAC-treated trees.

METHODS AND MATERIALS

Locations. Experiments 1 and 2, investigating the effects of different dosages of CAC, were undertaken in Wirral District of Merseyside County on the Leverhulme Estate; Experiment 3, investigating the effects of different pheromone components on bark beetle response to CAC-treated trees, was undertaken in Duke St. Cemetery, Southport, in the Merseyside Borough of Sefton.

Experiment 1. Specimens of Wheatley elm, (*Ulmus carpinifolia* f. *sarniensis* (Loudon) Rehder), were treated with CAC, sodium dimethyl arsinic acid (marketed as RAD-E-CATE 35®, Vineland Chemical Company, Vineland, New Jersey). Trees were pressure injected (2 bar/30 psi) with 500, 1000, or 2000 ml of CAC dissolved in an equal volume of water, dispensed

from ASL Polyspray® plastic garden sprayers modified for injection purposes. Each tree had 10 injection points 1 m above ground and about 15 cm apart, and the chemical solution was delivered through Nylon T pieces (Elm Research Institute, Harrisville, New Hampshire). All but three trees were injected using the ASL polysprayers, while trees number 1, 2, and 3, that comprised replicate 1, were injected using the 5-liter Tree Saver® metal containers (Tree Conservation Ltd., Wantage, Oxon, England), modified to take the T pieces.

Five replicates of the three treatments were initiated on June 2 and 3, 1982. Each replicate comprised three treated and one control tree of similar age, size, girth, and disease status. Trees in which it was hoped that the disease would cause a rapid decline similar to that caused by injection of CAC (O'Callaghan and Fairhurst, 1982) were favored as controls. All trees, including the controls, were baited with 4-methyl-3-heptanol (4M3H), a component of the aggregation pheromones of *S. multistriatus* (Pearce et al., 1975) and of *S. scolytus* (Blight et al., 1977). This was dispensed from Conrel® (Albany International, Needham Heights, Massachusetts) hollow-fiber dispensers, that release 400 mg of 4M3H per day for about six weeks.

To monitor bark beetle landings on the trees, two bands of white corrugated plastic sheeting, 10 × 65 cm, coated with Hyvis® (PLA Insect Monitoring, Southampton, England), were stapled to all trees at 2–3 m from ground level. These bands were changed six times at weekly intervals between May 27 and July 8, 1982, when the experiment ended.

Experiment 2. This experiment was initiated in August 1982 and was the same as experiment 1, except that there were three rather than five replications of three treatments and one control and no pheromone baits were used. Bands to monitor beetle landings were changed five times between August 5 and September 8, 1982.

Experiment 3. Specimens of pollarded Wych elm, *U. glabra* Hudson, were treated with 500 ml of CAC as described in experiments 1 and 2. The trees were unbaited, baited with 4M3H only, or with a "cocktail" of 4M3H plus α-cubebene, (−)-limonene, and α-pinene [all of which have been implicated in the aggregation attractant of *S. scolytus*, (Blight et al., 1980)]. Untreated and unbaited diseased Wych elms were used as experimental controls. Three replications of this experiment were initiated between June 2 and 10, 1982. As in experiments 1 and 2, all trees were fixed with sticky bands which were changed at biweekly intervals throughout the experiment until its conclusion on September 24, 1982.

Sampling Procedures. During the late autumn and winter of 1982, the treated trees were felled and samples removed for examination. Because the Wheatley elms on the Leverhulme Estates were being felled commercially for timber, it was not possible to cut disks from the boles at 1- or 2-m intervals

as in previous years (O'Callaghan and Fairhurst, 1983). Therefore 10-cm-thick disks were cut from the base at (at 35 cm diameter) and from that part of the bole beyond 8 m (at 8–15 cm in diameter). Bark sections averaging 60 × 60 cm were stripped from the lower 8-m section of bole at 2-m intervals. Four of the Wych elms in experiment 3 were felled and 10-cm-thick disks cut from the boles at 1-m intervals. The remaining eight trees had bark samples stripped for examination.

All samples were examined in the laboratory to determine the number of attacks, brood galleries, and brood larvae. The occurrence of *Phomopsis oblonga* was ascertained by the presence or abscence of the zone lines (Webber, 1981) and recorded.

Beetles were picked from the bands used to monitor landings and cleaned by soaking in odorless industrial kerosene (Rentokill, U.K. Ltd., Kirkby Industrial Estate, Liverpool) to dissolve the sticky material. After about a week, the kerosene was replaced and the beetles identified and sexed in clean kerosene.

Statistical analyses of the numbers of attacks, brood larvae, and beetles captured on bands were made using the chi-square test in paired comparisons.

RESULTS

Experiment 1. The untreated control trees sustained more attacks and produced significantly more brood than the treated trees (Table 1). Within treatments, trees injected with 500- and 1000-ml doses of CAC sustained approximately three times as many attacks as those injected with the 2000-ml dose, but the differences were not significant (Table 1). Trees receiving the 2000-ml dose of CAC produced significantly less brood than either the 500- or 1000-ml dosed trees (Table 1).

Approximately 2.5–3.5 times more beetles were taken on the bands of the treated trees than on those untreated controls (Table 1). Within the treatments, more beetles were trapped on the bands on trees treated with 500 ml of CAC than on either the 1000- or 2000-ml treatments, and these differences were significant ($P < 0.05$, Table 1) due mainly to catches of *S. scolytus*. The trends in beetle catches on bands were consistent for both *S. scolytus* and *S. multistriatus*.

Zone lines characteristic of *P. oblonga* were recorded in 95% of samples from treated trees, with no difference between the various doses. By contrast however, zone lines were recorded from about 40% of control samples taken.

Experiment 2. Unlike experiment 1, the untreated control trees sustained significantly fewer attacks than trees treated with 500 ml CAC and marginally fewer than those treated with 1000 ml CAC, but more than the 2000-ml treat-

TABLE 1. NUMBER OF ATTACKS AND BROOD LARVAE RECORDED PER dm^{2a} OF BARK SURFACE AREA, AND NUMBERS OF BEETLES OF BOTH SPECIES TRAPPED PER dm^2/STICKY BAND ON WHEATLEY ELMS TREATED WITH VARIOUS DOSES OF CACODYLIC ACID (CAC) AND BAITED WITH 4-METHYL-3-HEPTANOL (4M3H) ON LEVERHULME ESTATES in JUNE AND JULY 1982

Treatment	Attacks/dm^2 BSA[b]	Brood/dm^2 BSA	Beetles/dm^2 band[c]	*S. scolytus*/dm^2 band	*S. multistriatus*/dm^2 band
500 ml CAC + 4M3H	8.21 a[d]	30.5 a	266.15 a	183.85 a	82.31 a
1000 ml CAC + 4M3H	6.79 ab	48.58 ac	206.92 bc	126.92 bc	72.31 ac
2000 ml CAC + 4M3H	2.38 abc	8.62 bde	192.31 bce	127.69 bce	64.62 ace
Control, 4M3H only	11.24 abd	180.34 bdf	77.69 bdf	67.69 bdf	10.00 bdf

[a] $dm^2 = 1000\ cm^2/0.1\ m^2$.
[b] BSA = bark surface area.
[c] Band = Two 10×65-cm strips of white corrugated plastic sheeting coated with Hyvis.
[d] Numbers followed by the same letter are not significantly different at $P > 0.05$ (χ^2) in paired comparisons.

TABLE 2. NUMBER OF ATTACKS AND BROOD LARVAE RECORDED PER dm^{2a} OF BARK SURFACE AREA AND NUMBERS OF BEETLES OF BOTH SPECIES TRAPPED PER dm^2 OF STICKY BAND ON WHEATLEY ELMS TREATED WITH VARIOUS DOSES OF CACODYLIC ACID (CAC) ON LEVERHULME ESTATES IN AUGUST AND SEPTEMBER 1982.

Treatment	Attacks/dm^2 BSA[b]	Brood/dm^2 BSA	Beetles/dm^2 band[c]	*S. scolytus*/dm^2 band	*S. multistriatus*/dm^2 band
CAC					
500 ml	1.96 a[d]	1.08 a	106.15 a	90.01 a	16.15 a
1000 ml	1.42 bc	1.69 ac	62.31 bc	53.08 bc	9.24 ab
2000 ml	0.00 bcd	0.00 ace	27.69 bde	19.22 bde	8.47 abc
Untreated control	1.30 bcd	15.05 bdf	21.53 bdf	14.61 bde	6.93 abc

[a] $dm^2 = 1{,}000\ cm^2/0.1\ M^2$.
[b] BSA = bark surface area.
[c] Band = Two 10×65-cm strips of white corrugated plastic sheeting coated with Hyvis.
[d] Numbers followed by the same letter are not significantly different at $P < 0.05$ (χ^2) in paired comparisons.

ment (Table 2). As in experiment 1, the untreated trees produced significantly more brood than any of the treated trees (Table 2).

Although all trees in this experiment were unbaited, the bands on the treated trees generally trapped significantly more beetles than those on the untreated control trees (Table 2). Between three and five times as many beetles were trapped on the bands on the trees receiving the 1000 and 500-ml treatments, respectively, than on the control trees; the bands on the 2000-ml treatments caught only slightly more than those on the control (Table 2). Again, as in experiment 1, these trends were consistent for both species of beetle except that there was no significant difference between treatments and control for *S. multistriatus* (Table 2). Also, there was no difference in the occurrence of *P. oblonga* between any of the treatments. Control trees, however, were less colonized by this saprophyte.

Experiment 3. Trees treated with CAC and baited with 4M3H sustained more attacks than either those treated and unbaited with the pheromone cocktail, those treated and unbaited, and those untreated and unbaited, but only the differences between CAC and 4M3H and CAC only were significant (Table 3). There was, however, little difference in attack density between the unbaited, untreated controls; the unbaited but treated trees; and the treated, cocktail-baited trees (Table 3). Unlike experiments 1 and 2, the trees that received no CAC treatment produced significantly less brood than the treated trees (Table 3).

Between two and three times more beetles were trapped on the bands of the 4M3H baited trees than in any of the other treatments; similar numbers of beetles were taken on the bands on the CAC-treated trees that were cocktail-baited or unbaited (Table 3). These trends were similar for both species of beetle.

Again, as in experiments 1 and 2, *P. oblonga* was found in 95% of samples from CAC-treated trees. Unlike the previous experiments, however, this fungus was also found in the majority of samples from untreated trees.

DISCUSSION

In agreement with previous results (O'Callaghan and Fairhurst, 1983), the untreated control trees in experiment 1 sustained more attacks than the CAC-treated trees, irrespective of the dose applied. It is, however, interesting that the treated trees attracted substantially more beetles, as measured by the band catches, than did the untreated control trees. The results indicate that the CAC-treated trees are indeed attractive to flying beetles that arrive in large numbers and are stimulated to land. The suggestion that the CAC-treated trees are inherently attractive is supported by the results of experiment 2 (Table 2), i.e., bands on CAC-treated trees trapped significantly more

TABLE 3. NUMBER OF ATTACKS AND BROOD LARVAE RECORDED PER dm^{2a} OF BARK SURFACE AREA AND NUMBERS OF BEETLES OF BOTH SPECIES TRAPPED PER dm^2 OF STICKY BAND ON POLLARDED WYCH ELMS TREATED WITH CACODYLIC ACID (CAC) AND BAITED WITH VARIOUS PHEROMONE COMPONENTS IN DUKE ST. CEMETERY, SOUTHPORT, DURING 1982

Treatment	Attacks/dm^2 BSA[b]	Brood/dm^2 BSA	Beetles/dm^2 band[c]	*S. scolytus*/dm^2 band	*S. multistriatus*/dm^2 band
500 ml CAC + 4M3H[d]	74.80 a[f]	22.28 a	320.77 a	215.39 a	105.39 a
500 ml CAC + cocktail[e]	46.20 ac	41.93 ac	217.69 bc	152.31 bc	64.62 bc
500 ml CAC only	38.90 bcd	20.80 ace	187.69 bce	124.61 bce	63.08 bce
Untreated/unbaited	46.90 acd	1.50 bdf	93.08 bdf	78.46 bdf	14.62 bdf

[a] dm^2 = 1,000 cm^2/0.1 M^2.
[b] BSA = bark surface area.
[c] Band = Two 10 × 65-cm strips white corrugated plastic sheets coated with Hyvis.
[d] 4M3H = 4-methyl-3-heptanol.
[e] Cocktail = 4M3H, α-cubebene, (−)-limonene, and α-pinene.
[f] Numbers followed by the same letter are not significantly different at $P < 0.05$ (χ^2) in paired comparisons.

beetles than bands on the control trees, even though no pheromone baits were used in this experiment, but this was due to the numbers of *S. scolytus* trapped.

The results of experiment 3 on Wych elms further supports this argument as the bands on untreated, unbaited trees caught significantly fewer beetles than those on CAC-treated but unbaited trees (Table 3). In this experiment, however, trees baited with 4M3H only (as were all trees in experiment 1), attracted significantly more beetles than treated unbaited trees or those treated and baited with a cocktail of compounds including 4M3H. Interestingly, the cocktail-baited trees did not attract significantly more beetles than the CAC-treated, unbaited trees (Table 3).

The results of these experiments are not consistent with our previous findings (O'Callaghan and Fairhurst, 1983), i.e., that CAC-treated elms are unattractive to flying *S. scolytus*. However, these conclusions were based on attacks as the measure of attraction. Results of this study show that CAC-treated trees are actually quite attractive to beetles but many of the beetles that land on these trees do not bore into them. One of us (P.M.A.) has found that trees in Southport that were treated in 1981 attracted bark beetles in 1982. It seems, therefore, that trees treated with CAC are not only attractive to flying beetles but can remain so for at least a year.

The results of these experiments suggest that both *S. scolytus* and *S. multistriatus* prefer CAC-treated to untreated trees, and this ability seems to be independent of the presence of some pheromone compounds. The ability of *S. multistriatus* to detect and respond to uninfested elm logs has been well demonstrated by Peacock et al. (1971) as has its ability to discriminate between logs cut from different species of elm (Švihra and Koehler, 1981). The results of this study extend previous findings by demonstrating an ability of *S. multistriatus* and *S. scolytus* to discriminate between trees that are diseased and trees stressed by injection of CAC. Based on these results we can reemphasize the suggestion that host selection in the genus *Scolytus* is a directed, nonrandom process as defined by Wood (1982).

It is interesting that large numbers of beetles are attracted to and land on treated trees but fly off again and that these landings are not converted into attacks or attempts at colonization. The beetles probably ascertain the suitability for attack after penetrating the bark and "tasting" the phloem. At this point they detect the presence of the saprophyte *P. oblonga* (or compounds produced by it) in the inner bark. As this fungus makes bark unsuitable for beetle breeding (Webber, 1981), it is possible that its presence, promoted by injection of CAC (Edwards, 1981; O'Callaghan and Fairhurst, 1983), deters the beetles from attacking the tree and constructing brood galleries. Zone lines characteristic of *P. oblonga* were recorded in over 95% of samples with no difference in its occurrence between the various doses.

Attacks that result in the establishment of brood galleries are a result of continued boring by beetles, so it is possible that the CAC-treated trees lack the necessary feeding stimulants or contain feeding deterrents. It has never been shown, for example, that the pine engraver, *Ips paraconfusus* Lanier, requires feeding stimulants in order to continue boring into the phloem of host trees and that these are lacking in certain nonhost trees (Elkinton et al., 1980, 1981). That *S. multistriatus* uses such stimulants has been documented by Doskotch et al. (1970). Furthermore, it was first demonstrated that feeding by this species can be deterred by juglone (5-hydroxy-1, 4-napthoquinone), derived from nonhost trees such as shagbark hickory *Carya ovata* (Gilbert et al., 1967; Norris, 1977). It is possible, therefore, that treatment of Wheatley and Wych elms with CAC promotes changes in phloem chemistry that deters feeding and boring by *S. scolytus* and *S. multistriatus*. These changes could be the result of action by the CAC treatment or from changes induces by the presence of *P. oblonga*. This lack of boring by the beetles would result in reduced pheromone production as boring is normally necessary for pheromone production (Gore et al., 1977).

As in previous years (O'Callaghan and Fairhurst, 1983) and in agreement with results of the American trials of the trap-tree technique (O'Callaghan et al., 1980), the number of brood larvae produced was significantly reduced in all treated trees as opposed to the untreated, except for the Wych elms of experiment 3. These controls either failed to succumb rapidly to the disease or, following death, were completely colonized by *P. oblonga*. These facts may explain why the control trees in experiment 3 sustained fewer attacks and trapped fewer beetles than the 4M3H-baited and treated trees.

Conclusions. We conclude that CAC-treated elms are attractive to flying *S. scolytus* and *S. multistriatus* and can remain so for some time. However, following treatment, the condition of the phloem is altered such that most of the attracted bark beetles reject the trees as breeding sites and fly off in search of more suitable hosts. The data give no indication of the speed at which these changes occur, but it is possible that immediately following treatment, any beetles attracted would be stimulated to attack and colonize. These data reemphasize the suggestion that for treated trees to have the trap effect, the coincidence of beetle flight periods with CAC-treatment is necessary. Regardless of the time of treatment, the neutralizing effect of the treatment on possible brood trees still holds. The contribution of CAC treatments to DED control in northwest England still lies in their ability to neutralize the elms which cannot be easily felled.

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REDUNDANCY IN A CHEMICAL SIGNAL: Behavioral Responses of Male *Trichoplusia ni* to a 6-Component Sex Pheromone Blend

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Abstract—The flight response of male *Trichoplusia ni* was observed in a flight tunnel to a sex pheromone blend composed of six components: Z7-12:Ac, 12:Ac, Z5-12:Ac, 11-12:Ac, Z7-14:Ac, and Z9-14:Ac. The number of males reaching a 3000-μg source of this blend was $>95\%$, equal to that observed to female glands and significantly greater than with the previously identified two-component blend (Z7-12:Ac + 12:Ac). In subtraction tests, all five-component blends, with the exception of the blend lacking the primary component Z7-12:Ac, and several four-component blends elicited similar peak levels of upwind flight, source contacts, and hairpencil displays to that observed with the six-component blend. We characterize the substitution of certain minor components for one another as a form of redundancy in the chemical signal and suggest that it contributes to response specificity and signal recognition in males. The results also support the concept that the full blend of components acts as a unit to influence male behavior at all phases of the response. Individual minor components were not responsible for eliciting specific behaviors in the sequence.

Key Words—*Trichoplusia ni*, cabbage looper moth, Lepidoptera, Noctuidae, pheromone, redundancy, flight tunnel.

INTRODUCTION

There is now a widespread recognition of the importance in many moth species of sex pheromone compounds that occur in small or trace amounts (Roelofs, 1981; Steck et al., 1982). This has come about largely as a result of advances in instrumentation, principally capillary GLC (Klun et al., 1979) and airborne collections (Baker et al., 1981a; Pope et al., 1982), as well as

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more detailed behavioral tests (Baker and Cardé, 1979; Linn and Roelofs, 1983; Vetter and Baker, 1983).

In the cabbage looper moth, *Trichoplusia ni* (Hubner), the predominant component is (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac), and when first isolated this compound provided a useful tool in many basic studies of pheromone biology that utilized a simple activation bioassay (Shorey, 1974). Subsequently dodecyl acetate (12:Ac) was identified as a secondary component (Bjostad et al., 1980), and behavioral tests, using a more complex bioassay incorporating a sustained flight tunnel, showed that 12:Ac, in combination with *Z*7–12:Ac, enhanced the behaviors exhibited by males close to a source (Linn and Gaston, 1981). The components in this binary mix met the criteria of primary (*Z*7–12:Ac) and secondary (12:Ac) pheromone components as proposed by Roelofs and Cardé (1977).

In a recent report on the sex pheromone of the cabbage looper moth, Bjostad et al. (1984) identified several additional compounds from female gland extracts and airborne collections. Four of these compounds, (*Z*-5-dodecenyl acetate (*Z*5–12:Ac), 11-dodecenyl acetate (11–12:Ac), (*Z*)-7-tetradecenyl acetate (*Z*7–14:Ac), and (*Z*)-9-tetradecenyl acetate (*Z*9–14:Ac), when added to the previously identified two-component blend (*Z*7–12:Ac + 12:Ac) formed a six-component mix that elicited extremely high (95%) levels of male response (completed flights to source and hairpencil display) in a sustained-flight tunnel. The response to the six-component mix was equal to that observed to female glands and significantly greater than the two-component blend or *Z*7–12:Ac alone. An additional compound, (*Z*)-7-dodecenol (*Z*7–12:OH), was identified (Bjostad et al., 1984) from some gland extracts and airborne collections, but when added to the six-component blend resulted in a significant arrestment of the upwind flight response of males. It was suggested that the compound was not a component of the sex pheromone released by females but rather an artifact of the preparations.

Here we present the results of detailed studies designed to demonstrate the importance of each component in the proposed six-component blend for *T. ni*. The results of a series of subtraction assays with five-, four-, three-, and two-component blends showed that whereas individual components exerted differential effects on male behavior, the influence of any compound depended on the presence of certain other compounds, with several blend combinations eliciting peak response.

METHODS AND MATERIALS

Insects. Cabbage looper moths were reared on a semisynthetic medium (Shorey and Hale, 1965). Males were separated from females as pupae, kept as adults on a 14:10 light–dark photoperiod at 25–27°C, and provided with 8% sugar water solution.

Chemicals. The synthetic chemicals used in this study were the same as those in Bjostad et al. (1984). The proportions of each compound in the standard six-component mix were as follows: 12:Ac (6.8%), *Z*5–12:Ac (7.6%), *Z*7–12:Ac (100%), 11–12:Ac (2.3%), *Z*7–14:Ac (0.9%), and *Z*9–14:Ac (0.6%). Solutions of the desired blends (with each compound within 1.5% of the standard) were prepared in Skelly B (predominantly *n*-hexane) and checked on capillary GLC (45-m Carbowax 20 M column) to ensure purity (>99%), with a detection limit of 0.1%. A dilution series was then prepared for each treatment to be applied to a release source, either filter paper, rubber septum, or polyethylene cap.

Release Sources. The filter paper source was a 0.75-cm^2 piece of Whatman No. 1 (qualitative, lot No. 308306) positioned on the end of an insect pin placed in a cork stand. Solutions (1 $\mu g/\mu l$) were added with a disposable pipet and were prepared daily just prior to testing.

The rubber septa (Arthur H. Thomas Co., red, 5×9 mm) were prepared by adding solutions (10 $\mu g/\mu l$) to the wide open end of the septa. Septa were placed individually in 4-dram vials and held at $-10°C$ when not in use.

Polyethylene caps (OS-6 closures, American Scientific Products, McGaw Park, Illinois) were prepared by adding solution (10 $\mu g/\mu l$) to the inside surface of the cap. After 1 hr to allow evaporation of the solvent, the cap was closed and placed in a laboratory exhaust hood for 36 hr. Caps were then placed individually in glass vials and held at $-10°C$ when not in use.

In addition to synthetic mixtures individual female glands were also tested. Individual females (3–4 days old) were prepared as in Bjostad et al. (1984) using intact females and the holder utilized for volatile collections.

Test Procedures: Flight Tunnel. Individual 3- to 4-day old males were tested in the sustained-flight tunnel described by Miller and Roelofs (1978) during the fifth and sixth hours of the 8-hr scotophase period (Linn and Gaston, 1981). Males were placed in the room housing the flight tunnel at the beginning of the scotophase to acclimate to conditions in the tunnel: 0.1–0.3 lux, 50–55 cm/sec air velocity, 21–23°C, 50–70% relative humidity (as in Linn and Gaston, 1981).

Males were allowed 1 min to respond and were scored for the following behaviors: activation wing fanning and walking, taking flight, stationary flight near the release cage with the male oriented in an upwind direction, initiation of upwind anemotactic flight, upwind flight to the midpoint of the tunnel, close range approach and contact with the source, protrusion of hairpencils, and attempted copulations. In the present study, source contact is differentiated from a copulatory attempt by males exhibiting, in the latter case, a bending of the abdomen and eversion of the hairpencils, as described in Gothilf and Shorey (1976). Extension of abdominal claspers without the hairpencil display was difficult to observe in the low light conditions, and this was not recorded as a separate behavior. All behaviors were recorded on cas-

sette tape so that temporal aspects of the flight response could be analyzed at a later time. Males were tested only once and then discarded. At the end of each test all metal plates, release cages, and support stands were thoroughly rinsed in acetone and oven-dried at 100°C overnight.

The cluster analysis of male response for all treatments in the subtraction tests was performed as in Linn and Roelofs (1983). The similarity matrix was calculated using the coefficient described by Gower (1971), and the clustering was done using an average linkage analysis with unweighted means, according to Sneath and Sokal (1974) and was performed on a PRIME 400 computer using a program in the GENSTAT statistical package (Alvey et al., 1977).

RESULTS

Dose Response to Six-Component Blend on Filter Paper, Rubber Septa, and Polyethylene Caps. Peak response for male *T. ni* to the six-component blend was observed to a 1-μg dosage on filter paper and to a 3000-μg dosage on polyethylene caps (Figure 1). In contrast, response levels were significantly lower when rubber septa were used, never reaching the near 100% levels observed with caps or filter paper. In addition, males were observed to locate the filter paper source and hairpencil more easily than with rubber septa or caps when the latter were placed in the usual position in the center of the 15 × 15-cm platform (see Linn and Roelofs, 1981). Males appeared to have difficulty reaching the rubber septum or polyethylene cap by landing and walking to them, preferring to fly up to the source, as with the filter paper, which was elevated several centimeters above the surface of the platform. Further tests showed that males exhibited higher numbers of displays relative to source contacts when the caps were placed at the downwind edge of the platform. In this situation, males made continuous flights up to the source, touching the cap with their forelegs and antennae while hanging and vibrating their wings, then bending the abdomen vertically and everting the hairpencils. As a result of these tests, closed polyethylene caps containing 3000 μg of synthetic compounds were used in the subtraction tests to follow, with the source placed on the downwind edge of the platform.

Tests with Five-Component Blends. Removal of Z7–12:Ac from the six-component blend resulted in significant decreases in male activity, with none of the males initiating upwind flight to the source (Figure 2, treatment 7). In contrast, removal of any other component did not affect male behavior. Further tests with the five-component blends (with the exception of the blend lacking Z7–12:Ac) at two lower concentrations, 300 and 1000 μg (Table 1), showed no significant difference in the number of males reaching the source between the five-component blends and the six-component blend at each concentration.

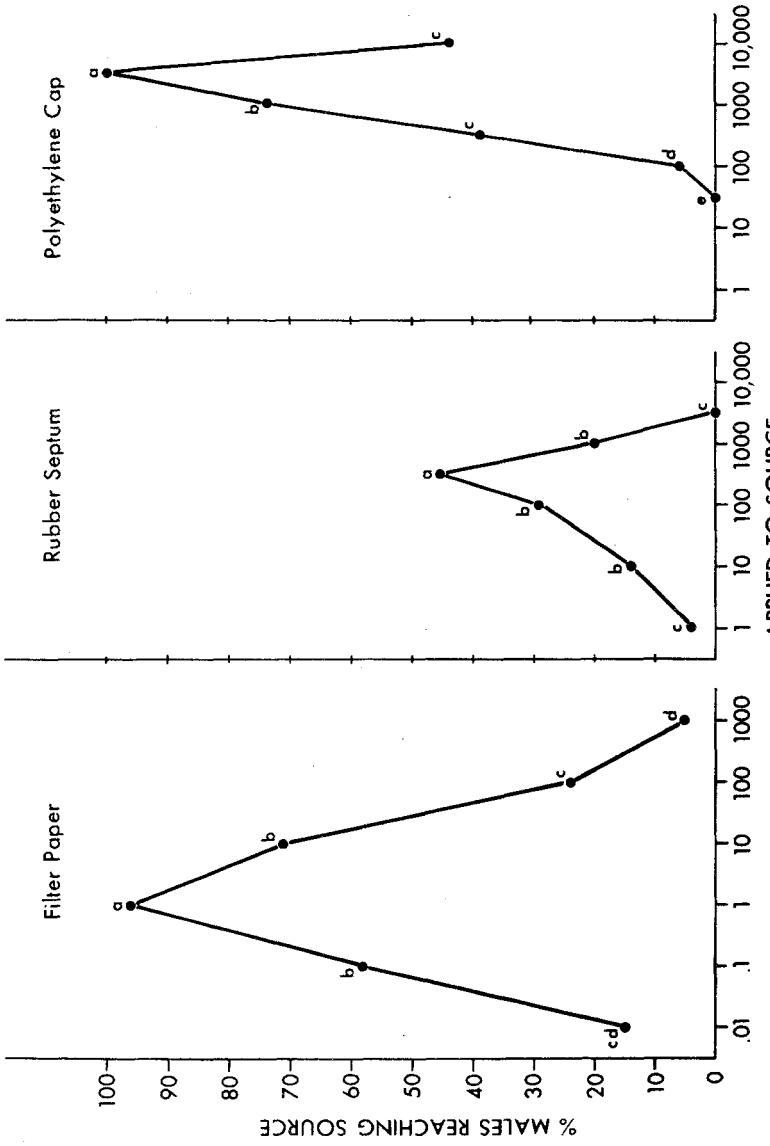

FIG. 1. Percentage of male *T. ni* making source contact with three different pheromone sources containing the six-component blend. For each source type, values having different letters are significantly different ($P < 0.05$) according to the method of adjusted significance levels for proportions (Ryan, 1960). $N = 100$ males for each concentration and source type.

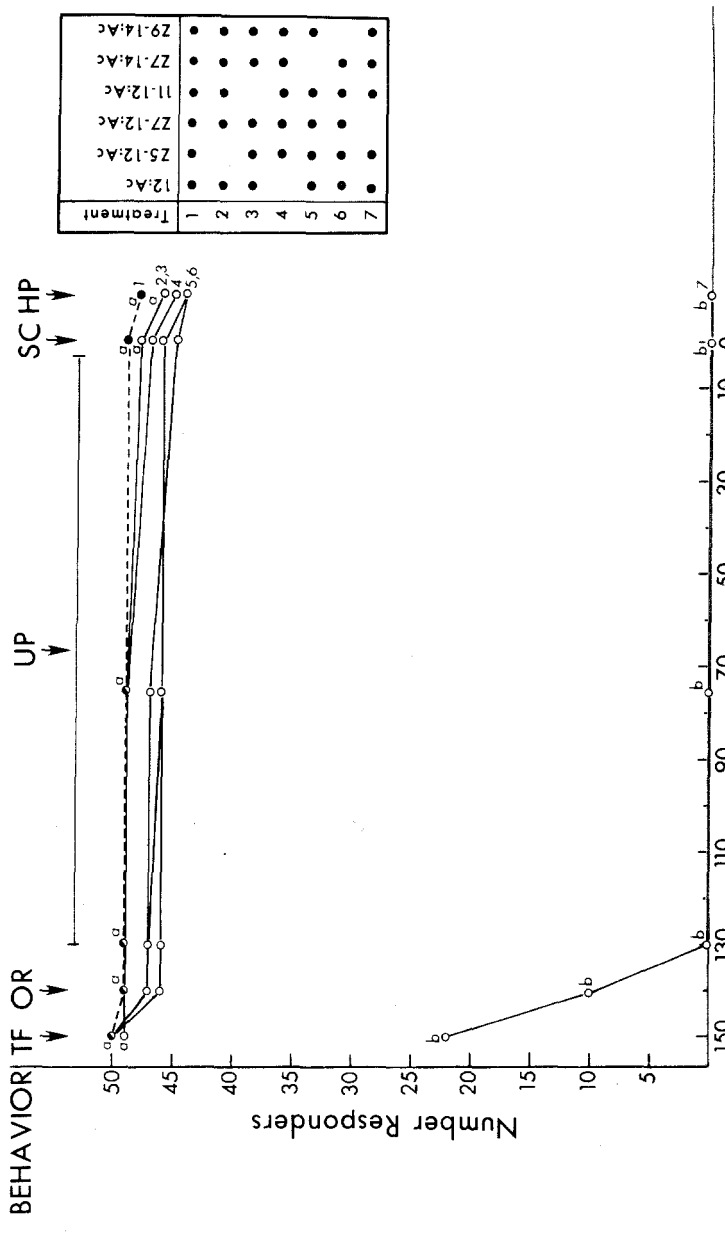

FIG. 2. Response of male *T. ni* to the six-component blend and all five-component blends. Behaviors are taking flight (TF), stationary orientation flight (OR), upwind flight (UP), source contact (SC), and hairpencil display (HP). For all treatments the concentration was 3000 μg. Significant differences for values at each behavior as in Figure 1. $N = 50$ for each treatment.

TABLE 1. PERCENT MALE *T. ni* REACHING SOURCE WITH THREE CONCENTRATIONS OF 6-COMPONENT AND FIVE OF 5-COMPONENT BLENDS[a]

Treatment	Concentration (μg)		
	300	1000	3000
1. 6-component	34a	76a	96a
2. − 12:Ac	38a	70a	90a
3. − Z5-12:Ac	26a	72a	94a
4. − 11-12:Ac	32a	68a	94a
5. − Z7-14:Ac	26a	60a	88a
6. − Z9-14:Ac	30a	78a	86a

[a] $N = 50$ for each concentration. Values in each column with different letters are significantly different ($P < 0.05$) according to the method of adjusted significance levels for proportions (Ryan, 1960).

Tests with Four-Component Blends. The number of males reaching the source was significantly decreased to four of the 10 treatments containing four components when compared to the six-component mix (Figure 3). With the treatment lacking both 14-carbon acetates (treatment 8), male response was most affected in the early part of the upwind flight response. The remaining three treatments (9, 10, and 11) significantly affected the earlier orientation flight to the plume as well as the upwind flight response. In addition, treatments 10 and 11 significantly decreased the level of hairpencil displays when compared to the number of males making contact. Treatments 9, 10, and 11 (Figure 3) all share the common property of lacking two of the following components: Z5-12:Ac, 11-12:Ac, or Z7-14:Ac.

Tests with Three-Component Blends. Male response to all three-component blends was significantly decreased at all stages in the sequence with the exception of the initial response, taking flight, when compared to the six-component blend (Figure 4). The patterns in Figure 4 also show that response to the three-component blends was not significantly different for any blend until the final step, hairpenciling and copulatory attempts. The level of hairpenciling was most affected by treatments lacking combinations of Z7-14:Ac, 11-12:Ac, Z5-12:Ac, or 12:Ac, with no single component or binary combination essential to the success of the display.

Tests with Two-Component Blends. As anticipated from the results in Figure 4, male response was significantly decreased to all two-component combinations when compared to the six-component blend (Figure 5). This figure also shows the response to Z7-12:Ac alone.

Cluster Analysis of All Treatments. Examination of the response patterns in Figures 2, 3, 4, and 5 suggested that certain treatments, which varied

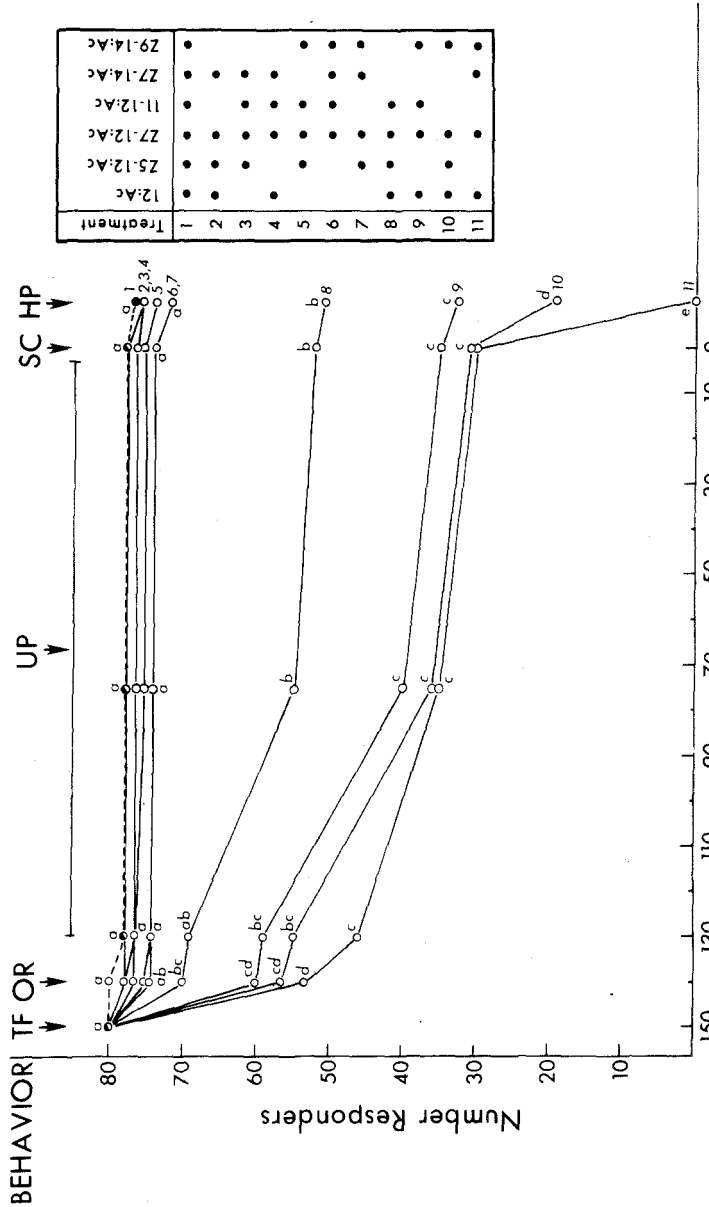

FIG. 3. Response of male *T. ni* to a 3000-μg concentration of the six-component blend and all four-component blends containing Z7–12:Ac. Behaviors and significant differences as in Figure 2. $N = 80$ for each treatment.

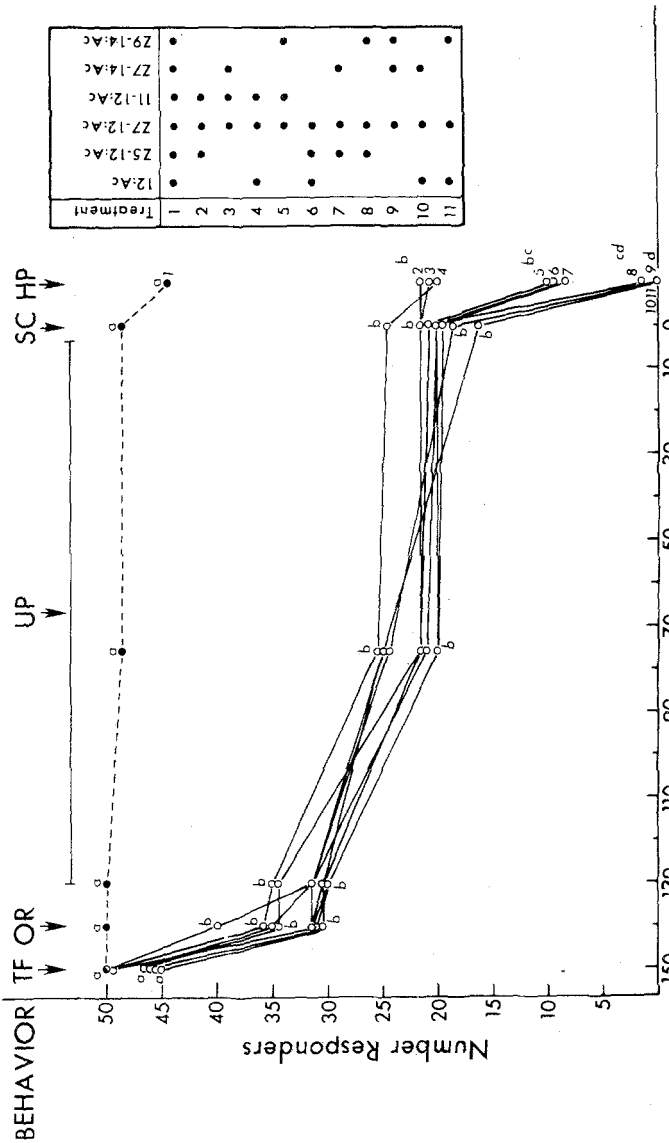

FIG. 4. Response of male *T. ni* to a 3000-μg concentration of the six-component blend and all three-component blends containing Z7–12:Ac. Behaviors and significant differences are in Figure 2. $N = 50$ for each treatment.

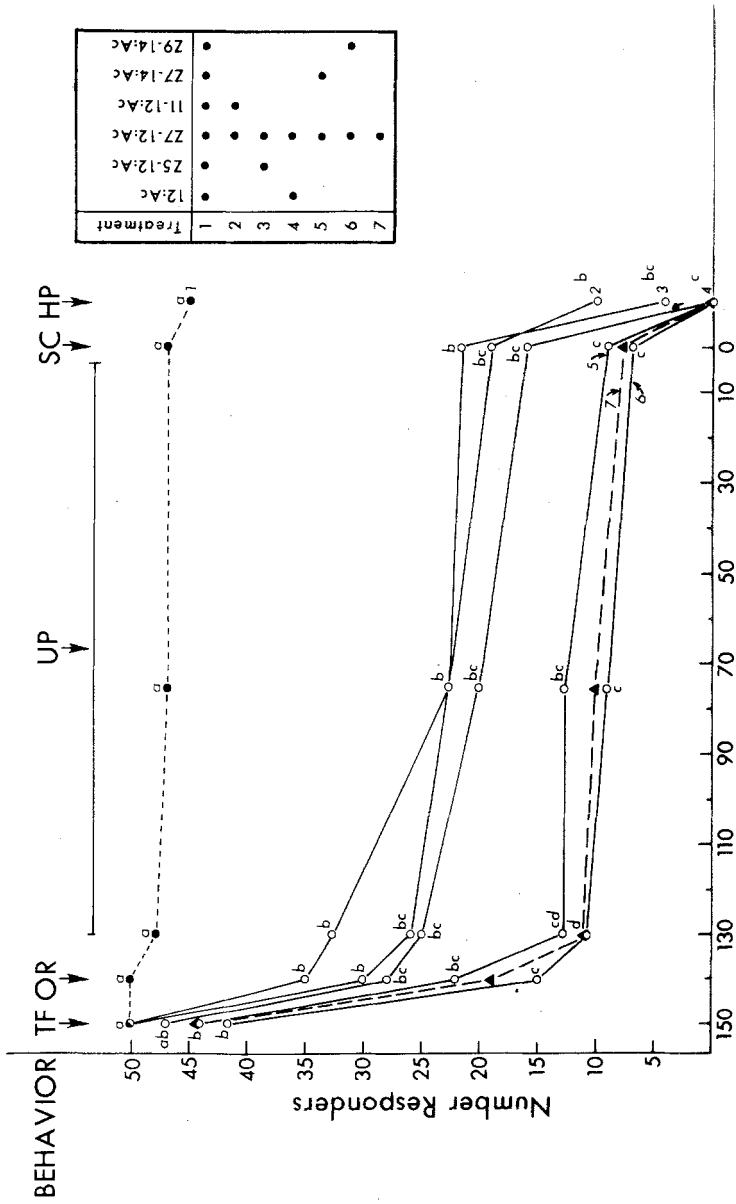

FIG. 5. Response of male *T. ni* to a 3000-μg concentration of the six-component blend, all two-component blends containing Z7–12: Ac, and to Z7–12: Ac alone. Behaviors and significant differences as in Figure 2. $N = 50$ for each treatment.

in the number of components, elicited similar levels and patterns of response. To determine the similarity of treatments, the table of response values for seven observed behaviors to the 33 treatments was subjected to hierarchical cluster analysis. The dendrogram shown in Figure 6 is derived from analysis of four behaviors (taking flight, upwind flight, source contacts, and hairpencil displays), as additional behaviors did not add significant information to the analysis. The response patterns for five identified clusters are shown in Figure 7, and the treatments within each cluster are displayed in Table 2.

From the data presented in Table 2 several important relationships can be noted. Most evident is that *Z*7–12:Ac is essential for any significant level of upwind flight to occur. Cluster III represents a number of treatments containing two, three, or four components, all of which significantly increased the number of males successfully initiating upwind flight and making contact with the source over that observed with treatments in cluster IV. The subdivision of cluster III was made first on the basis of the cluster analysis differentiating increased levels of hairpenciling (levels IIId, c, b, a) and second on the basis of visual inspection of Table 2 indicating the presence of specific combinations of components that were associated with increasing levels of hairpenciling (demarcated by the dashed lines). Treatments in cluster IIId, for example, lack *Z*5–12:Ac and 11–12:Ac and represent those for which no hairpenciling was observed. Treatments in clusters IIIc, b, and a show the effect on hairpencil displays of adding *Z*5–12:Ac or 11–12:Ac to the treatments in IIId.

Cluster II represents a single four-component blend resulting from addition of 12:Ac to *Z*7–12:Ac + *Z*5–12:Ac + 11–12:Ac, and lacking both of the 14-carbon acetates. Significant increases in source contacts compared to treatments in cluster III occurred as a result of an increase in the number of males successfully initiating upwind flight.

Peak levels of response occurred to treatments in cluster I, comprising the six-component blend, five of the five-component blends, and several four-component blends. Examination of treatments in cluster I leads to a set of associations, shown in Figure 8, describing all possible signals eliciting peak response.

Analysis was also made of several other quantitative aspects of the flight response and the hairpencil display, using the treatment clusters in Table 2. Males were observed to take significantly longer to fly upwind to the source to treatments in clusters III and IV compared to I and II (Figure 9). There was no difference in this temporal measure for the subdivided treatment groups within cluster III. With respect to the hairpencil display, males spent significantly longer periods at the source and exhibited greater numbers of displays to treatments in clusters I, II, and IIIa, compared to those in IIIb, c, d; IV; or V (Figure 10).

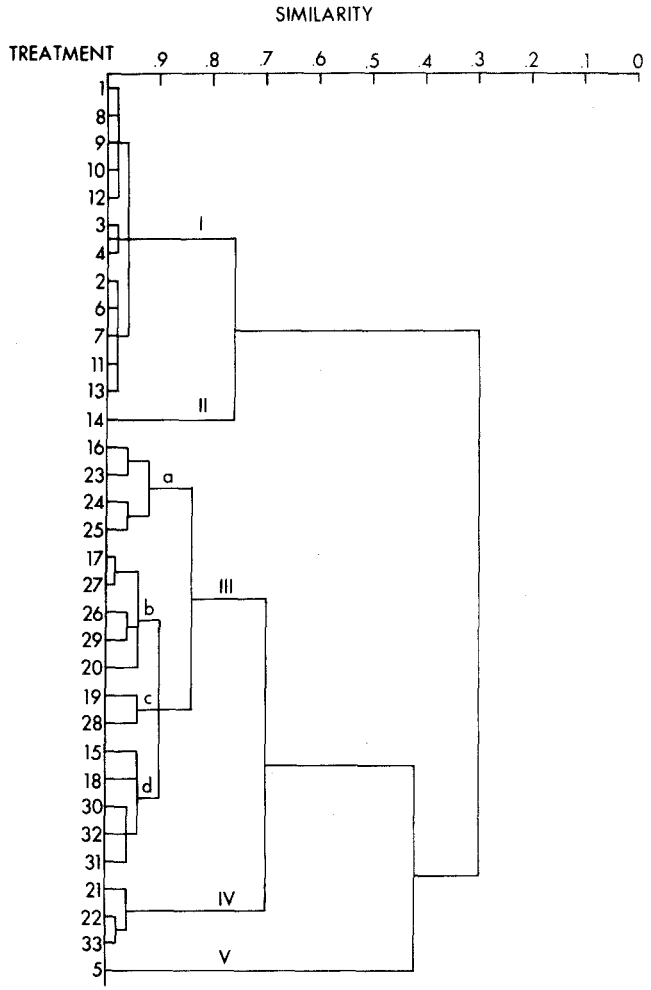

FIG. 6. Dendrogram resulting from hierarchical cluster analysis of response values for four behaviors (TF, UP, SC, HP; see Figure 2) to the 33 treatments tested in subtraction assays.

Z7–12:OH and Male Behavior. Response levels to the six-component blend on filter paper were found to be greater at all dosages tested when compared to the six-component blend with 0.5% Z7–12:OH added (Figure 11). Male response to the six-component blend was also found to be significantly affected during the upwind flight phase of the sequence as increasing amounts of Z7–12:OH were added to the 1-μg dosage (on filter paper) (Figure 12).

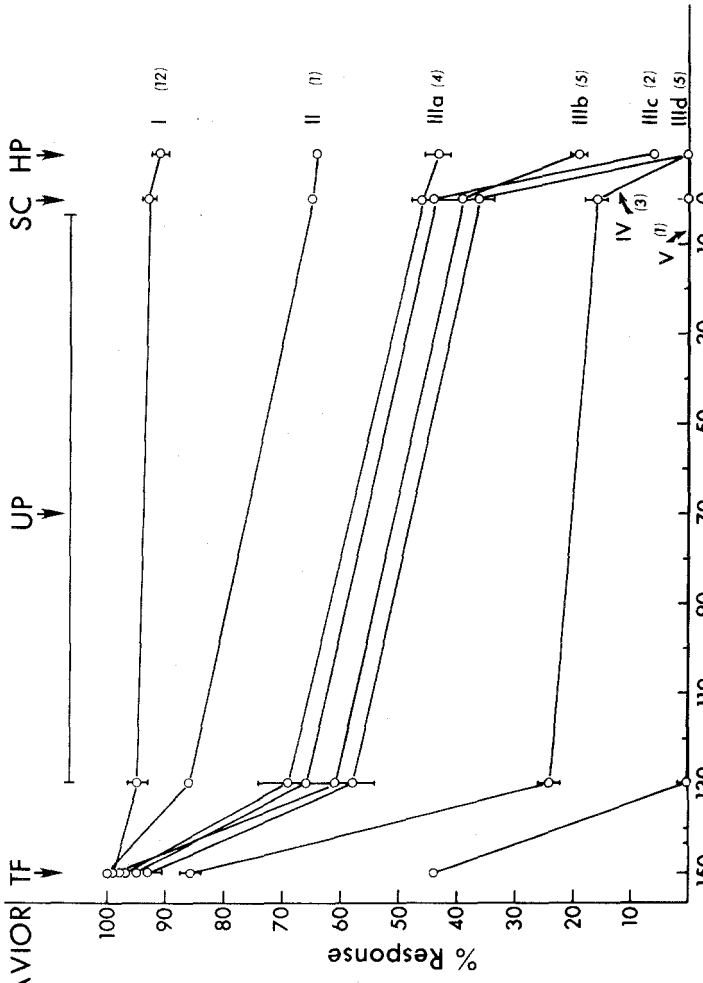

FIG. 7. Response patterns for clusters of treatments from dendrogram in Figure 6. Response values are the mean (±SD) for each behavior (as in Figure 2) in the sequence. Values next to numerals indicate the number of treatments in each cluster.

TABLE 2. PERCENT RESPONSE OF MALE *T. ni* TO 33 TREATMENTS SHOWN IN FIGURE 6[a]

Treatment	Taking flight (%)	Upwind flight (%)	Source contacts (%)	Hairpencil displays (%)	12:Ac	Z5-12:Ac	Z7-12:Ac	11-12:Ac	Z7-14:Ac	Z9-14:Ac		Cluster
1	100	98	98	96	*	*	*	*	*	*	6	
3	100	98	94	96		*	*	*	*	*	5	
2	100	94	92	92	*	*	*	*	*	*	5	
4	100	98	96	92	*	*	*		*	*	5	
6	100	94	90	88	*	*	*	*		*	5	
7	100	92	92	88	*	*	*	*	*		5	
11	100	93	93	90			*	*	*	*	4	I
13	100	92	92	90		*	*		*	*	4	
12	100	96	95	92		*	*	*		*	4	
9	100	96	96	95		*	*	*	*		4	
10	100	96	96	95	*		*	*	*		4	
8	100	98	98	95	*	*	*		*		4	
14	100	86	65	64	×	×	×	×			4	II
25	94	62	46	46		●	●	●			3	
16	100	74	44	41	◒		◒	◒		◒	4	IIIa
23	100	70	48	40	◒		◒	◒			3	
24	92	68	46	44			◒	◒	◒		3	
26	96	60	40	20			◒	◒		◒	3	
20	100	62	38	16			◒	◒			2	
17	100	69	39	19	○	○	○			○	4	IIIb
27	100	66	40	18	○	○	○				3	
29	98	60	38	16		○	○		○		3	
28	90	66	44	6		○	○			○	3	IIIC
19	100	66	44	7		○	○				2	
32	90	62	34	0	◒		◒			◒	3	
31	94	66	36	0	◒		◒		◒		3	
15	100	57	37	0	◒		◒		◒	◒	4	IIId
18	94	50	32	0	◒		◒				2	
30	90	60	40	0			◒		◒	◒	3	
21	88	26	18	0			+		+		2	
22	84	22	14	0			+			+	2	IV
33	87	23	15	0			+				1	
5	44	0	0	0	●	●		●	●	●	5	V

[a]Treatments are arranged in clusters as characterized in Figure 6, and listed within each cluster to show relationships between blend composition and response level. See text for details. $N = 80$ for the four-component blends, and 50 for any others.

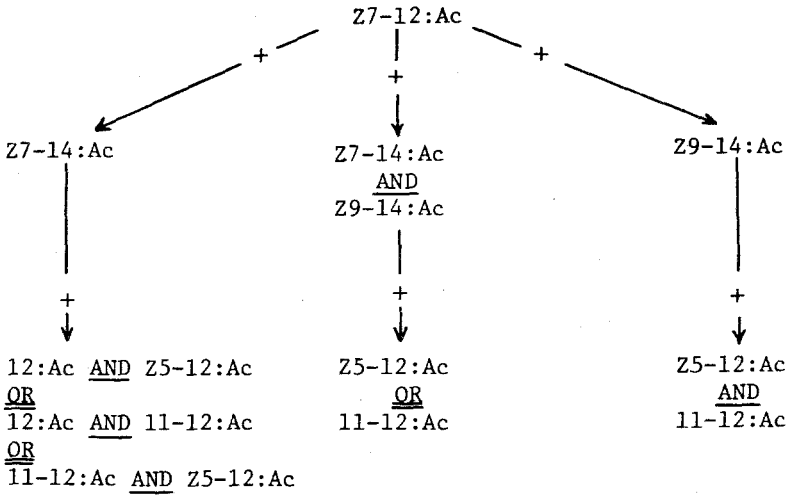

FIG. 8. Flow chart showing the association of components into blends that will elicit peak response.

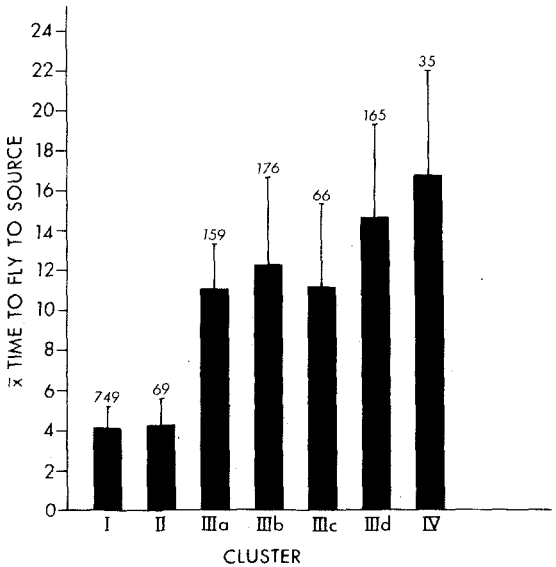

FIG. 9. Time (sec) for male *T. ni* to fly upwind 130 cm to the source to treatments within each cluster identified in Figure 7. Values are the mean (±SD), with the number of responders shown above each column.

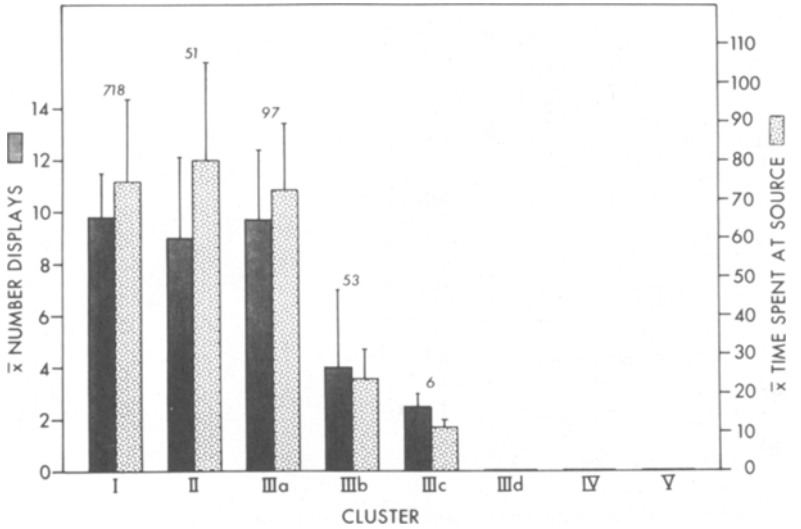

FIG. 10. Time (sec) spent by male *T. ni* at the source, and the number of hairpencil displays exhibited. Values are means (±SD), with the number of responders shown above each column.

Increasing levels of Z7–12:OH resulted in a significant number of males that initiated upwind flight exhibiting arrested flight patterns in the first half of the flight downwind in the tunnel. This arrestment was assessed as an abrupt slowing of upwind flight to the stationary zig-zag pattern with no net upwind movement.

DISCUSSION

Pheromone Component Functions and Redundancy in a Chemical Signal. We propose, on the basis of the present study, that all six components are involved in the sex pheromone communication system of *T. ni* and that the observed redundancy with respect to component substitutions and interactions comprises a new and important element of pheromone studies. All of the five-component blends (except the one lacking Z7–12:Ac) and several four-component blends elicited peak response levels as high as those observed with the six-component blend. The evidence implies that none of the five secondary components is absolutely required for peak response. It appears instead that the pheromone is polythetic in character, in that a combination of any four minor components can compensate for the lack of the fifth, and in some cases combinations of the three minor components can compensate for the lack of the other two. The results also show that as re-

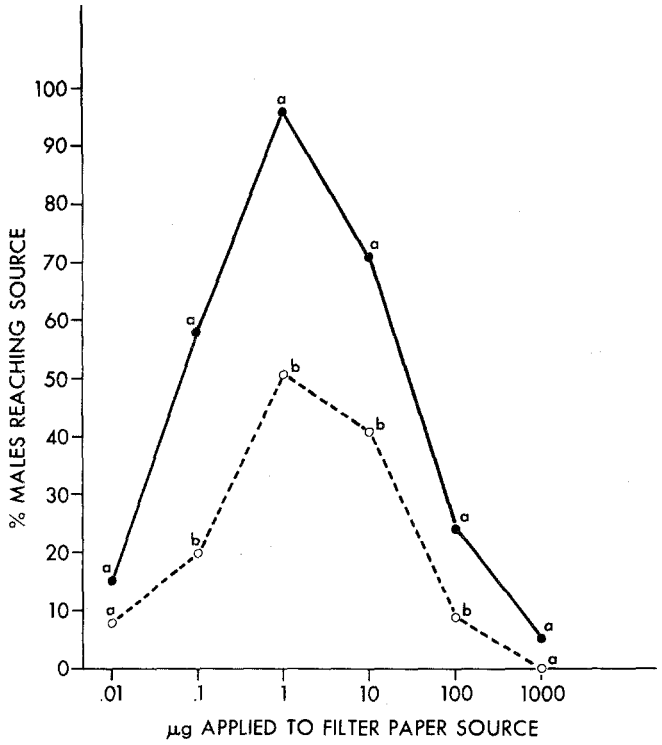

FIG. 11. Response of male *T. ni* to the six-component blend alone (solid line) and with 0.5% Z7-12:OH added (dashed line). Significant differences as in Figure 2. $N = 100$ for each concentration and treatment.

sponse levels increased with increasing blend complexity, the importance of individual components as functional elements associated with specific behaviors diminished dramatically. The conclusion is that it was not possible to assign specific behavioral functions to any of the individual compounds.

Our results raise some important points concerning the classification of a given compound as a pheromone component. In the classic view of a pheromone complement, if subtraction of a compound from a candidate blend fails to diminish the response to the blend, the compound is not considered a pheromone component. Our conclusion that all six acetates are pheromone components in *T. ni*, despite the fact that any of the five minor components can be omitted without diminishing male response, is a significant departure from this view. In drawing this conclusion, we were careful to satisfy two criteria that we propose are essential: that male responses to females or female extracts should be high, indicating that the assay conditions are appropriate, and that the male response to the best synthetic blend should be equally high.

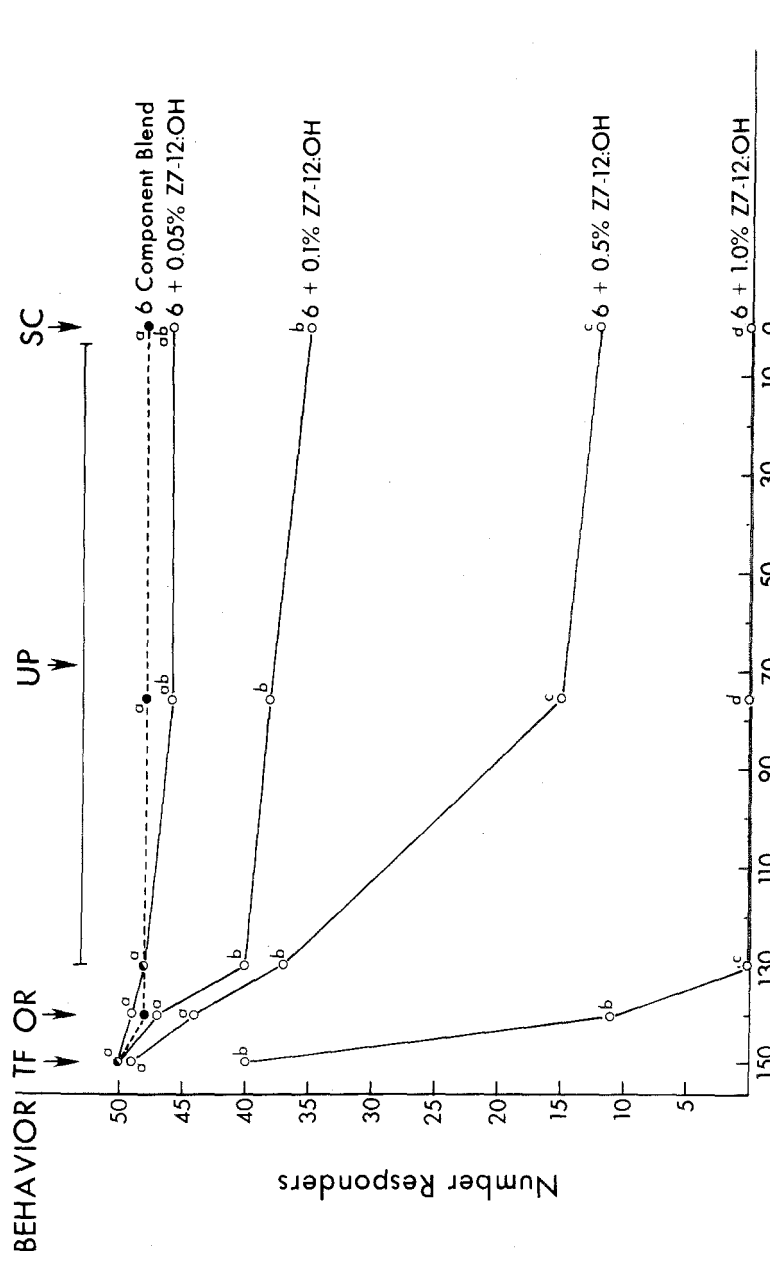

FIG. 12. Response of male *T. ni* to a 1-μg dosage of the six-component blend on filter paper with four added proportions of Z7-12:OH. Behaviors and significant differences as in Figure 2. $N = 50$ for each treatment.

Our analysis supports the concept that it is the blend of components acting as a unit that is critical in effecting optimal behavior in males (Linn and Roelofs, 1982, 1983; Baker and Cardé, 1979; Baker et al., 1981b). Studies with *Grapholitha molesta* have shown that males are more sensitive to the natural blend of components compared to single components (Linn and Roelofs, 1983) and that they are very sensitive to slight changes in blend. The resulting sensitivity is presumably adaptive since it enhances the males ability to detect, recognize, and respond rapidly to the airborne signal, thus optimizing the males' likelihood of locating a mate.

Recognition involves an assessment by the male of the chemical signal and a decision as to whether the signal is being released by a female of his species. Inherent in the decision process is a possibility that the male will make a wrong choice (Wiley and Richards, 1982), either by not responding to the appropriate signal or perhaps by responding to a similar signal of a closely related species. The uncertainty involved is greatly reduced by utilizing complex blends of chemicals, because the decision to respond can be made dependent on perception of a specific blend of components.

While complex blends aid in achieving response specificity and increased mating success, the presence of redundant elements in the signal is not a necessary feature. In the present study redundancy was evidenced by the fact that certain minor components could substitute for each other without any observed change in male behavior. We would propose that the specific substitutions observed for the minor components reflect interactions, at the level of the peripheral antennal receptors, which significantly influence neural processing of the chemical signal. The presence of redundant elements could add to the efficiency of the processing by allowing some minor components to interact at several receptor sites, resulting in an amplification of the stimulus induced by specific minor components, or the major component.

Examples from neurophysiological studies indicate that there are a number of possibilities for component–receptor site interactions at the peripheral level. It has been established for a number of species that individual senilla trichodea contain from two to five receptor types (Preisner, 1983). In the *Yponomeuta* complex of species, for instance, response spectra of sensilla trichodea cells showed that two or three types of sensilla containing two to three different cells could be distinguished. Up to six different sensilla could thus occur in one species, and this did not take into account the added variability in sensitivity as a function of concentration (Van Der Pers, 1982). There can also be considerable variability in response across receptor neurons to any one compound, and across compounds to any neuron (O'Connell, 1975), presumably due to intrinsic properties of the neuron, such as kind, number, and location of the different receptor sites. In general, while the receptors are specific for individual compounds (Preisner, 1979), interactions can occur, and it is not always certain whether one is dealing

with distinct sites or a quantitative difference in binding strength between different chemicals at a common site (O'Connell, 1975). In some cases there appears to be a competition between compounds for the same receptor site, as in *Adoxophyes orana* (Den Otter, 1977). In this species one cell (the B cell) is excited by Z9–14:Ac alone, whereas another cell (the B cell) is excited by Z11–14:Ac and to much lesser extent Z9–14:Ac. The A cell shows a decrease in response to Z11–14:Ac when Z9–14:Ac is present, and it was concluded that the two compounds compete for the same sites in the A cell membrane, with the binding of Z9–14:Ac resulting in a smaller potential change than occurs with Z11–14:Ac. In other cases, an interaction between compounds can occur at the same receptors, as appears to be the case in *Spodoptera litura* (Aihara and Shiboya, 1979). In both *S. litura* and *S. exempta* (Steinbrecht, 1982), minor components do not elicit strong responses unless the major components are present. This is also the case with *Argyrotaenia velutinana*, in which dodecyl acetate gives a weak response when presented alone, but strongly potentiates the response to Z11–14:Ac (O'Connell, 1972).

While our hypothesis is speculative in nature, it is testable. We would propose, for example, that differential adaptation studies as well as further behavioral tests involving preexposure of males to individual components (as in Linn and Roelofs, 1981), as well as specific combinations of components illustrated in Figure 8, would help elucidate receptor–component interactions.

With respect to the female, the releaser in the system, studies on the biosynthesis of the *T. ni* sex pheromone (Bjostad and Roelofs, 1983) suggest that all of the secondary components are products of a common pathway and thus the probable expense of producing and releasing a six-component vs. a two-component blend is slight. At the same time, producing a complex blend provides the female with a mechanism for selecting mates that preferentially respond to her and also avoiding hybridizing with closely related species.

Complex pheromone blends that include redundant elements are one end of a spectrum of possibilities that insects might use in their communication systems. We do not propose that all species will have complex pheromone blends, and we recognize that this will depend on several factors. One involves consideration of the biosynthetic route for the primary component(s) and the potential for additional components from precursors in the pathway (Bjostad and Roelofs, 1983; Roelofs an Brown, 1982). Another is the inter- and intraspecific pressure on males to utilize a highly specific blend. It is hoped that future studies will address the importance of additional components in optimizing male behavior and that greater consideration will be given to ecological questions concerning male competition for mates (as in Parker, 1978) and the importance of interspecific interactions (see Tumlinson, 1982).

Experimental Design and Peak Response. The success of the flight tunnel as a sensitive assay for this complex blend was not anticipated based on previous experience with this insect (Linn and Gaston, 1981). *T. ni* males are very sensitive to movement as well as several environmental stimuli during the scotophase activity period, and it was found in the previous study that an acclimation period to flight-tunnel conditions was essential for male flight to the pheromone. In the present study, a longer acclimation period (4 hr vs. 1 hr) was incorporated to aid in handling of the insects, and yet even with all precautions, male response levels to the two-component blend, Z7–12:Ac + 12:Ac, were highly variable from day to day when tested over a one-month period, as was done in preliminary tests. This variability (which was not observed with the six-component mix) had also been observed in previous work (Linn and Gaston, 1981) and necessitated establishing in that study a daily response criterion of at least 50% of the moths taking flight for the results to be considered in the final analysis. This resulted in assay data for many days not being included, and in what now can be recognized as an artificially high response value reported to the two-component blend [>70% in Linn and Gaston (1981) compared to 30–40% in the present study, Figure 5].

We propose that the poor response to rubber septa was the result of an inappropriate ratio of compounds being released from this substrate. Analysis of two airborne collections of the six-component mix on rubber septa showed that Z7–14:Ac and Z9–14:Ac were released in much lower proportions than was applied to the source (0.02 and 0.01% vs. 1.0 and 0.6%, respectively). Polyethylene caps were not tested due to physical limitations of the airborne collection apparatus.

Close Range Behavior and Hairpencil Displays. The hairpencil display observed in the present study was very similar to that reported by Gothilf and Shorey (1975). As reported by them and discussed in Colwell et al. (1978), females of several species characteristically release pheromone while wing fanning and hanging from a substrate. Males typically fly up to the female and initiate courtship while flying in a hovering position. Examples include *I. ni*, *Lymantria dispar* (L.), and *Pectinophora gossypiella* (Saunders) and are in sharp contrast with species with as *A. velutinana* (Walker) and *Grapholitha molesta* (Busck), which typically land and walk to the female. This basic difference in close range flight behavior proved critical in the hairpencil display observed here with *T. ni.* Males performed the display more often and with more intensity when they could fly up to the source and hang on it while wing-fanning.

The hairpencil display was the behavior most affected by subtle changes in blend composition. Males did not hairpencil to the two-component blend containing Z7–12:Ac and 12:Ac, and the minimum blend for optimal display was found to depend on the presence of 11–12:Ac and either 12:Ac,

Z5-12:Ac, or Z7-14:Ac (cluster IIIa, Table 2). The compound 11-12:Ac, however, was not essential since within cluster I treatments containing both Z5-12:Ac and Z7-14:Ac were equal to any containing 11-12:Ac. As noted earlier, the conclusion is that no single component can be labeled as a close range or hairpenciling component, when evaluated in the context of the response to the full blend.

Z7-12:OH and Male Behavior. The identification of a sex pheromone component has typically involved some behavioral test, either field testing or a laboratory bioassay. In all cases, confirmation of the compound's status has depended on a positive or enhancing effect on male behavior (Cardé, 1979). At the same time, several studies have demonstrated that males of certain species are very sensitive to compounds that are related to known pheromone components but are not released by females. Such is the case with Z7-12:OH in *T. ni*. Field tests (Tumlinson et al., 1974) and flight-tunnel studies (McLaughlin et al., 1974) provided evidence that Z7-12:OH significantly decreased the level of upwind flight in male *T. ni*. Electrophysiological studies (O'Connell et al., 1983) also support the fact that male *T. ni* can perceive this compound.

It was thus of some interest to us to observe this compound in some of the female airborne collections (Bjostad et al., 1983). Data from the present study confirm that, under all conditions tested, addition of Z7-12:OH resulted in decreased male response. The weight of evidence suggests that its occasional presence of about 0.5-1.0% in gland extracts and airborne collections is an artifact, perhaps due to a small amount of hydrolysis of Z7-12:Ac during handling. While we have concluded that the presence of this compound in the airborne collections is an artifact, the question concerning the biological relevance of the observed activity remains. It most likely has biological significance in interspecific interactions, wherein *T. ni* males have reduced behavioral response to other cohabitating species utilizing Z7-12:OH.

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Book Review

Plant Resistance to Insects. Paul A. Hedin (ed.), ACS Symposium Series No. 208. Washington, D.C.: American Chemical Society, 1983. $53.95, 375 pp.

There seems to be a spate of new books on insect herbivory or insect–plant coevolution at the present time, and there may be some danger of overproduction in this field. Yet the present volume, based on the proceedings of a symposium held in Las Vegas in the spring of 1982, would seem to be justified.

Although directed at the practical application of knowledge on plant–insect interactions to pesticide development, the contributors are all research oriented and possible applications are only briefly mentioned. In many ways, this book could be regarded as an updating of Rosenthal and Janzen's *Herbivores: Their Interaction with Secondary Plant Metabolites*, which, although dating only from 1979, has become something of a classic. Indeed, there are a number of contributors in common. For example, D.F. Rhoades here presents evidence for pheromonal sensitivity in willows when under attack by tent caterpillars, while C.A. Ryan describes his latest experiments on the accumulation of proteinase inhibitors in tomato leaves after wounding. Induced resistance mechanisms are also discussed by J.C. Schultz in a more general review of the defensive chemistry of forest trees.

One of the more popular theories of plant defense, that apparent species are chemically protected from insects in different ways than nonapparent species, has come under fire recently, especially regarding the defensive role of condensed tannins. That the defense strategies of plants are much more complex than this theory suggests is made clear in several of these essays. R.G. Cates, for example, in studying the interaction between the Douglas fir and western spruce budworms, finds that foliar terpenes are major determinants in limiting feeding, while changes in nitrogen and polyphenol levels have little effect. Similarly, in a study of the cotton plant–tobacco budworm interaction, P.A. Hedin reports that condensed tannin concentrations in the terminal leaves are not correlated with resistance. Instead, cyanidin 3-glucoside is as effective as gossypol in limiting larval growth, both on the plant and in laboratory feeding experiments.

One other general point emerging from several chapters, and notably that of J.C. Reese, is that all plants, even susceptible varieties, appear to be defended against insect feeding in such a way that insects do not thrive as

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well on their host plants as on artificial diets. Thus, even under favorable circumstances, specialist feeders may be restricted in their growth and fecundity, probably due to as yet little-understood nutrient-allelochemical interactions.

Space does not allow mention of all the various aspects of plant resistance mentioned in the 20 chapters of this book. The contributions vary from those which are entirely reviews (e.g., the role of lipids in plant resistance to insects) to those which present recent research results (e.g., on the oviposition stimulants in solanaceous plants to the tobacco hornworm). While some of the material is very familiar (e.g., phytoecdysones, polyphenol-protein interactions, cytochrome P-450 detoxification), there is still much that is new. Two of the problems in books produced from camera-ready copy are a lack of uniform style and the fact that many errors that an editor would pick up go undetected. While both these are true in this book, there seem to be no serious faults. Overall, this is a welcome addition to the literature on plant-insect biochemistry and it will surely be of interest to most readers of this journal.

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STRUCTURE–ACTIVITY RELATIONSHIPS BETWEEN STIMULUS MOLECULE AND RESPONSE OF A PHEROMONE RECEPTOR CELL IN TURNIP MOTH, *Agrotis segetum*

Modifications of the Acetate Group

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Abstract—The response of an antennal receptor cell of the turnip moth, *Agrotis segetum,* was recorded during stimulation with a series of (*Z*)-7-dodecenyl acetate analogs with structural variations of the acetate group. The investigated receptor cell is known to be highly selective to (*Z*)-7-dodecenyl acetate. All parts of the acetate group were found to be of great importance for full biological activity. The results indicate very strict requirements on the shape of the polar functional group, as well as on its electron distribution for a successful interaction with the antennal receptor cell.

Key Words—Structure–activity relationships, single-cell recordings, turnip moth, *Agrotis segetum,* Lepidoptera, Noctuidae, molecular shape, dipole moments.

INTRODUCTION

The interaction between moth sex pheromone components and their target sensory cells in male moth antennae is distinguished by its high selectivity (Priesner, 1979). This indicates very strict requirements on the molecular properties of the substrate molecule for a successful interaction with the presumed macromolecular receptor located in the dendritic membrane. The

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high selectivity in the pheromone perception process makes it of interest to study the details of the transduction mechanism as well as the molecular properties responsible for a productive substrate–receptor interaction. The aim of such studies is to make it possible to explain the observed selectivity in terms of molecular structure and intermolecular forces. A basic question in this context is which parts of the pheromone component molecule are essential for full biological activity. If such parts can be identified, their relative contributions to the selectivity may be studied.

Straight-chain olefinic acetates constitute a large and important group of molecules employed by female moths as sex pheromone components (Steck et al., 1982). The corresponding alcohols and aldehydes are also frequently found in pheromone mixtures. The models used in attempts to understand the substrate–receptor interaction for this type of molecules generally focus on three different parts of the stimulus molecule: (1) the polar functional group (acetate, aldehyde, or alcohol), (2) the carbon–carbon double bond, and (3) the terminal methyl group. Thus, Kafka and Neuwirth (1975) proposed a static three-point model, using these parts of the most active compound for the species investigated, to simulate three "active sites" on the hypothetical receptor surface. A similar but more qualitative model has been described by Roelofs and Comeau (1971). Bestmann and Vostrowsky (1982) advocate a dynamic model for the substrate–receptor interaction, in which the substrate molecule is bound stepwise to the active site of the receptor. In all models, the polar functional group plays an important role. Kafka and Neuwirth use a point dipole to model its interaction with the corresponding "active site." Roelofs and Comeau assume binding sites for the acetate carbonyl, as well as for the acetate methyl group. In the model put forward by Bestmann and Vostrowsky, the polar functional group is responsible for the primary recognition of the substrate molecule by the receptor site, forming an initial nucleation complex, while other parts of the molecule are responsible for the excitation function.

The selection of the polar acetate group as a candidate for important interactions with the receptor is understandable in view of its capacity to engage in strong binding through electrostatic forces (dipole–dipole interactions) and/or through hydrogen bonds to both ester oxygen atoms.

To investigate the selectivity of the receptor with respect to the polar functional group of straight-chain olefinic acetates, we recorded the electrical responses of a specific receptor cell during stimulation with a series of (*Z*)-7-dodecenyl acetate (*Z*7–12 : Ac) analogs in which the acetate group had been modified. Olfactory receptor cells specifically tuned to *Z*7–12 : Ac are present in antennal sensilla trichodea type SW1 (Hallberg, 1981) of the turnip moth, *Agrotis segetum* (Löfstedt et al., 1982; Van Der Pers and Löfstedt, 1983).

Data on structure–activity relationships in insect olfaction have been previously obtained by recording behavioral responses in the field and in the laboratory or by electrophysiological methods. Evaluation of the results is more reliable with the latter methods, in which interference of unknown factors is low. Two electrophysiological recording methods are in common use in studies on insect olfaction: the electroantennogram (EAG) and the single-cell technique. An EAG response is assumed to reflect the combined activity of many olfactory cells on the antenna (Schneider, 1957; Boeckh et al., 1965), whereas single-cell recording deals with the electrical activity of a single receptor cell only (Boeckh et al., 1965; Schneider and Steinbrecht, 1968; Kaissling, 1971). At present, the single cell recording technique offers the most direct method in the study of structure–activity relationships.

The antennal olfactory receptor cells of *Agrotis segetum* are easily accessible for single-cell recording (Löfstedt et al., 1982; Van Der Pers and Löfstedt, 1983).

The polar functional groups of the molecules chosen for this study are all formally derived from the acetate group, with modification(s) of one of the parts of the functional group which may possibly interact with a complementary structure at the active site of the receptor. The compounds are shown in Figure 1.

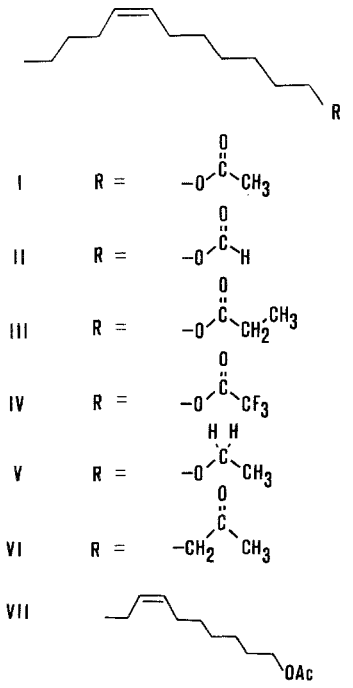

FIG. 1. Compounds studied.

Compound VII was included to allow comparisons with results of modifications at the nonpolar end of compound I. The results of electrophysiological measurements using I–VII as stimulus molecules will be discussed in terms of molecular shape and electron distribution.

METHODS AND MATERIALS

Chemicals. (*Z*)-7-Dodecenyl acetate (I), (*Z*)-7-dodecenyl formate (II), (*Z*)-7-dodecenyl propionate (III), (*Z*)-7-dodecenyl trifluoroacetate (IV), and (*Z*)-7-dodecenyl ethyl ether (V) were all synthesized from the same precursor, (*Z*)-7-dodecenol. This alcohol was prepared from 1-hexyne and 6-bromo-1-hexanol through hydrogenation of the resulting acetylenic compound over Lindlar catalyst. The corresponding esters were obtained by well-established reactions with acid chloride, acid anhydride or, in the case of the formate II, with methyl formate. For the synthesis of the ethyl ether V, the alcohol was first converted to the bromide and then reacted with ethoxide ion.

The methyl ketone, (*Z*)-10-pentadecen-2-one (VI), was prepared by the reaction of methyl lithium with (*Z*)-9-tetradecenoic acid.

(*Z*)-7-Decenyl acetate (VII) was obtained from commercial sources. The small amounts of *E* isomer obtained in the hydrogenation step were removed by argentation liquid chromatography (Houx et al., 1974). After further purification by preparative GC, all compounds were at least 99.5% pure.

Calculations. Electron distributions, dipole moments, and directions of dipole moments were calculated using the semiempirical quantum chemical CNDO/2 method (Pople and Beveridge, 1970). Energy-minimized molecular geometries were obtained from molecular mechanics calculations employing the MM2 program developed by Allinger and coworkers (Allinger and Yuh, 1980; Burkert and Allinger, 1982). The space-filling van der Waals models were calculated and plotted by a locally modified PLUTO program (Allen et al., 1979) using the same van der Waals radii as employed by the MM2 program.

Electrophysiology. Electrophysiological recordings were made from single antennal sensilla trichodea, type SW1 (Hallberg, 1981), on excised antennae of male *Agrotis segetum* moths. Each sensillum trichodeum type SW1 contains three receptor cells, as could be established on the basis of morphological investigations (Hallberg, 1981), as well as electrophysiological studies (Van Der Pers and Löfstedt, 1983). The moths were obtained from a laboratory culture used by Löfstedt et al. (1982). The action potentials (spikes) generated by the receptor cells associated with these sensilla were recorded by the tip recording technique (Kaissling, 1974) as modified by Van Der Pers and Den Otter (1978). Chemical stimuli were

applied from disposable plastic 5-ml syringes, which served as odor sources. The method of stimulation was essentially the same as that described by Van Der Pers (1982). One set of odor syringes was prepared containing a piece of filter paper loaded with 100 μg of each test chemical. Another series of syringes was prepared containing filter papers loaded with compound I in decadic steps from 10^{-2} to 10^{2} μg per syringe.

Two electrophysiologically different types of SW1 sensilla occur on the male antenna of *Agrotis segetum*. One type constitutes about 30% of all sensilla SW1 and contains a receptor cell highly sensitive to *Z*-7-dodecenyl acetate (Löfstedt et al., 1982). This cell can easily be distinguished from the other two present in the sensillum by its large spike amplitude. Recordings were made from sensilla containing this type of cell. To check the presence of such a cell, the sensillum was stimulated with a short puff from an odor source containing *Z*-7-dodecenyl acetate previous to the measurements. During one test program, the preparation was stimulated with each of the test compounds II–VII in a quantity of 100 μg per odor source. The same sensillum was subsequently stimulated with compound I at increasing stimulus strength, from 10^{-2} to 10^{2} μg per odor source in decadic steps. The stimulation time was 1 sec alternating with intervals of 1 min. Each test program was carried out on a previously nonstimulated, fresh preparation. Between the test program the sequence of stimulation with compounds II–VII was changed randomly. All responses were recorded on magnetic tape and subsequently transferred to paper using an electrostatic recorder.

The responsiveness of the receptor cells was expressed as the number of spikes generated during the second of stimulation. The responses to compound I were averaged and combined in a dose–response diagram. The responses to the other compounds were expressed as the average number of spikes per second of stimulation with 100 μg per odor source. Recordings were made from 15 receptor cells.

RESULTS

Receptor Cell Responses. The results of the electrophysiological experiments are presented in Figure 2. The average response threshold of the 15 receptor cells tested with compound I is about 10^{-2} μg in the odor source. The responses increase gradually with increasing stimulus concentrations. At a concentration of 10^{2} μg of compound I, the receptor cells respond with an average of 135 spikes per second. At this concentration, all other test compounds elicit considerably lower responses. Compound VII evokes on the average about 65 spikes per second, followed by compound VI with 31 spikes per second. Compounds II, III, IV, and V evoke less than 10 spikes per second.

The effectiveness of the analogs II–VII relative to the acetate, com-

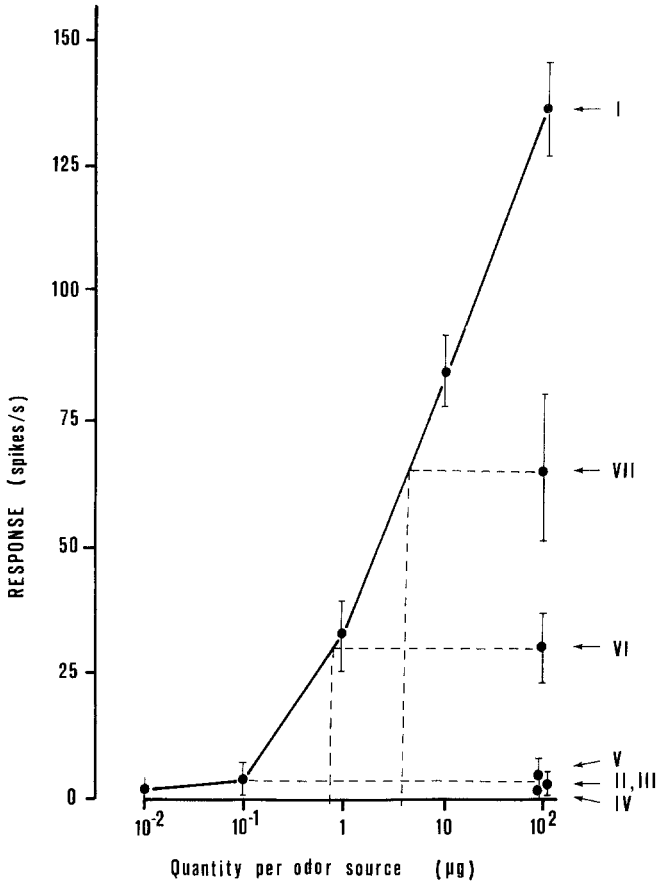

FIG. 2. Single olfactory cell responses (spikes/sec) evoked during 1 sec of stimulation with increasing (10^{-2}–10^{2} μg) quantities of test compound I. Dots designated by II–VII indicate receptor cell responses during 1 sec of stimulation with 10^{2} μg of test compounds. Small vertical lines: standard error of mean value. Number of replicates = 15.

pound I, can be expressed as the ratio of the quantities needed to evoke the same number of spikes, as is illustrated by dotted lines in Figure 2. About 25 times the quantity of compound VII has to be applied in order to evoke the same response as that evoked by a given amount of compound I. For compound VI this value is about 100, and for compounds II, III, IV, and V about 1000.

The standard errors in the measured averages are of a reasonable order of magnitude for experiments like these. The differences in effectiveness between compound VII, compound VI, the group of compounds II–V, and

the natural pheromone component, compound I, are clearly significant at a stimulation strength of 10^2 μg.

However, the significance of the differences in effectiveness between compound VII, compound VI, and the group II–V is lost, if these compounds are applied at a tenfold lower intensity.

DISCUSSION

The shape and the electron distribution are two molecular properties of decisive importance in structure–biological activity relationships (Dodd, 1976; Lehmann, 1978). A molecule that is larger than optimal may exceed the available receptor space and cause severe repulsions with the receptor "wall," prohibiting a correct orientation for binding to the active site of the receptor. A molecule smaller than optimal may show low affinity due to reduced interactions with the complementary receptor structures. This is of special importance for those parts of a molecule engaged in short-range interactions, for instance interactions by nonpolar parts through dispersion forces.

When heteroatoms are present in the molecule, the electron distribution may give rise to a sizable permanent dipole moment. This is a vector quantity, and its interaction with dipoles at the receptor site may have a strong influence on the orientation of the molecule at the receptor site. A permanent dipole in the substrate molecule may also play a dominant role in the recognition step, since the dipole–dipole interaction is quite strong and acts at much longer distances from a binding site than, for instance, the dispersion forces.

Before going into the discussion of these properties for the compounds studied in the present work, it must be borne in mind that the details of the transduction process are still largely unknown. The most thorough studies of this process for pheromone reception have been made by Kaissling on the bombykol receptor of *Bombyx mori* (Kaissling, 1974, 1976, 1977). These studies indicate the possibility of a two-step process, in which the binding of the ligand to the receptor is followed by a formation of an activated complex. The formation of the activated complex is connected with changes in the conductivity of cell membrane. It is conceivable that the two steps have different chemical selectivities. The general validity of these observations remains to be determined, but it cannot be excluded that an observed selectivity is a combination of selectivities in different steps of a multistep process.

Molecular Shape. As the molecular shapes of compounds I–VI are identical with respect to the hydrocarbon chain, it is sufficient to study the shapes of the different polar functional groups. These may be represented by

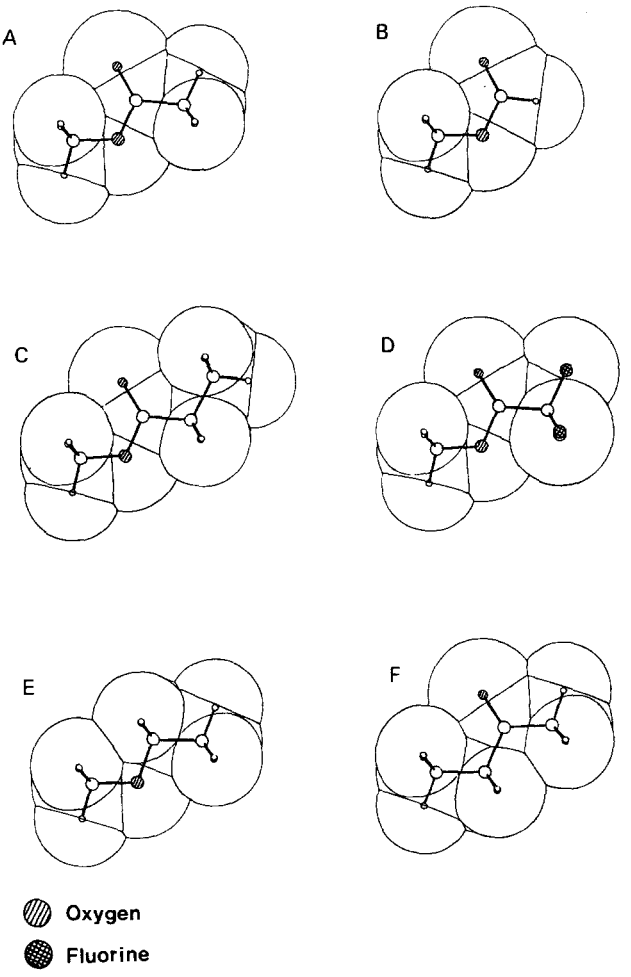

FIG. 3. Space-filling van der Waals models of (A) methyl acetate, (B) methyl formate, (C) methyl propionate, (D) methyl trifluoroacetate, (E) ethyl methyl ether, and (F) 2-butanone.

the van der Waals (vdW) space-filling models of methyl acetate and its analogs corresponding to the functional groups in II–VI. These models are shown in Figure 3. Ball-and-stick models of the molecules have been superposed to facilitate the identification of different parts of the vdW models. The positions of the atomic nuclei constitute centers of vdW spheres with radii as proposed by Allinger and used in molecular mechanics calculations (Burkert and Allinger, 1982). The molecules shown in Figure 3 are all in their thermodynamically preferred conformations and have

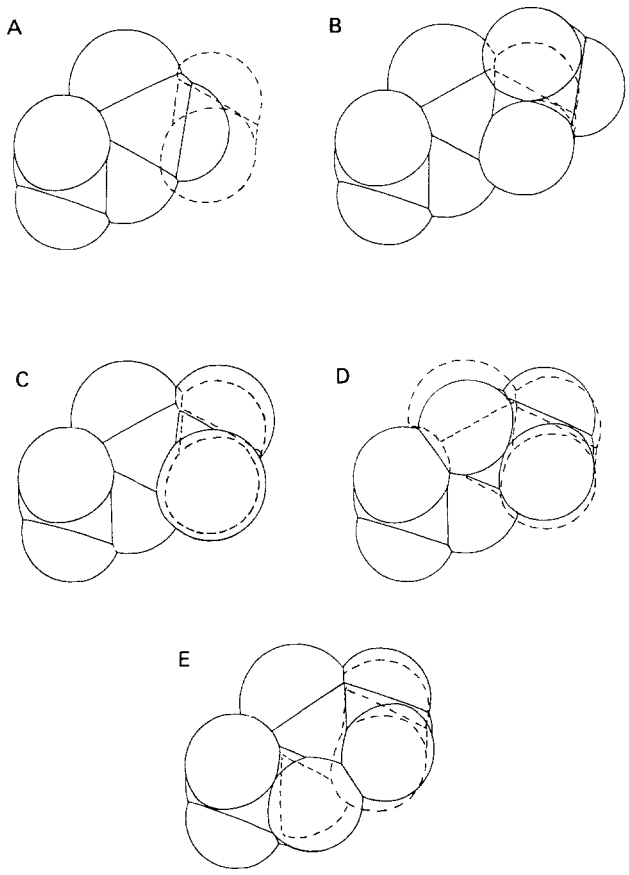

FIG. 4. Superpositions of the van der Waals model of methyl acetate (dashed line) with those of (A) methyl formate, (B) methyl propionate, (C) methyl trifluoroacetate, (D) ethyl methyl ether, and (E) 2-butanone.

energy-minimized geometries calculated by the molecular mechanics method. To qualitatively compare the shapes of the different acetate analogs, the vdW model of methyl acetate has been superposed on those of the functional groups of compounds II-VI. The results are shown in Figure 4.

The trifluoroacetate, the ethyl ether, and the methyl ketone (Figure 4C, D, and E, respectively) all have molecular shapes showing a high degree of similarity to the acetate group. It is thus less probable that the receptor would reject these functional groups due to their sizes or shapes. They should all fit reasonably well into a receptor cavity with a shape that is complementary to that of the acetate group. Note that the boundary of a

vdW sphere describes the region in space of maximum van der Waals attraction between a given sphere and another approaching sphere. The two spheres must thus penetrate somewhat into each other (a few tenths of an Ångström) before appreciable van der Waals repulsion takes place.

The largest deviations from the vdW shape of the acetate group are shown by the formate and propionate groups (Figure 4A and B, respectively). The propionate group is clearly significantly more bulky in the vicinity of the carbonyl group than is the acetate group. Since the two functional groups have very similar properties in all other respects (see below), the very low activity of compound III in the single-cell measurements (Figure 2) indicates a very strict requirement for a correct shape in this region. The receptor-active site should thus be highly complementary to an acetate methyl in this region. A similar result was obtained in an EAG study by Bestmann and Vostrowsky (1982) on analogs of (*Z*)-5-decenyl isovalerate, a sex pheromone component of *Nudaurelia cythereae.* The isovalerate compound was shown to be the most active compound of a number of ester group analogs with modifications of the ester alkyl group, irrespective of the length of the olefinic hydrocarbon chain.

In experiments with the moth *Ochropleura plecta,* Priesner (1980) reported a 100-fold lower activity of the formate analog with respect to the activity of the acetate. A difference of similar magnitude was found between the effect of II or III and the acetate in *Trichoplusia ni* and *Autographa gamma* (Priesner, personal communication). The differences between the results reported by Priesner (1980, and personal communication) and the results of the present study are most likely to be explained by the different methods of recording and stimulation.

The formate group in compound II is significantly smaller than the acetate group (Figure 4A), and should thus not exceed the limits of the space available at the receptor-active site. The formate group, however, lacks the possibility to engage in short-range binding through dispersion forces, with the receptor structure that is complementary to the acetate methyl. It is of interest to compare the effect on the biological activity of shortening and lengthening of the ester alkyl group, with the effect of the corresponding modifications at the nonpolar end of a pheromone component molecule. There are several examples from field studies that homologs with one carbon unit shorter or longer at the nonpolar end may replace the natural component in a pheromone blend without eliminating the biological activity (Steck et al., 1982; Cardé and Roelofs, 1977; Struble, 1981). It has also been shown in single-cell studies that elimination of a methyl group at the nonpolar end only moderately reduces the cell activity (Priesner, 1979). The same behavior is shown by the Z7–12 : Ac receptor of *Agrotis segetum,* studied in the present work. As can be seen in Figure 2, the homolog

Z7–12 : Ac (VII), which is two carbon units shorter than the natural component, still shows an appreciable biological activity, significantly higher than that of the formate compound II.

The interaction between the nonpolar end and the receptor should be due to dispersion forces. This implies that the very low activity of the formate compound II is probably not exclusively due to lack of hydrophobic (dispersion) binding to the "methyl site" of the receptor. Thus, the acetate methyl seems to play a more complex part in the substrate–receptor interaction than does the methyl group at the nonpolar end.

That a shape of the functional group that closely mimicks the shape of the acetate group is a necessary requirement for biological activity is demonstrated by the inactivity of the propionate compound III. The low activities of compounds IV, V, and VI furthermore demonstrate that a high degree of similarity with the shape of the acetate group is not a sufficient condition for activity.

Electron Distribution. Modifications of the polar acetate group may not only lead to changes in molecular shape, but also to significant changes in the electron distribution. Compounds I–VI all have permanent dipoles located at the polar functional group. Interactions between such dipoles and corresponding receptor dipoles may be of crucial importance for a proper orientation of the molecule at the active site of the receptor. The dipole–dipole interaction is maximally attractive when two dipoles are in an antiparallel orientation. Parallel dipoles are maximally repulsive. Dipole moments and dipole moment directions have been calculated by the quantum mechanical CNDO/2 method for methyl acetate and its analogs corresponding to the different functional groups in compounds II–VI. The results are shown in Figure 5. Experimental data are available for methyl formate (Curl, Jr., 1959), ethyl methyl ether (Hayashi and Kuwada, 1975), and 2-butanone (Pierce et al., 1969). These values are shown as dashed vectors in Figure 5. The excellent agreement between calculated and experimental results increases our confidence in the calculated moments and directions for the remaining compounds.

As expected, the acetate and the propionate have almost identical dipole moments and dipole moment directions. This supports the conclusion above that the low activity of the propionate compound III is due to a "bulk effect." However, although methyl formate and methyl acetate have very similar dipole moments, 1.77 and 1.69 D, respectively, in the vapor phase, the directions of the moments differ significantly (Figure 5). This difference may be a contributing reason for the unexpectedly low activity of compound II. Interestingly, the most active compound of the acetate group analogs studied, the methyl ketone VI, is calculated to have a dipole moment direction very close to that of the natural pheromone component I

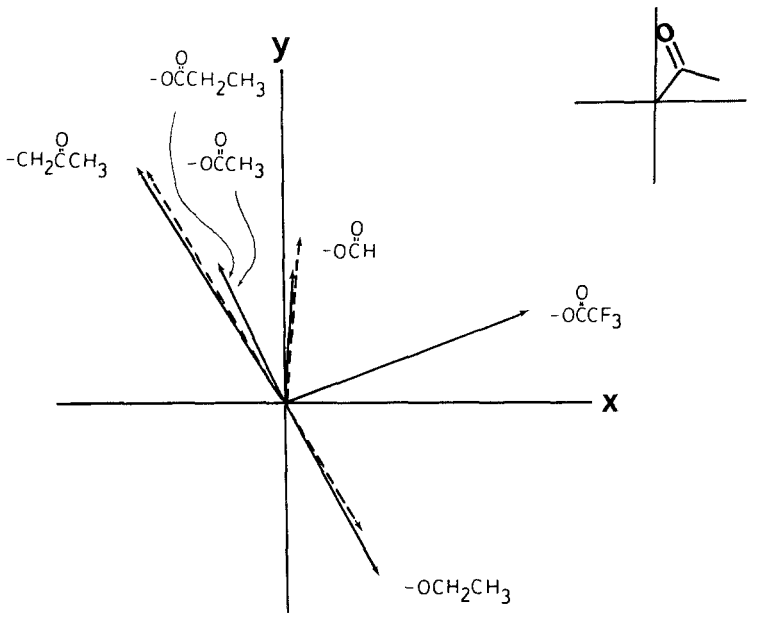

FIG. 5. Calculated (this work, solid line) and experimental (literature data, dashed line) dipole moment directions. The length of the vectors are proportional to the size of the dipole moments.

(Figure 5). This may indicate that the direction of the dipole moment vector is of importance in the substrate–receptor interaction. The activity of compound VI is, however, quite low, which demonstrates that both ester oxygens are necessary for full activity. The fact that the ketone VI shows some activity on the receptor precludes the possibility that the mechanism of the transduction process involves hydrolysis of the ester group of the natural component.

Methyl trifluoroacetate and ethyl methyl ether both have dipole moment directions very different from that of methyl acetate (Figure 5). Both of these directions lead to repulsive interactions with a dipole antiparallel to that of compound I, if IV and V are oriented as in Figure 4C, and D. This fact is sufficient to understand why the activities of compounds IV and V are so low, in spite of their similarity in shape to the natural pheromone component I. In addition, V also, of course, lacks the possibility to engage in hydrogen bonding at the position corresponding to the acetate carbonyl group.

The quite low activity of the methyl ketone VI on the *Z*7–12 : Ac receptor should be contrasted to the reported results on the reverse modification of an aldehyde to a formate group:

SCHEME I

In *Heliothis* species the replacement of the natural aldehyde component by the corresponding formate compound has been shown to be compatible with full biological activity. This has been demonstrated in behavioral experiments (Tumlinson, 1979) as well as in EAG and single-cell measurements (Priesner, 1979). The replacement of a methylene group in the natural compound by an oxygen atom is a less serious modification than the reverse one, as there is no possible hydrogen bonding site or polar interaction site affected.

CONCLUSIONS

The part of the Z7–12 : Ac receptor of *Agrotis segetum* that interacts with the acetate group shows a very high selectivity. All portions of the acetate group seem to be of great importance for full biological activity, as measured by the single-cell technique. There is a very strict requirement on the shape of the polar functional group, as well as on its electron distribution. A one-point model for the acetate group or the structure of the receptor complementary to the acetate group is a gross oversimplification.

Although this investigation only deals with one receptor cell in one moth species, there are previous indications that different receptor cells in different moth species exhibit similar behavior with respect to molecular modifications. The results obtained in this work may therefore be of more general validity. However, since receptors selective for Z7–12 : Ac are very common in moth species, compounds I–VII could be employed to probe structure–activity relationships in other moth species for comparison.

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CONVENIENT METHOD APPLICABLE TO SINGLE INSECTS FOR COLLECTION AND MEASUREMENT OF BLEND RATIOS OF AIRBORNE PHEROMONES FROM ARTIFICIAL SOURCES

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Abstract—A simple, fast, and sensitive technique has been developed for collecting airborne pheromones with potential application to a live and free-moving single insect. The surface within a disposable glass (Pasteur) pipet is sufficient to trap low nanogram quantities of sex pheromones, and molecules sensitive to oxidation such as (*Z*)-9-tetradecenal and (*Z,E*)-9,12-tetradecadienyl acetate are not significantly degraded during the chosen periods of aerial trapping. Rapid measurements of blend ratios for synthetic pheromone mixtures and detection at the low picogram level have been achieved by GC-MS.

Key Words—airborne pheromones, collection, GC-MS, single insect, glass surface, pheromone composition, (*Z,E*)-9,12-tetradecandienyl acetate, aldehydes, alcohol.

INTRODUCTION

"If the technical problems involved in quantifying sex pheromones volatilized from female glands can be solved. . ." (Olsson et al., 1983) is an often repeated statement by entomologists, electrophysiologists, chemists, and others concerned with pheromone research and use, and it is a reflection of the inadequacies of many of the current methods of measuring airborne pheromones.

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There were three main requirements for our studies of pheromone blends released from single insects: the insect should not be disturbed or harmed while the pheromone was collected so that it could be used for other experiments; the trapping method for the pheromone should be simple, efficient, and rapid; and instrumentation for detection should maximize the information that could be gained in many individual experiments.

Most techniques in the past and still in use today have been based on accumulating pheromones from several insects, and thus the quantities and ratios of individual components represent only averages. Pheromone collecting from single insects, usually female moths, is beset by many difficulties, mainly because of the very minute quantities released. The available methods include minor surgery of glands (Miller and Roelofs, 1980); extraction, dipping, or washing of glands in a solvent (Webster and Cardé, 1982); and passing air or inert gas over the insect or its pheromone gland and trapping the volatiles on an adsorbent such as Porapak Q or Tenax (Bjostad er al., 1980; Ma et al., 1980; Morse et al., 1982), on glass wool (Baker et al., 1981; Mistrot Pope et al., 1982), or glass beads (Charlton and Cardé, 1982), followed by washing with a relatively large volume of solvent. All include an evaporation stage to remove most of the solvent before GC analysis (Ma and Schnee, 1983) or other detection techniques (Morse et al., 1982; Golub et al., 1983).

Each experiment in these published procedures usually requires separate handling for collection and solvent evaporation, and when the determination of the pheromone blends is performed using detection that is nonselective in its response to components (e.g., a flame ionization detector, FID), the conclusions can be equivocal. Procedures other than aerial collection for sampling the pheromone gland composition were not appropriate since they lead to death of the insect and adulteration of the resultant solutions with many compounds other than the target pheromones. In addition, there is the considerable uncertainty as to whether the pheromone composition within the gland reflects that in the vapor phase in nature (Baker er al., 1980; Tumlinson and Teal, 1982; Olsson et al., 1983).

In this paper we describe and validate methods and equipment that we have developed for a project to investigate the blend ratios of pheromones released aerially from single insects. The experiments described here deal mainly with calibration of the methods using synthetic sex pheromone compounds; those concerned with live females are reported elsewhere (Shani et al., in preparation).

METHODS AND MATERIALS

General. Standard solutions of (*Z*)-9-tetradecenyl acetate [(*Z*)-9-(C_{14}OAc] (I), (*Z,E*)-9-tetradecanadienyl acetate [(*Z,E*)-9,12-C_{14}OAc] (II),

(*Z*)-9-tetradecenal [(*Z*)-9-C_{14}Ald] (III), (*Z*)-9-hexadecenal [(*Z*)-9-C_{16}Ald] (IV), and (*E,E*)-8,10-dodecadienol [(*E,E*)-8,10-C_{12}OH] (V) were prepared from HPLC purified chemicals (>99%) in hexane (HPLC grade, Waters Associates) and kept at −5°C when not in use. Separate syringes were used for specific chemicals or mixtures to avoid cross-contamination. All glassware was cleaned by chromic acid solution, washed in tap water, soaked in saturated $NaHCO_3$ solution, again washed in tap water, and then dried in air. Glass wool was heated at 180° for 24 hr. Disposable glass Pasteur pipets (Corning, catalog No. 70958, 1.3 mm ID) were heated briefly in the blue oxidizing portions of a flame prior to use. Instrument grade air (CIG, Australia) was filtered twice through 100-mm glass tubes (5 mm ID) containing Porapak Q immediately upstream of the manifold, which was constructed with ground-glass ball joints (Figure 1).

Collecting System. The leading arm (650–800 mm long) of the all-glass manifold (Figure 1) holds 20 extensions with B14 (or B19) Quickfit sockets [Figure 2(a), 30–40 mm separation] to attach 20 glass cages. Each cage (70–80 mm long) comprises a B14 (or B19) cone, a bulbous region (10–25 mm ID) suitable for containing a live and free-moving insect, and a

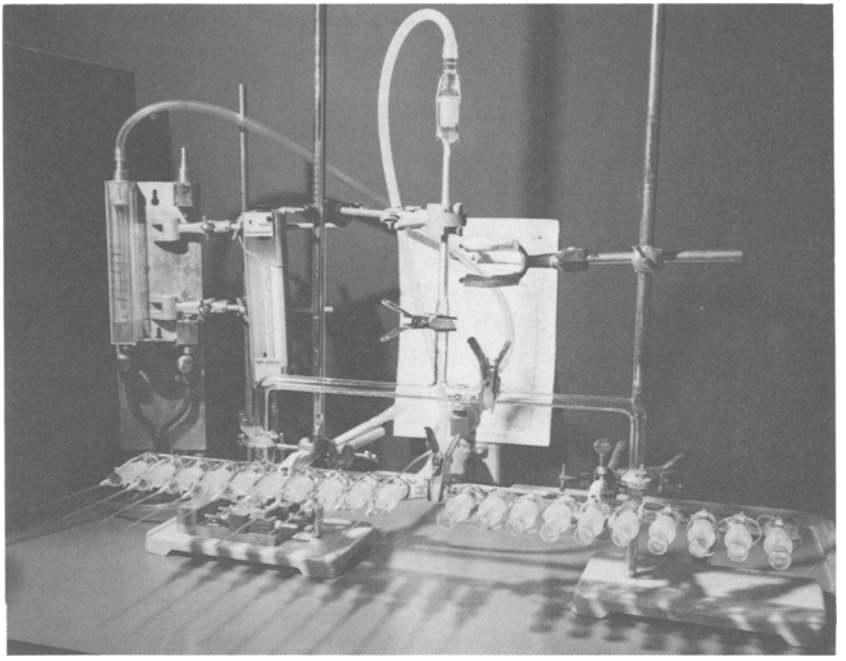

FIG. 1. All glass collecting system showing arrangement of manifold, cages, and pipets for trapping nanogram levels of airborne pheromones from single insects and artificial sources.

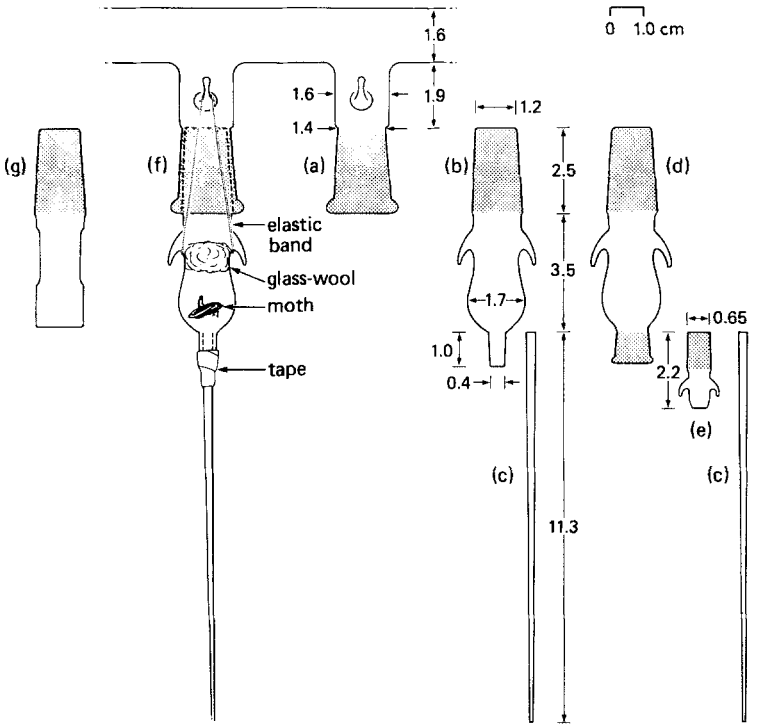

FIG. 2. Schematic of all-glass collecting system showing: (a) extension from leading arm of manifold; (b) cage with outlet constriction to hold (c) capillary section of disposable glass Pasteur pipet; (d) alternative cage with socket and (e) cone for exchanging pipets during course of a collection; (f) cage and pipet in position for collection; and (g) stopper for unused extensions of manifold.

constricted outlet (Fig. 2, b). The constriction (10–12 mm length, 2–3 mm ID) allows the separated capillary portion (Fig. 2, c, 100–120 mm long) of a disposable glass Pasteur pipet to be wedged firmly in place when introduced from within the cage. Any movement of the pipet during experiments was prevented by applying a little adhesive tape to the outside of the joint (Fig. 2, f).

In those cases where we wished to exchange the pipet for a clean one during the course of a collection period, a modified outlet was used, constructed from a B7 Quickfit socket and cone (Fig. 2, d and e). A glass wool plug was introduced into the cage upstream of the artificial source or live insect (Fig. 2, f), both to prevent the live insect from leaving the cage and entering the manifold and to serve as an extra filter for organics that might escape the Porapak Q filter. Air from the cylinder was admitted through an adjustable flowmeter (0–1200 ml/min) at a rate of 35–40 ml/min per cage

[separate experiments confirmed that all cages received the same amount of air (±5%)].

In experiments in which Dry Ice was used as coolant, two aluminum trays with cuttings to fit the pipets were introduced from below and were kept full with Dry Ice for the duration of the experiment. Dimmed light (1.5–1.7 lux) was used in all the experiments. Temperatures in the preparation laboratory were 19 ± 1°C and in the collecting room 22 ± 1°C.

Release and Trapping of Pheromones. Two alternative artificial sources were effective for release of the synthetic pheromones, namely, the surface of the mouth of the pipet and an artificial "gland." In the former case, a measured volume of the required solution (3 μl) was introduced into the mouth of the pipet from inside the cage, allowed to dry, and the cages were then mounted on the manifold. Air was admitted and, for experiments with cooling, Dry Ice was placed into the trays. The artificial gland (a glass bead 1 mm diameter) was made by fusing the tip of a 20-mm length of capillary tube. The required solution was applied to the surface of the bead and, after evaporation outside the cage, the bead was placed at the mouth of the pipet within the cage. When air of low humidity was used, ice formation within the cold zone of the pipets did not present any problems, but when the air was wetter, droplets of water accumulated and froze. In the latter case, the air flow diminished slowly from 35–40 ml/min to a very low rate and sometimes stopped completely; the Dry Ice tray had then to be removed, whereupon the flow rate was restored within 1–2 min after thawing. The air flow was maintained at various rates as indicated in Tables 1–4 for 15 min, 1 hr, or 2 hr.

After experiments with application at the mouth of the pipet, the outlet of the pipet was fused and the pipet cut into two parts; the lower section, at least 15 mm away from the limit of spreading of the test solution, and the upper part that had been treated with test solution. The lower section previously sealed at its outlet was marked as "volatiles" and washed with hexane (HPLC grade, Waters Associates) (3× 3–4 μl). The upper part, marked as "washings," was rinsed with hexane (3× 5 μl) into another pipet sealed at the outlet. When a glass bead was used as the source of the test solution, it was similarly rinsed into a sealed pipet. Finally, all pipet sections were sealed at their upper ends and kept at −5°C until the contents could be assessed by gas chromatography–mass spectrometry (GC-MS).

Determination by GC-MS. The measurements were made using a Varian 1400 Series GC coupled to a VG Micromass 70/70 mass spectrometer.

GC conditions were on-column injection, packed glass column (10% Silar 10C, 1.8 m length × 3 mm ID), helium carrier gas 30 ml/min, column temperature isothermal 160° (I–IV) and 180°(V). Retention times were 2.92 min for I, 3.76 min for II, 2.30 min for III, 3.92 min for IV, and 2.20 min for

V. The column was used for pheromone determinations from aerial collections exclusively.

The GC-MS interface was a single-stage glass jet separator.

MS conditions were 70 eV EI, source temperature 190°, trap current 200 μA, mass resolving power adjusted to 650 (10% valley). Ions were monitored using the VG multiple ion detection (MID) module, in electric field switching mode for solutions of I/II and of V, and in magnetic field switching mode for solutions of III/IV. Monitored ions were $(M\text{-}HOAc)^{+}\cdot$ for I and II, $(M\text{-}H_2O)^{+}\cdot$ for III and IV, and $M^{+}\cdot$ for V. The limits of detection (signal-to-noise ratio 2 : 1) using the packed column for compounds I, II, III, IV, and V were 20, 50, 30, 80, and 120 pg, respectively. The mass lock channel used the column bleed peak of m/z 207. The analog output from the MID was directed to a Servogor 460 multichannel recorder.

GC-MS Procedure. Conditioning of the GC column prior to the measurements was effected with injections of nanogram quanitites of the standards. The GC column temperatures were such as to secure separation of the peak maxima for the components of at least 0.75 min and complete elution of both components within 4.5 min. Under such conditions, on-column injections of samples and standards could be made at 5-min intervals because the selectivity of the GC-MS procedure nullified cross-talk from any coeluting impurities. The resolution of the components of the mixture by the GC enabled the use of single-ion monitoring for the appropriate mass channel as each peak eluted, rather than the less sensitive procedure of repetitive channel switching.

Because of the frequency (5-min intervals) with which the GC-MS measurements could be performed, the blend ratios were assessed from consecutive injections of sample and standard mixtures. At least two injections (each 4–5 μl) were available per sample solution, and each determination was followed by one for injection of a measured volume of the appropriate standard mixture. Quantities were taken to be proportional to peak heights.

RESULTS AND DISCUSSION

In order to validate the methodology, trace quantities of representative molecules (acetates, aldehydes, alcohol) were trapped within pipets after release from various sources to provide information about chemical and physical effects on the proportions recovered. The acetates were (*Z*)-9-C_{14}OAc (I) and (*Z,E*)-9,12-C_{14}OAc (II), the two components of the sex pheromone of *Ephestia cautella* (Brady, 1973); the aldehydes were (*Z*)-9-C_{14}ald (III) and (*Z*)-9-C_{16}ald (VI), components of the *Heliothis* complex (Klun et al., 1980); and the alcohol was (*E,E*)-8,10-C_{12}OH (V), the main

component of codling moth (Roelofs et al., 1971). Compounds II, III, and IV are sensitive to oxidation and rapid degradation (Goto et al., 1974; Shaver and Ivie, 1982; Dunkelblum et al., 1984), while V can be oxidized by singlet oxygen (Ideses et al., 1982). The quantities of model compounds and ratios for the acetates released in the present experiments were of the same order as those released aerially from single insects.

The first series of experiments to release I and II in different ratios (15 : 85, 8 : 92, 5 : 95) in quantities of 0.2–3 ng from glass wool (20–30 mg) and the glass surface of the cage itself (1–2 $cm^2 = 10^{16} Å^2$) with air flows of 30–50 ml/min over a 2-hr period were unsuccessful, since the quantities of trapped pheromones were generally below the limits of detection, and much of the original mixture could be recovered at source by extraction with hexane (compare Mistrot Pope et al., 1982). Application of the solution of I and II to the mouth of the pipet permitted a more efficient release (Table 1) by reducing the effective surface area at source (ca. 10^{14}–$10^{15} Å^2$). Control experiments showed that 20–40% of the original mixture of I and II was lost through evaporation of the solvent during the application (Baker et al., 1980) and drying period. In addition we could not detect any II in a pipet mounted immediately downstream of the trap used for the larger quantities at source (Table 1, entries 3 and 4).

Our inferences from the results in Table 1 are: the ratio between I and II in "volatiles" is essentially the same as that applied at source; most of I and II at source can be volatilized in a low flow rate of air and adsorbed again within the pipet; and total recovery is high when the evaporative losses on application are taken into account. Because these losses at source were variable and breakthrough was negligible, a more accurate assessment of the average mass balance was not attempted.

The use of air of higher humidity led to icing and occasional blockage in the cooled zone of the pipet, such that much of the mixture was retained at the source and could be recovered by washing (Table 2). The ratio of I and II was constant in volatiles and washings, demonstrating that significant differences in the volatilization or trapping of I and II did not occur during the period of aeration. The agreement was not as good as in Table 1 between the ratio of recovered pheromones and that applied at source, but an important observation was that cooling appeared to be unnecessary to retain the volatilized acetates within the pipet (Table 2, entry 3).

It was possible that the results for pheromone release from the mouth of the pipet (Tables 1 and 2) could have been perturbed either by migration of the acetates along the glass surface (without volatilization) or by solvent entrainment of the acetates during the initial evaporation, with subsequent adsorption of a fraction within the collection zone. A control experiment without air flow (10 min) failed to show any transport of the applied mixture away from the mouth of the pipet. Moreover, when a small glass

TABLE 1. RELEASE OF (*Z*)-9-C_{14}OAc (I) AND (*Z,E*)-9,12-C_{14}OAc (II) (STANDARDS) FROM GLASS SURFACE[a]

	Applied at source			Volatiles recovered					Washings recovered			
				I		II			I		II	
Entry	I(pg)	II(pg)	Ratio(%)	pg	%	pg	%	Ratio(%)	pg	%	pg	%
1	210	1152	15.4 : 84.6	40	19	300	26	12.1:87.9(2.0)	ND[b]		140	12
2	210	2304	8.4 : 91.6	110	52	1260	55	8.0:92.0(1.6)	ND		ND	
3	210	4032	5.0 : 95.0	120	57	2100	52	5.1:94.9(0.8)	ND		ND	
4	210	5760	3.5 : 96.5	90	43	2980	52	3.1:96.9(0.7)	ND		220	4

[a]The test solution (3 μl) was loaded at the mouth of the disposable pipet from inside the cage, evaporated for 5–10 min, and then an air flow (dry) was maintained at 35–40 ml/min for 2 hr. Dry Ice was used for trapping the volatiles. Quantities of recovered pheromones, rounded off to the nearest 10 pg (I) or 20 pg (II), were estimated by external calibration and are the sum total of at least two GC-MS determinations per sample solution. Ratios for recovered pheromones are the mean of a least two GC-MS measurements per sample solution (range in parentheses).
[b]Not detected: below the limit of detection per GC-MS measurement of 20 pg for (*Z*)-9-C_{14}OAc (I) and/or 50 pg for (*Z,E*)-9,12-C_{14}OAc (II).

TABLE 2. RELEASE OF (*Z*)-9-C_{14}OAc (I) AND (*Z,E*)-9,12-C_{14}OAc (II) (STANDARDS) FROM GLASS SURFACE[a]

	Applied at source			Volatiles recovered					Washings recovered				
				I		II			I		II		
Entry	I(pg)	II(pg)	Ratio(%)	pg	%	pg	%	Ratio(%)	pg	%	pg	%	Ratio(%)
1	210	1152											
		DI, 1 hr	15.4 : 84.6	ND[b]	—	140	12	—	140	67	660	57	19.1:80.9(1.7)
2	210	1152											
		DI, 2 hr	15.4 : 84.6	ND	—	ND	—	—	140	67	560	49	20.7:79.3(4.3)
3	210	2304											
		NDI, 1 hr	8.4 : 91.6	40[c]	19	300[c]	22	10.4 : 89.6[c]	170	81	1500	65	10.3:89.7(0.6)
4	210	2304											
		DI, 2 hr	8.4 : 91.6	80	38	620	27	12.0:88.0(0.4)	60	29	440	19	12.8:87.2(0.9)

[a]The test solution (3 μl) was loaded at the mouth of the disposable pipet from inside the cage, evaporated for 5–10 min, and then an air flow (wet) was maintained at an average rate of 15–25 ml/min for 2 hr. DI, Dry Ice was used for trapping the volatiles; NDI, Dry Ice was not used. Quantities and ratios for recovered pheromones were estimated as indicated in Table 1, footnote (*a*).
[b]Not detected: below the limit of detection per GC-MS measurement of 20 pg for (*Z*)-9-C_{14}OAc (I) and/or 50 pg for (*Z,E*)-9,12-C_{14}OAc (II).
[c]Results from a single GC-MS determination.

bead (nominal surface area 10^{14} Å^2) was used instead as the source of the test solution, the initial evaporation was effected outside the collection pipet and physical contact between the source and the trapping surface was negligible. We conclude that migration of the model compounds from the source to the collection zone of the pipet occurs through the vapor phase.

Experiments with the glass bead as source of I and II showed that much of the initial mixture was retained by the bead after 1–2 min evaporation of the solvent (Table 3, entries 1 and 2). The acetates were successfully released from the source in an air stream and could be trapped within a pipet without cooling (Table 3, entry 3), while the amount of II retained in a pipet mounted immediately downstream of the trap was undetectable. Agreement was obtained between the ratios for the recovered pheromones and those applied at source, and the efficiency of trapping appears to be high after allowance is made for the losses during the initial application and evaporation.

The ease of the trapping technique described is demonstrated by the fact that it has proved possible to use the apparatus to collect sex pheromones (mixtures of I and II in nanogram to subnanogram quantities) from several hundred individual females of *E. cautella* (laboratory and wild strains) and to measure the ratio of the pheromone constituents by GC-MS in the space of only two months (Shani et al., in preparation). Other insects are under investigation. As an illustration, we trapped nearly a nanogram of the major component II and 150 pg of I over 1 hr from a live female *E. cautella* contained in the glass cage (Table 3, entry 4). We could not detect any pheromone within a second pipet mounted immediately downstream of the collection pipet, demonstrating, as expected, that breakthrough had not occurred during this period. The amount of particulate matter transferred from the moth to the trap was negligible because the mean wind velocity in the cage was low. In addition, the selectivity of the GC-MS procedure precluded interference from other molecules adventitiously trapped with I and II.

The collection technique was repeated for the aldehydes III and IV, which differ more significantly in volatility than the acetates I and II. The initial evaporation led to enhanced loss of III over IV (Table 4, entries 1, 2, and 5). Furthermore, the more volatile III was generally more abundant in the volatiles for the samples and less abundant in the washings (Table 4, entries 3, 4, and 6). It is evident that oxidation of III and IV had not occurred significantly within the period of evaporation and aeration (compare Tumlinson and Teal, 1982), and that recovery appears to be high when based on losses of 20–60% (Table 4) during the initial application and evaporation. In agreement with this conclusion, the amount of IV in a pipet mounted downstream of the trap (Table 4, entry 3) was undetectable.

TABLE 3. RELEASE OF (*Z*)-9-C_{14}OAc (I) AND (*Z,E*)-9,12-C_{14}OAc (II) FROM GLASS BEAD[a] AND SINGLE LIVE INSECT[a]

	At source			Volatiles recovered					Washings recovered				
				I		II			I		II		
Entry	I(pg)	II(pg)	Ratio(%)	pg	%	pg	%	Ratio(%)	pg	%	pg	%	Ratio(%)
Glass bead evaporation													
1.	210	2304	8.4 : 91.6						180	86	1680	73	9.9:90.1(0.8)
2.	210	4032	5.0 : 95.0						120	57	2160	54	6.2:93.8(2.0)
Glass bead in cage													
3.	210	2304	8.4 : 91.6	140	67	1240	54	10.0:90.0(0.9)	ND[b]		140	6	—
	NDI, 1 hr												
E. cautella in cage[c]													
4.	—	—		150	—	940	—	13.5:86.5(0.4)					
	NDI, 1 hr												

[a]The glass bead was loaded with the test solution (3 μl), dried outside the cage (for 60–120 sec), and either washed immediately (entries 1, 2) or mounted at the mouth of the disposable pipet from inside the cage (entry 3). Airflow (wet) was maintained at average rate of 35–40 ml/min without Dry Ice (NDI, entries 3, 4) for 1 hr. Quantities and ratios for recovered pheromones were estimated as indicated in Table 1, footnote *a*.
[b]Not detected; below the limit of detection per GC-MS measurement of 20 pg for (*Z*)-9-C_{14}OAc (I) and/or 50 pg for (*Z,E*)-9,12-C_{14}OAc (II).
[c]The insect exhibited calling behavior for most of the collection period.

TABLE 4. RELEASE OF (Z)-9-C_{14}ALD (III) AND (Z)-9-C_{16}ALD (IV) (STANDARDS) FROM GLASS BEAD AND GLASS SURFACE[a]

	Applied at source			Volatiles recovered					Washings recovered				
				III		IV			III		IV		
Entry	III(pg)	IV(pg)	Ratio(%)	pg	%	pg	%	Ratio(%)	pg	%	pg	%	Ratio(%)
Glass bead evaporation													
1	1770	7560	19.0 : 81.0						1440	81	8160	108	15.1:84.9(0.2)
2	1770	7560	19.0 : 81.0						640	36	5600	74	10.3:89.7(0.4)
Glass bead in cage													
3	1770	7560	19.0 : 81.0	1280	72	4120	54	23.6:76.4(0.1)	100	6	1640	22	6.1:93.9(0.8)
	Air, NDI, 15 min												
4	1770	7560	19.0 : 81.0	600	34	2400	32	20.7:79.3(3.2)	200	11	1760	23	10.3:89.7(1.0)
	N_2, NDI, 15 min												
Surface evaporation													
5	1770	7560	19.0 : 81.0						540	31	3040	40	15.3:84.7(0.1)
Surface in cage													
6	1770	7560	19.0 : 81.0	560	32	2240	30	20.4:79.6(2.8)	280	16	1880	25	12.9:87.1(1.2)
	Air, NDI, 15 min												

[a]The glass bead was loaded with test solution (3 μl), dried outside the cage for 30–120 sec, then either washed immediately (entries 1, 2) or mounted at the mouth of the disposable pipet and exposed to an air or nitrogen stream (entries 3, 4). Alternatively, the mouth of the pipet inside the cage was loaded with test solution (3 μl), dried for 5–10 min, then either washed immediately (entry 5) or exposed to an air stream (entry 6). Air (wet) or nitrogen was maintained at an average flow of 35–40 ml/min. Dry Ice was not used (NDI) for trapping in these experiments. Quantities of recovered pheromones, rounded off to the nearest 20 pg (III) or 40 pg (IV), were estimated by external calibration and are the sum total of two GC-MS determinations per sample solution. Ratios for recovered pheromones are the mean of two GC-MS measurements per sample solution (range in parentheses).

When a hexane solution of (*E,E*)-8,10-C_{12}OH (V) was applied to the glass bead and the collection technique repeated, 40–45% of the original 1.7 ng was recovered in the volatiles over 1 hr with Dry Ice cooling and 5–10% was recovered from washings of the bead. On the basis of these results and those obtained for I–IV, we infer that oxidation of V did not occur to any marked degree during this period of trapping.

The above observations demonstrate both the ability of a glass surface as source to release molecules of pheromones (and probably other organic compounds) and the tenacity of the more extensive glass surface within the pipet when dealing with a few nanogram and subnanogram quantities. To the best of our knowledge, all other studies of this type refer to quantities of several nanograms and even micrograms at source, where aerial trapping by a limited amount of glass could not be detected. In the method described here the inner glass surface of the pipet is estimated to be sufficient to retain a few nanograms of pheromones, and they are readily removed with a small volume of solvent (10–15 μl or even less), thus considerably simplifying the work-up and minimizing the possibility of contamination by solvent impurities that might interfere with the measurements. The losses of pheromone components that might occur during evaporation of large volumes of solvent and the probable resulting distortion of fractional composition are completely eliminated by our procedure. If greater accuracy is required for the measurement of blend quantity, internal standards would be used instead of solvent to remove the trapped pheromones.

The procedure of trapping within pipets can be utilized wherever small quantities of volatiles need to be collected from insect or other sources (dissected pheromone glands, pheromone dispensers, leaves, flowers, etc.), using cooling, elongated, or tandem capillary traps, and/or inert gas streams where appropriate. It could be also used to accumulate larger quantities of pheromones from many individual insects, held in separate cages, by rinsing the pipets with the same small volume of solution sequentially. This would give an average blend ratio of the pheromones in one analysis and would permit complete mass spectra to be recorded for qualitative identification of pheromones. This particular procedure for accumulation may be especially useful in identification of minor volatile components, which otherwise are barely detectable, and for characterization of functional groups by chemical modification.

There is major difference between the experiments in the present study involving release from a glass surface as source and those involving actual insects. With an insect releasing pheromones from a gland exposed to the air stream, the target molecules are considered to be already in the vapor phase, and the pheromone mixture is retained by the surface within the pipet with minimal alteration to its composition. In contrast, desorption processes are

involved to produce the vapor stream from a glass surface as source, and these processes differ from compound to compound.

Unlike many previously used techniques, the insect would be minimally disturbed in the trapping method described here, and this reduces the likelihood of distortion or interference with pheromone release. After the ratio of the pheromone constituents has been measured, the intact insect can be used in correlative experiments on behaviour or electrophysiology. We can handle up to 20 female insects in a single experiment (in principle, more could be studied at the same time). This is another attribute of our strategy, since little time is needed for the whole process from mounting the pipets into the cages, through the collecting period and minimal work-up, to the GC-MS measurement. If an insect releases a large amount of pheromone per hour (50–100 ng), shorter periods would be used for trapping to avoid breakthrough. In most cases a short, packed column may be sufficient for rapid and adequate separation of the blend, permitting single ion monitoring for each selected component or repetitive channel cycling where lower sensitivity is possible. In cases of fine structural differences (such as geometrical isomers), high-resolution GC and internal standards would be necessary in the GC-MS measurements, but the total time should still be less than an hour per insect. If it is necessary to carry out the determination of blend ratios by high-resolution GC alone, the rinsing of the pipets could be effected with small quantities of CS_2 containing internal standards.

Because of the sensitivity of the GC-MS determination, contamination by the same or similar molecules must be avoided. It is essential not to handle large (for example microgram) quantities of the pheromone constituents under study before handling the glass equipment (cages, pipets, syringes) during the collecting period. Another source of impurities is the phthalates, although the selectivity of the GC-MS analysis precludes their interference in the measurements. Care should be taken not to touch any plastic materials while handling the glassware, to avoid using disposable gloves, and not to use plastic tubing in washing and aeration.

Typical pheromone molecules are very fragile in an EI mass spectrometer source at 70 eV, and thus the ions selected for monitoring in the present GC-MS measurements represented less than 1% of the total ion current per compound. Despite this, we readily achieved limits of detection for I–V at the low picogram level. For instance, a 2.5-μl injection of a 7 pg/μl solution of I gave a detectable response. Background noise in the ion currents recorded is due almost entirely to bleed from the packed GC column and limits the amplification possible for the MS and recorder under these conditions.

Higher selectivity and sensitivity for even smaller amounts of pheromones can be achieved by using chemical ionization (CI) and, in addition,

the signal-to-noise ratio is then increased because the response of the MS to the column bleed is suppressed. The adoption of selected CI modes that concentrate the total sample ion current in molecular parent ions can permit the monitoring of low picogram or subpicogram levels. Additional specificity in the MS experiment is possible using selected reaction monitoring (GC-MS-MS) (McLafferty, 1983). Such procedures are currently under investigation.

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SELECTIVE PREDATION ON CHEMICALLY DEFENDED CHRYSOMELID LARVAE
A Conditioning Process

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Abstract—Laboratory experiments showed that female *Tenthredo olivacea* prefer to prey upon insects of a previously encountered species, instead of upon unknown ones. This has been observed when comparing two natural prey of the sawfly, the larvae of *Phratora vitellinae* and those of *Plagiodera versicolora*. The two species secrete copious amounts of defensive secretion, the first salicylaldehyde, and the latter a mixture of cyclopentanic monoterpenes. The predator appears less reluctant when encountering a species whose secretion has been previously experienced. A selective pressure might thus exist favoring rare secretions, which is consistant with the well-known diversity of defensive compounds among sympatric insects.

Key Words—Coleoptera, Chrysomelidae, leaf-beetle larva, *Phratora vitellinae, Plagiodera versicolora,* Hymenoptera, Tenthredinidae, sawfly, *Tenthredo olivacea,* predation, conditioning, defensive secretion.

INTRODUCTION

Many chrysomeline larvae are protected by nine pairs of eversible glands. When the insect is disturbed, droplets of secretions are produced and form a chemical barrier against enemies (Figure 1A). These secretions are chemically diverse: so far, six different methylcyclopentanoid monoterpenes, five aromatic compounds, three alkenyl acetates, one alkyl acetate, and one hydrocarbon have been identified in various species. This diversity is even greater when the proportions of the compounds are considered (Pasteels et al., 1982, 1984) and remains poorly understood. Host-plant chemistry has

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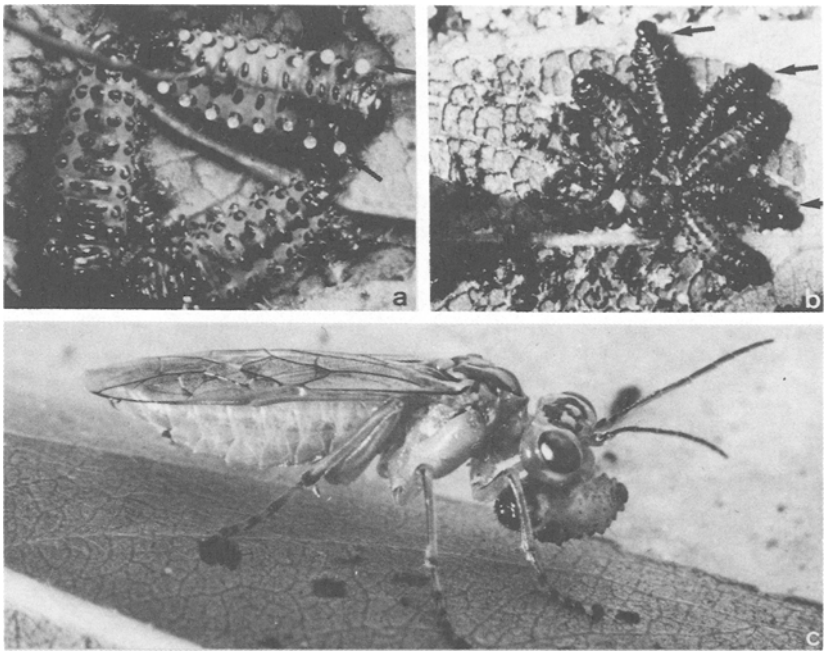

FIG. 1. (A) A third instar *Phratora vitellinae* larva has been disturbed with a seeker and has everted its abdominal glands, secreting droplets of a salicylaldehyde–water emulsion (arrows) (× 10). (B) Larvae of *Phratora vitellinae,* and *Plagiodera versicolora* (arrows) forming a common aggregate on a *Salix* leaf, in the laboratory. Larvae of both species are more or less the same size and shape and look very much alike (× 5). (C) A female *Tenthredo olivacea* holding freshly captured prey (*Phratora vitellinae*) in its mandibles (× 5).

some influence (Rowell-Rahier and Pasteels, 1982; Pasteels et al., 1983b), but the selective pressure exerted by predators remains totally ignored. Generalized predators like ants are effectively repelled, whatever the nature of the secretion (Blum et al., 1972, 1978; Pasteels et al., 1983b; Sugawara et al., 1978; Wallace and Blum, 1969). Other insect predators, however, are able to overcome the chemical defense of chrysomelid larvae and even seem to specialize on these prey (Fabre, 1891; Clausen, 1940; Jolivet, 1950; Whitehead and Duffield, 1982).

We demonstrate here that at least one of these predators, the female sawfly, *Tenthredo olivacea* Kl., shows some preference between sympatric chrysomeline larvae. This differential predation, however, is not intrinsic, but depends on previous experiences with a given prey species.

METHODS AND MATERIALS

The sawflies were observed and field-collected in a poplar nursery belonging to the Rijksstation voor Populierenteelt, at Geraardsbergen, Belgium. Female adults were kept singly in the laboratory, in Petri dishes (30 cm diameter) half filled with moist plaster. They were fed with full grown chrysomelid larvae, *Plagiodera versicolora* Laich. and *Phratora vitellinae* (L.) These larvae fed on a *Salix* leaf fastened in the dish so that the sawfly had access to its two sides.

During the first three days, the sawflies were fed with only one or the other chrysomelid species. Three larvae were given the first day and the consumed larvae replaced each morning. The number of larvae consumed was counted four to five times a day, and the following morning before the replacement of missing larvae. During the 4th day, the sawflies were given the choice between three larvae of both species, and the missing larvae were replaced after each count.

RESULTS

Female adult sawflies were observed in a nursery exploring the leaves of young poplars to find prey and to oviposit. These poplars were infested with the chrysomeline larvae, *P. vitellinae,* which were preyed upon by the sawflies. While feeding (Figure 1C), the predators completely chewed the larvae and left only small pellets of cuticle. Behaving in this way, they cannot avoid being contaminated by the copious secretions of a salicylaldehyde–water emulsion (Wain, 1943).

In other circumstances, *T. olivacea* was observed exploring *Salix* spp. infested by larvae of another chrysomeline, *P. versicolora.* This latter species has not been observed in the nursery. Both chrysomelines, however, are sympatric, and they have been observed in other locations feeding on the same *Salix* tree. In the laboratory at least, they even form common aggregates (Figure 1B). They look very much the same, but *P. versicolora* differs considerably from *P. vitellinae* in the chemistry of its defensive secretion, producing a water emulsion of a mixture of two methylcyclopentanic monoterpenes, plagiodial and plagiolactone. The precise composition of the secretion depends on the geographical origin of the larvae. In larvae collected in Belgium, plagiodial amounts to 70% of the volatiles and plagiolactone for 30% (Pasteels et al., 1982).

Predation on third-instar larvae of both chrysomelines by female sawflies collected in the nursery was compared in the laboratory. The sawflies were divided into two groups of 30 and fed during three days with

larvae of either *P. vitellinae* or *P. versicolora*. In all, 217 *P. vitellinae* larvae, and only 168 *P. versicolora* larvae were consumed ($P < 0.001$, χ^2). This could suggest that the latter species is better protected from *T. olivacea* predation, its defensive secretion being more efficient. A closer look at the results, however, indicates another possibility.

A comparison of the daily consumption rate in the two groups shows that, during the first and second days, predation was much higher on *P. vitellinae* than on *P. versicolora* (Figure 2). This indicates that at first the sawflies were reluctant to feed on *P. versicolora*. With nothing else to eat

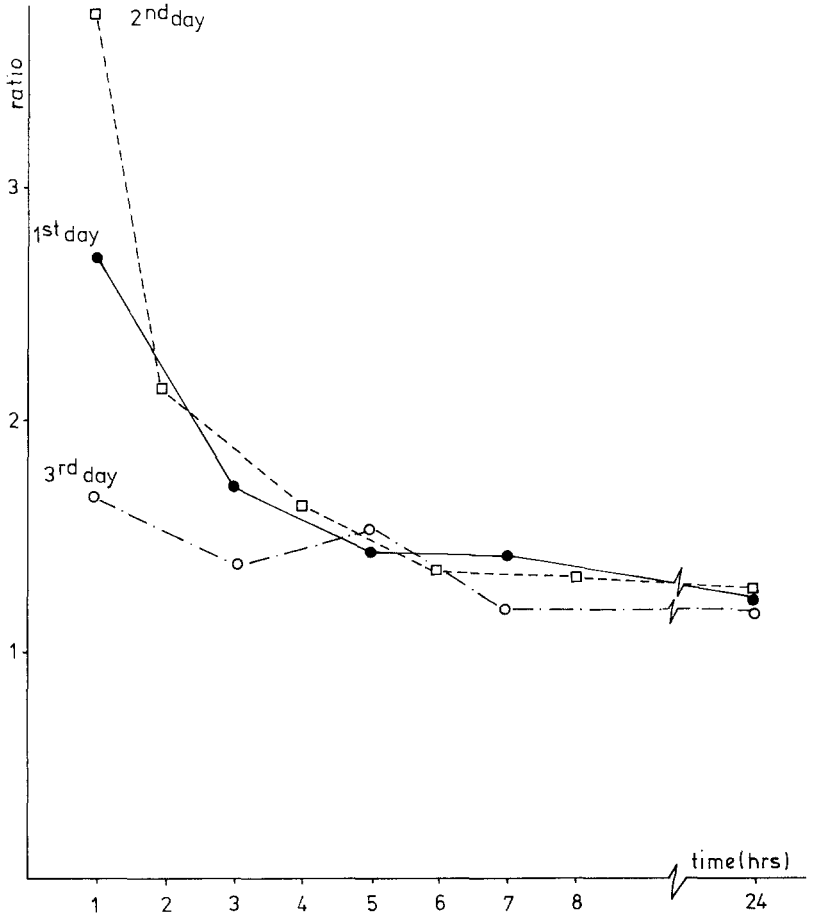

FIG. 2. Ratio of the number of *Phratora vitellinae* to the number of *Plagiodera versicolora* preyed upon by two groups of *Tenthredo olivacea*, during three successive days.

they finally attacked *P. versicolora,* with the result that the differences in consumption decreased from the beginning to the end of the day. Interestingly, the behavior of both groups of sawflies was less dissimilar at the start of the day 3, even if the predation rate on *P. versicolora* did not reach that on *P. vitellinae.* This suggests that the distaste for *P. versicolora* larvae could decrease after previous experience with these larvae. As the sawflies were collected in a poplar nursery heavily infested by *P. vitellinae,* but in

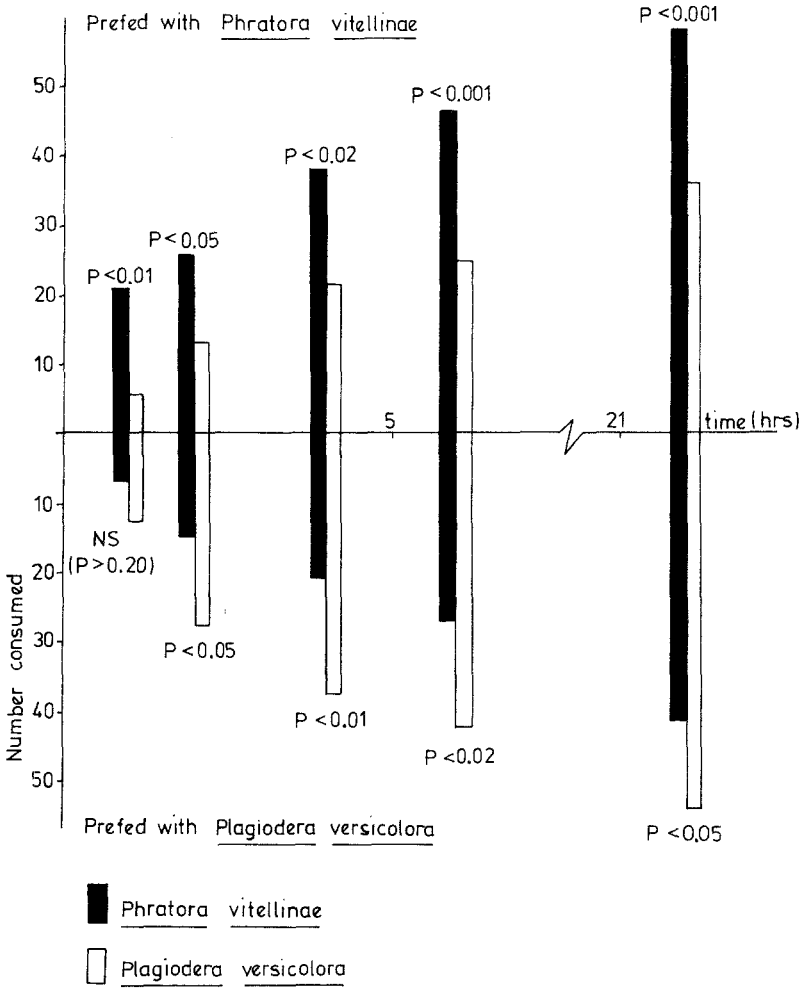

FIG. 3. Differential predation by *Tenthredo olivacea* prefed with the one or the other prey. Black bars: *Phratora vitellinae;* white bars: *Plagiodera versicolora.* Probabilities were calculated by means of the χ^2 distribution.

which *P. versicolora* was absent or rare, their marked preference for the first could be due to some conditioning process rather than being intrinsic.

This latter hypothesis was tested by giving the sawflies the choice between both species (three larvae of each) during the 4th day. The results were striking: the sawflies prefed with *P. vitellinae* larvae showed, as before, a preference for this prey, but those prefed with *P. versicolora* preferred *P. versicolora* (Figure 3). These results demonstrate that prey selection in this predator is not absolute but depends upon previous experience.

DISCUSSION

It is known that conditioning may influence food choice of phytophagous insects (Jermy et al., 1968) and host choice of parasitoid insects (Arthur, 1981). Differential predation due to learning is well known among vertebrates (Tinbergen, 1960; Holling, 1961). The formation of a "search image" has supposedly led to polymorphism in cryptic, nonchemically defended prey species (ref. in Edmunds, 1974). Similarly, vertebrate predators learning to recognize and avoid aposematic, chemically defended prey have been considered as exerting a strong selective pressure towards mimicry.

This is the first time that food conditioning has been demonstrated in an insect predator and for chemically defended prey. We have no indication, so far, that *T. olivacea* actually develops a search image of the larvae on which they prey. The defensive secretion could act as an obvious signal to recognize a specific prey. We were unable, however, to demonstrate that the defensive secretion becomes an attractant or a phagostimulant for the predator. It simply appears that the sawflies were less hesitant when confronted with the secretion after frequent exposure to it. The conditioning process appears to be an habituation, as recently described for some phytophagous insects repeatedly exposed to plant deterrents (Jermy et al., 1982).

So far, we have been unable to experiment with naive insects. Adults are difficult to obtain in the laboratory, due to an obligatory diapause stage. It is not possible to assess the strength of the conditioning when adults are fed from emergence with only one kind of prey. Our experiments indicate, however, that in nature, where diversity of prey is always available, conditioning is reversible. This reversible conditioning seems advantageous for the predator. It allows it to tolerate the chemical defenses of abundant prey, without being strictly dependent on a particular species which could become scarce. It is also possible that conditioning to abundant prey could lower the density of major competitors for the sawfly larvae, which also feed on poplar leaves.

Despite their spectacular chemical defense, chrysomeline larvae are preyed upon by 42 European insects belonging to 17 families and five orders (Fabre, 1891; Clausen, 1940; Jolivet, 1950). If habituation leading to prey preference proved to be frequent, a strong selective pressure would exist for the chemical polymorphism of defensive secretions commonly observed in insects (ref. in Pasteels et al., 1983a). It would be premature, however, to draw general conclusions from experiments with a single predator: in phytophagous insects, it has indeed been demonstrated that repeated exposure to feeding deterrents can lead either to habituation or aversion learning (Jermy et al., 1982).

It should be stressed that mimicry and polymorphism in chemical defense, which lies beyond external appearance, are in no way mutually exclusive. These two strategies could be used simultaneously by the same species: mimicry against vertebrate predators, long-lived and with a high learning capacity, and chemical polymorphism against arthropod predators able to become tolerant to defensive compounds. In fact, the two prey species studied here may well be using both strategies simultaneously.

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SEX ATTRACTANT FOR THREE SPECIES OF THE GENUS *Oncocnemis: O. chandleri* (GRT.), *O. cibalis* (GRT.), AND *O. mackiei* (B. & BENJ.) (LEPIDOPTERA: NOCTUIDAE)

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Abstract—*Oncocnemis chandleri, O. cibalis,* and *O. mackiei* were attracted to chemically baited traps in the field. In all three cases, (5*E*,7*Z*)-dodecadienyl acetate was a key component for attraction. Attraction of *O. chandleri* to traps baited with the (5*E*,7*Z*)-dodecadienyl acetate was inhibited by addition of (*Z*)-7-dodecenyl acetate. *O. cibalis* required both (5*E*,7*Z*)-dodecadienyl acetate and (*Z*)-7-dodecenyl acetate for attraction. Electroantennogram responses for the three species are also reported.

Key Words—Lepidoptera, Noctuidae, *Oncocnemis chandleri, Oncocnemis cibalis, Oncocnemis mackiei,* (5*E*,7*Z*)-5,7-dodecadienyl acetate, (*Z*)-7-dodecenyl acetate, sex attractant.

INTRODUCTION

The genus *Oncocnemis* is found in the north temperate zone of both hemispheres, but most species are found in the arid southwest of North America (Forbes, 1954). Hodges et al. (1983) list 86 species for North America. There is little published on most species, and the immature stages of many are unknown (J.D. LaFontaine, personal communication). Mixtures of monoolefinic acetates have been reported as sex attractants for *O. lepipuloides* (McD.) and for *O. piffardi* (Wlk.) (Steck et al., 1982).

We report here attraction of *O. chandleri* (Grt.), *O. cibalis* (Grt.), and *O. mackiei* (B. & Benj.) male moths to field traps baited with synthetic chemical lures. The three species have an overlapping August–September

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flight period, and *O. chandleri* can be isolated from *O. cibalis* by a chemically defined lure. The electroantennogram (EAG) responses of the three species are also reported.

METHODS AND MATERIALS

The chemicals used in this investigation were synthesized in this laboratory. All four geometrical isomers of 5,7-dodecadien-l-ol (5,7–12:OH), 5,7-dodecadienal (5,7–12:Ald), and 5,7-dodecadienyl acetate (5,7–12:Ac) were prepared and purified to 98% or better by methods previously described (Chisholm et al., 1981). The synthetic monoolefins had a purity of 99% (Steck et al., 1977).

Electroantennogram (EAG) response of the insects' antennae to the synthetic chemicals were recorded (Chisholm et al., 1975) using field trapped males. The moths used for analyses were recovered from the traps within 12 hr of capture. All *E* and *Z* isomers of dodecenyl acetate with double bonds from positions 3 to 11, and all four geometrical isomers of 5,7–12:Ac, 5,7–12:OH, and 5,7–12:Ald were tested. The chemical dose on each filter paper disk was 1 μg, except as otherwise noted. Each response was corrected for mechanical stimulation.

The field trials were carried out near Saskatoon, Canada (52.3°N, 106.5°W). The chemical lures were dispensed from red rubber septa (Arthur H. Thomas, No. 8753-D22) placed within Pherocon 1-CP traps. The traps, placed 15 m apart and 1 m above the ground, were hung in a randomized block design on a fence line. The fence line separated experimental plots of cereal and forage crops from native grassland containing patches of trees and shrubs. Moths were removed from the traps twice a week and their number recorded.

A 40-W black-light trap, located near the test area and examined five times weekly, was used to monitor the annual variation in the numbers of the *Oncocnemis* species.

The data from the replicated field tests were transformed $\sqrt{x+1}$ and then analyzed by an analysis of variance test and significantly different means were separated by Duncan's multiple range test.

RESULTS AND DISCUSSION

The EAG antennal response of *O. chandleri, O. cibalis,* and *O. mackiei* to the *E* and *Z* isomers of dodecenyl acetates and to all four geometrical isomers of 5,7–12:Ac, 5,7–12:OH, and 5,7–12:Ald are presented in Figure 1. In the monoene series *Z*7–12:Ac, *Z*5–12:Ac and *E*7–12:Ac elicited a similar response from all three species. In the diene series, compounds with

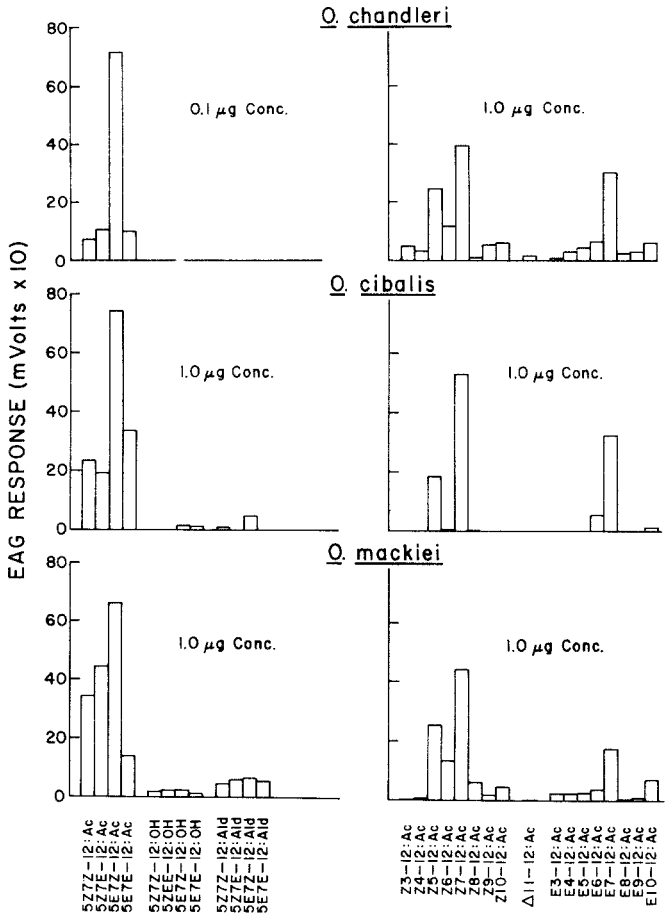

FIG. 1. Electroantennogram responses of *Oncocnemis* males to dodecenyl acetates and dodecadienyl acetates, alcohols, and aldehydes.

the acetate function gave relatively high activity and for each species, *5E,7Z*–12:Ac gave the greatest response. However, *O, chandleri* was an order of magnitude more sensitive to the *5E,7Z*–12:Ac than either *O. cibalis* or *O. mackiei.* When a 1-μg concentration was applied to *O. chandleri,* the antennae became refractory to further stimulation, so it was necessary to reduce the amount of *5E,7Z*–12:Ac applied to the filter paper disk to 0.1 μg.

All four geometrical isomers of 5,7–12:Ac, 5,7–12:OH, and 5,7–12:Ald were field screened as possible sex attractants for moths during the summer of 1979. Twelve traps were set out, each baited with one of the 12 compounds. The initial dose was 100 μg chemical per lure. Between August 28 and September 16, *O. chandleri* males were captured by four of the traps

baited with the following compounds: 5*E*,7*Z*–12:Ac caught 16, 5*Z*,7*E*–12:Ac caught two, 5*Z*,7*Z*–12:Ac caught 2, and 5*E*7*E*–12:Ac caught one male.

During the 1980 flight period, replicated field trapping tests were carried out using 5*E*,*Z*7–12:Ac at initial doses of 1, 10, and 100 μg/bait. The number of *O. chandleri* captured at the various dose levels was not significantly different. Near the end of the flight period 5*E*,7*Z*–12:Ac at 10 μg/lure was tested in combination with 10 μg of various geometrical isomers of 5,7–12:OH, 5,7–12:Ald, and 5,7–12:Ac. In this test (3× replicated), the two component lure containing 5*Z*,7*Z*–12:OH captured more moths than any other treatment, but was not significantly different from the number of moths captured by 5*E*,7*Z*–12:Ac alone.

In 1981 lures composed of 5*E*,7*Z*–12:Ac + 5*Z*,7*Z*–12:OH were field tested alone (Table 1, tests A and B) and in combination with one of the following monoenes: (*E*)-5-dodecenyl acetate (*E*5–12:Ac), (*Z*)-5-dodecenyl acetate (*Z*5–12:Ac), (*Z*)-7-dodecenyl acetate (*Z*7–12:Ac), (*Z*)-5-dodecen-1-ol (*Z*5–12:OH), and (*Z*)7-dodecen-1-ol (*Z*7–12:OH). The purpose of these tests was to find a chemical compound that would either synergize the two-component lure or inhibit attraction of *O. chandleri*. The lure containing *Z*7–12:Ac excluded *O. chandleri* and attracted another moth species in the

TABLE 1. CAPTURE OF *O. chandleri* AND *O. cibalis* BY TRAPS BAITED WITH LURES COMPOSED OF MONOENE AND DIENE COMBINATIONS

Lure composition (μg)	Males captured per trap[a]	
	O. chandleri	*O. cibalis*
Test A		
5*E*,7*Z*–12:Ac(100) + 5*Z*,7*Z*–12:OH(100)	10.3a	0.0
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10)	13.0a	0.0
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10) + *E*5–12:Ac(10)	13.0a	0.0
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10) + *Z*5–12:Ac(10)	12.0a	0.0
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10) + *Z*7–12:Ac(10)	0.0b	3.3
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10) + *Z*5–12:OH(10)	15.3a	0.0
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10) + *Z*7–12:OH(10)	17.0a	0.0
Test B		
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10)	1.7	0.0b
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10) + *Z*7–12:Ac(10)	0.0	8.7a
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10) + *Z*7–12:Ac(1)	0.0	0.0b
5*E*,7*Z*–12:Ac(10) + 5*Z*,7*Z*–12:OH(10) + *Z*7–12:Ac(0.1)	0.7	0.0b
5*E*,7*Z*–12:Ac(10) + *Z*7–12:Ac(10)	0.0	10.3a
5*Z*,7*Z*–12:OH(10) + *Z*7–12:Ac(10)	0.0	0.0b
*E*5–12:Ac(10) + *Z*7–12:Ac(10)	0.0	0.0b

[a]Three replicates per test: 1981; test A, Aug. 7–Sept. 14; test B, Aug. 21–Sept. 14. Means followed by the same letter are not significantly different ($P = 0.05$).

same genus, *O. cibalis* (Table 1, test A). The other four monoenes tested had no effect. In a subsequent test (Table 1, test B) treatments composed of *Z*7–12:Ac in combination with 5*E*,7*Z*–12:Ac + 5*Z*,7*Z*–12:OH or with 5*E*,7*Z*–12:Ac alone were equally attractive to *O. cibalis*. In addition 5*Z*,7*Z*–12:OH did not elicit an EAG response from *O. cibalis* (Figure 1), and therefore it probably is not a lure component. Test B (Table 1) also was designed to determine if *Z*7–12:Ac in low-integral ratios with the 5*E*,7*Z*–12:Ac + 5*Z*,7*Z*–12:OH would act as a trace coattractant (Steck et al., 1980) for *O. chandleri*. No definite conclusion can be drawn from this aspect of the test as *O. chandleri* numbers were declining before this test was initiated. However, distribution of the few moths that were captured suggests that *Z*7–12:Ac is not a trace coattractant for *O. chandleri*. Test B also shows that neither *O. chandleri* or *O. cibalis* is attracted by a bait composed of *E*5–12:Ac + *Z*7–12:Ac in a 1:1 ratio.

Data from our 1982 field test (Table 2) confirmed our previous finding for *O. cibalis*. Traps baited with 200 μg of 5*E*,7*Z*–12:Ac + *Z*7–12:Ac in 1:1 ratio attracted significantly more *O. cibalis* than traps baited with a 20-μg dose. Only a few *O. chandleri* males were captured during the 1982 test, probably because its flight was declining before the test was set in the field. In 1981, *O. chandleri's* flight peaked on August 14.

In 1983 sixteen treatments, composed of mixtures of *E*5–12:Ac and *Z*7–12:Ac and the various dienes, captured 46 *O. chandleri* and 169 *O. cibalis*. In addition, 17 *O. mackiei* were captured; 16 of these males were taken by traps containing 5*E*,7*Z*–12:Ac as part of the lure. The best lure 5*E*,7*Z*–12:Ac (100 μg) + *E*5–12:Ac (100 μg) captured five moths, and blank traps caught no moths. This trapping data, in conjunction with the EAG profile (Figure 1),

TABLE 2. CAPTURE OF *O. chandleri* AND *O. cibalis* BY TRAPS BAITED WITH LURES COMPOSED OF MONOENE AND DIENE COMBINATIONS

Lure composition (μg)	Males captured per trap[a]	
	O. chandleri	*O. cibalis*
5*E*,7*Z*–12:Ac(100)	0.7	0.0c
5*Z*,7*Z*–12:Ac(100)	0.3	0.0c
5*Z*,7*Z*–12:OH(100)	0.0	0.0c
5*E*,7*Z*–12:Ac(100) + 5*Z*,7*Z*–12:OH(100)	0.7	0.0c
5*E*,7*Z*–12:Ac(100) + 5*Z*,7*Z*–12:Ac(100)	1.7	0.0c
5*E*,7*Z*–12:Ac(100) + 5*Z*,7*Z*–12:OH(100) + *Z*7–12:Ac(10)	0.0	6.3b
5*E*,7*Z*–12:Ac(100) + *Z*7–12:Ac(100)	0.0	28.0a
5*E*,7*Z*–12:Ac(100) + *Z*7–12:Ac(10)	0.0	2.3bc
5*E*,7*Z*–12:Ac(10) + *Z*7–12:Ac(100)	0.0	2.0bc
5*E*,7*Z*–12:Ac(10) + *Z*7–12:Ac(10)	0.0	6.3b

[a]Three replicates: 1982, Aug. 24–Sept. 23. Means followed by the same letter are not significantly different ($P = 0.05$).

TABLE 3. YEAR-TO-YEAR CAPTURE OF *Oncocnemis* BY A BLACK-LIGHT TRAP AT SASKATOON

Year	Total moths captured		
	O. chandleri	*O. cibalis*	*O. mackiei*
1979	0	1	1
1980	0	0	3
1981	0	2	33
1982	1	0	15

suggests 5*E*,7*Z*–12:Ac could be a sex attractant lure component for *O. mackiei.*

Noctuid moths rarely respond to conjugated dienes (Steck et al., 1982), and we are not aware of any reference of them responding to 5,7-dodecadienes. Table 3 shows only a few *Oncocnemis* were captured by a black-light trap from 1979 through 1982. These data, in conjunction with moth capture data in Tables 1 and 2, suggest the chemical lure is a much stronger attractant than light and thus offers a better tool to study the occurrence and habits of *O. chandleri* and *O. cibalis* than a light trap.

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ALERT ODOR FROM SKIN GLAND IN DEER

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Abstract—Black-tailed deer (*Odocoileus hemionus columbianus*) discharge an odor from the metatarsal (MT) gland, located on the hindleg, when disturbed or alarmed. Freely moving, captive deer were exposed to the MT odor by means of a remote-release apparatus. Responses by males and females to MT odor from both sexes were recorded with a coding system and a video camera. When the odor was present, females became more alert and left the site more often than in the presence of control odors, odorless air, or deer urine. It is concluded that the MT secretion provides an alert signal, placing the odor into the class of alarm pheromones. There is no evidence that the closely related white-tailed deer, *O. virginianus,* possesses this alert (or alarm) odor system to the same degree.

Key Words—Alert odor, bioassay, black-tailed deer, Cervidae, deer, *Odocoileus hemionus columbianus.*

INTRODUCTION

"Alarm" is defined as "a signal warning of danger," "a device that signals a warning of danger," "the terror caused by danger," or "a warning notice" (Webster). Many invertebrates, from sea anemones (Howe and Sheikh, 1975) to snails (Snyder, 1967; Atema and Burd, 1975), earthworms (Ressler et al., 1968), and social insects (Maschwitz, 1964) have odors that communicate alarm between conspecifics. In vertebrates, the fright substance of fish (Frisch, 1941) and tadpoles (Pfeiffer, 1974) are well-known examples of alarm odors.

Mammals have been reported to produce "stress odors" that are avoided by conspecifics. In mice (*Mus musculus*) such an odor is contained in the urine (Müller-Velten, 1966). Other "stress odors" that are related to fighting and dominance (Carr et al., 1970; Jones and Nowell, 1974) are not

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alarm pheromones. Rats discriminate between odors of stressed and nonstressed rats (Valenta and Rigby, 1968; Stevens and Koster, 1972), as do hamsters (Sherman, 1974).

Some mammalian species are known to discharge odors when alarmed or pursued. Pronghorns (*Antilocapra americana*) release an odor from their sciatic glands during flight (Buechner, 1950; Müller-Schwarze and Müller-Schwarze, 1972). When handled, an odor emanates from shrews of the genus *Suncus* (Dryden and Conaway, 1967). No effects on conspecifics are known in these cases.

Among cervids, footprints with interdigital gland secretion may carry an alarm signal. Lent (1966) reported caribou (*Rangifer tarandus*) avoiding the track of an alarmed conspecific, and Kurt (1967) observed roedeer (*Capreolus capreolus*) bucks fleeing from footprints of other male roedeer. The latter case may be an individual avoidance response rather than warning behavior in the face of interspecific danger.

We now report an alert response by conspecifics to an odor emanating from a highly specialized gland, the metatarsal (MT) gland in black-tailed deer (*Odocoileus hemionus columbianus*). The gland is located on the outside of the hindfoot, measures 6×4 cm, and produces a strong, skunk- or garlic-like odor. The odor is discharged when deer flee or are disturbed, cornered, caught, handled, or in strange surroundings (Müller-Schwarze, 1971). The MT gland is active and the odor is present in both sexes, year round, and some newborn fawns had a faint MT odor when handled.

The closely related mule deer (*O. h. hemionus*) has a metatarsal gland of similar size (Cowan, 1956; Quay and Müller-Schwarze, 1971), while the gland is less developed in white-tailed deer, *O. virginianus* (Hershkovitz, 1958; Quay, 1959).

The first behavioral effect demonstrated for the metatarsal secretion was inhibition of feeding in a consecutive-choice test (Müller-Schwarze, 1971; Müller-Schwarze and Volkman, 1977). To test behavioral effects of metatarsal secretion in freely moving deer engaged in natural behavior, a new bioassay was developed. The bioassay and apparatus used, along with preliminary results, were reported earlier (Müller-Schwarze, 1980).

METHODS AND MATERIALS

Animals. The test animals were mixed groups of males and females, each in a pen 30×30 m large. A total of six males and 18 females in six groups were used, ranging from two males and two females to one male and six females. The odor donor was sedated with Rompun, the MT hair was shaved off, and the secretion was squeezed ("milked") from the gland and then wiped off both metatarsal glands with cotton swabs held with a hemostat.

Odor Delivery Apparatus. The secretion-soaked cotton balls were placed immediately in an airtight stainless-steel cylinder (4 cm diameter, 4 cm high) which is part of an odor delivery apparatus (Figures 1 and 2) described elsewhere (Müller-Schwarze, 1980). The steel container has two air valves, opening in opposite directions. The valves can be opened, and thus odor delivered, by an airstream through Tygon tubing from a compressed-air tank, 10 m away. The air flow is regulated by a pressure gauge regulator and a flowmeter (Victrometer) on the air tank. To avoid contamination by rubber, the pressure gauge was custom-made and has a

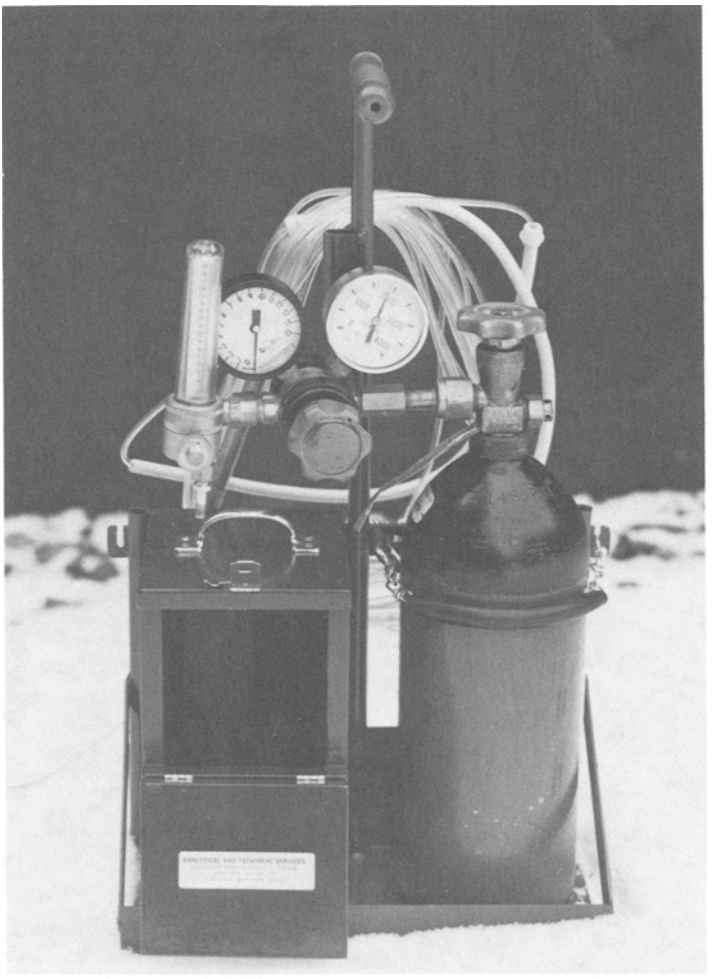

FIG. 1. The odor delivery apparatus in the field. On lower left the odor container in protective cover. On right compressed air tank with gauges and flowmeters.

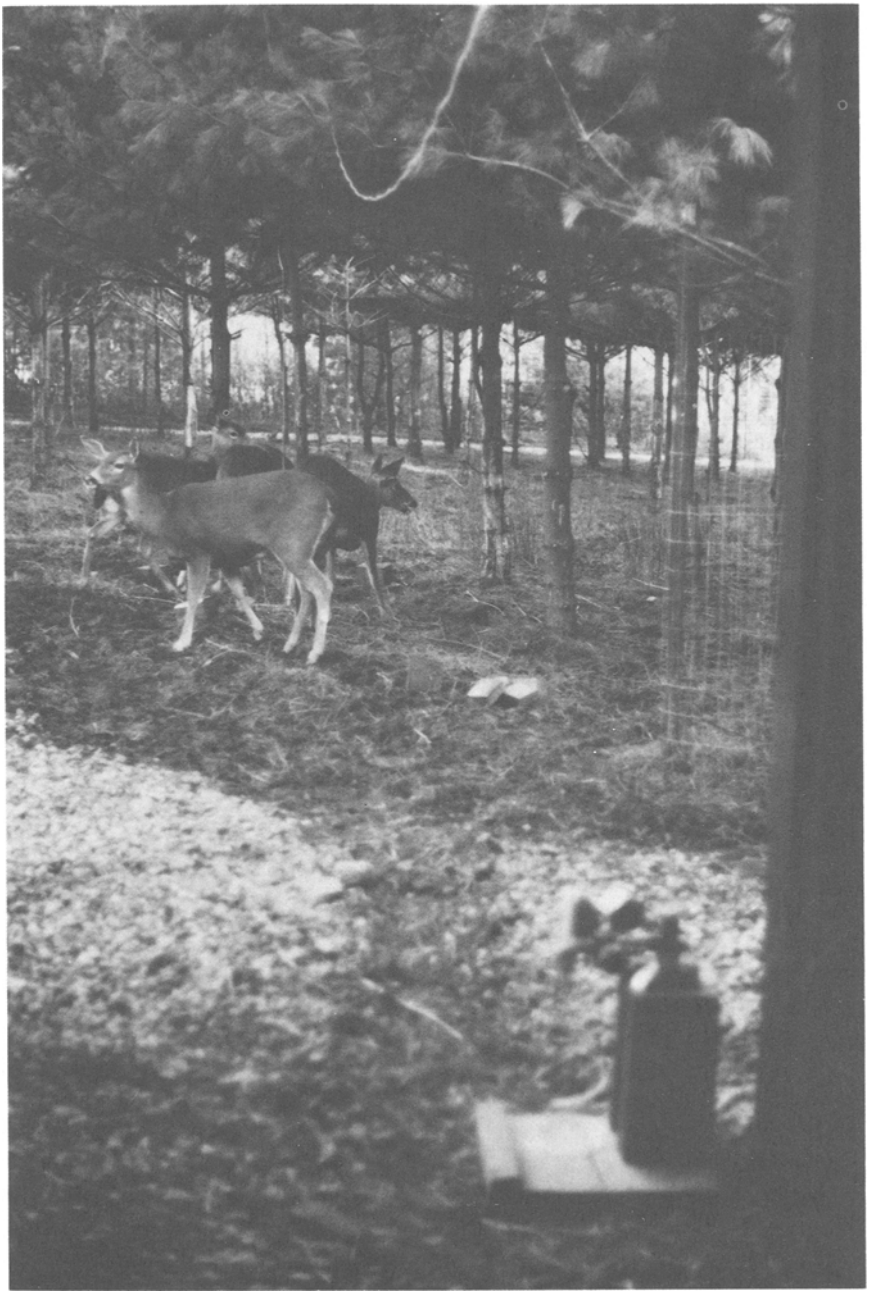

FIG. 2. Deer at feeding station during odor release. Note that alert deer look in different directions, instead of focusing on a particular disturbance.

brass body, brass gauge, stainless-steel diaphragm, and Kel-F seat. Actual flow through the steel container is confirmed by a flowmeter (Victrometer) at the exit of the steel cylinder. The flowmeter is monitored with the aid of binoculars. The amount of secretion collected for one test was two gland equivalents (2 GE), its volume 50–100 μl (measured with micropipettes), and it contained an estimated 10–100 ng of the major volatile components (measured with GLC: A. Singer, personal communication).

Odor Presentation. The steel container with the odor was set up at the base of a post or tree in the interior of the enclosure. Food preferred by the deer was placed around the odor source. The preferred food changed with the seasons and included apples, apple leaves, hay, white pine twigs (*Pinus strobus*), sugar maple, blackberry leaves, and white cedar. As soon as at least two individuals were feeding at the station, recording of their behavior commenced. After 1 min of baseline observation (preodor period), the airflow (120 ml/min) from a cylinder in a shed 12 m away was turned on for 2 min (odor period). After that, behavior was observed for 2 min with valves closed. For comparisons of the general readiness to assure the alert posture in the absence of any odor stimulation, the first preodor period (valves closed) of each day was used, and termed blanks. When feeding, the deer were with their noses 5–100 cm from the odor outlet, i.e., the odor was diluted by about 1.57 m^3 air (1 GE/0.70 m^3 air). Tests were run at ambient temperatures ranging from $-5°$ to $+20°$C and relative humidity from 36 to 100%.

A series of control odors was chosen that ranged from conspecific (deer urine) to other animal odors (beaver castor, muskrat musk) and nonbiological odors (gasoline, coffee).

Each day metatarsal odor (M), air (A), and a control odor (C) were tested, and their sequence counterbalanced, so that one day the sequence M-A-C was used, the next C-A-M, and so on, until all possible permutations had occurred. The sequence used on a particular day was randomly chosen.

Five series of tests were run. MT I: 16 tests; control odors: butylmercaptan, beaver castor, hydrogen sulfide, apple juice, female black-tailed deer urine. MT II: 13 tests; control odors: hydrogen sulfide, gasoline. MT III: 18 tests; control odors: gasoline, beaver castor, female deer urine; tests run at dusk. MT IV: 16 tests; control odors: gasoline, male dog urine, muskrat musk, male black-tailed deer urine. MT V: 12 tests; control odor: male and female deer urine; responses filmed on videotape.

Since the responses to the different control odors did not differ significantly, they were lumped as "control odors." Only hydrogen sulfide and beaver castor caused some elevated alertness.

The behaviors recorded are feeding, full alert, half alert, sniffing the odor outlet or apparatus, and leaving the site.

The behavior was recorded with a notation system on data sheets for

four series of tests and simultaneously on data sheets and with a video camera for the fifth. Both systems of recording yielded the same results, with, of course, the video recordings being more precise in real time. Interobserver reliability was 90%.

The notation system distinguished four intensities of being alert which received point scores: "Full alert" (with head held up high) of 3 secs duration or more (2 points), "low alert" (head lifted, but eyes not above shoulder line) of 3 sec or more (1.5 points), short "full alert" (below 3 sec; 1 point), and short "low alert" (below 3 secs; 0.5 points). The points were added for males and females separately and divided by the number of animals that were present and the number of half-minute periods these were present. Thus, a final measure of "alerts per animal minute" is derived. This is used as the dependent variable in the graphs.

RESULTS

The MT odor has an effect on conspecifics: black-tailed deer are more alert when MT odor is present than when there is no odor, odorless air, or control odors. The level of alertness (weighted for two different intensities and two different durations) increases by 40% above base level, on the average (Figure 3). In some tests, the level of alertness was twice the base level.

Sex Differences of Gland Size. The metatarsal glands of males are larger than those of females, but related to body size, they are slightly smaller in males than in females. This difference, however, is not significant (Table 1) so that the MT glands can be considered to lack sexual dimorphism in size. ($N = 7$ females; 6 males.)

Sex Differences of Response. Only females show increased alertness in the presence of MT odor. In response to female MT odor, females are 35% more alert ($N = 13$; $P < 0.05$), than during the preodor period. No such differences were found between the alertness levels of the preodor and odor conditions when control odors and air were presented (Figure 1). Males do not become more alert when exposed to MT odor; both when compared to the preodor level and to that of control odors or air (Figures 4 and 5).

There is a slightly higher increase (40.5%; $N = 18$, $P < 0.05$) of females' alertness from the preodor level to that of MT when the odor donors were males (Figure 6). This may be due to the larger gland size in males, which possibly results in more odoriferous material being present in one gland equivalent (GE) of MT secretion.

All measurements were subjected to an analysis of covariance. For female responses to female MT odor, the difference between treatments ($df = 2$) is significant at $P < 0.001$ ($F = 20.72$). For female responses to

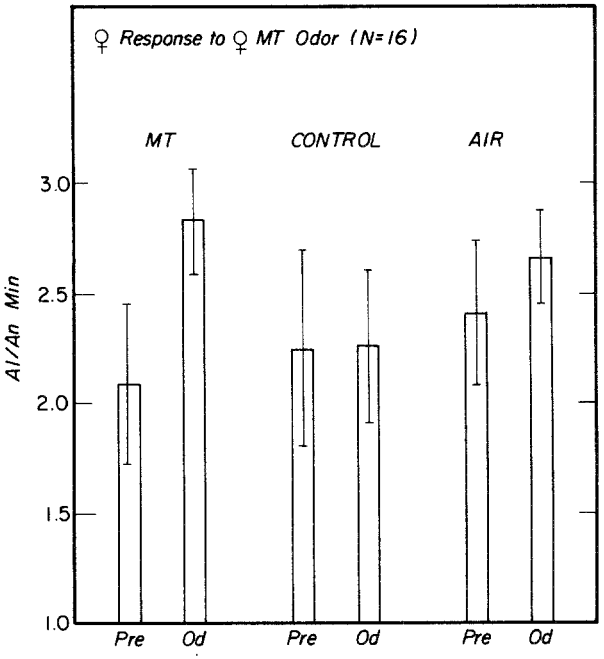

FIG. 3. Alert responses (means with standard errors) by females to female metatarsal odor, control odors, and air. Abscissa: Preodor response level (valves closed; Pre) compared with responses during odor flow (Od). Ordinate: weighted alerts per animal minute.

male MT odor (used in four tests), F ranges from 1.27 to 89.72 and P from not significant to < 0.001 (Tables 2 and 3).

Because of the lack of response by males to MT odor, the remainder of this report deals with responses by females only.

TABLE 1. SIZES OF METATARSAL GLANDS IN EXPERIMENTAL BLACK-TAILED DEER

	Gland		Body		
Sex	Length (cm)	Width (cm)	Total Area (cm^2)	Weight (BW) (kg)	Area (cm^2)/kg BW
Male	7.89 ± 0.52 (SD)	2.53 ± 0.30 (SD)	18.7	74.6	0.271* ± 0.10
Female	6.2 ± 0.57	2.26 ± 0.26	14.0	50.8	0.249* ± 0.01

*Sex difference: $t = 0.418$, not significant.

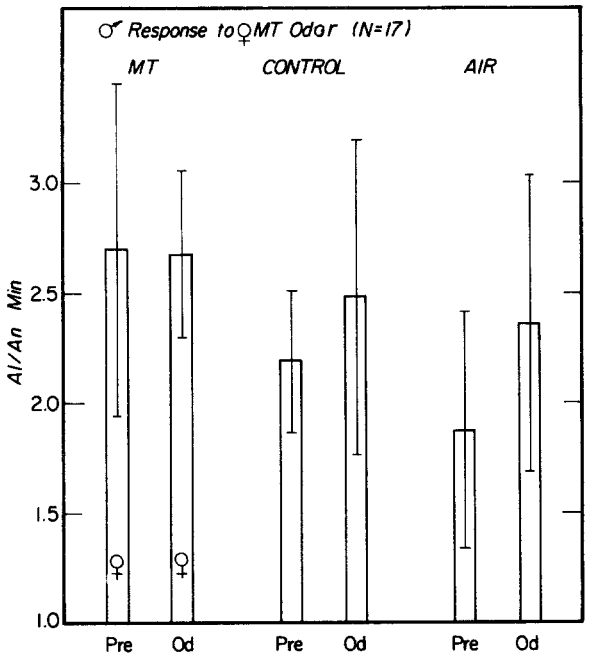

FIG. 4. Alert responses of males to female metatarsal odor; as in Figure 3.

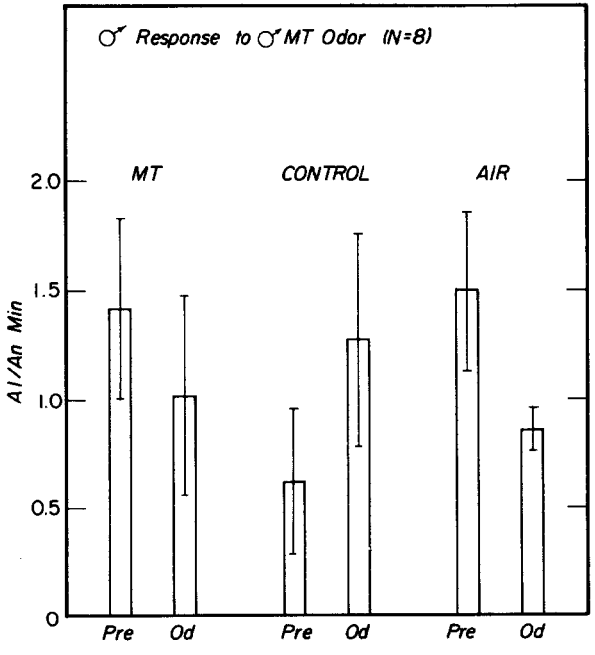

FIG. 5. Alert responses of males to male metatarsal odor; as in Figure 3.

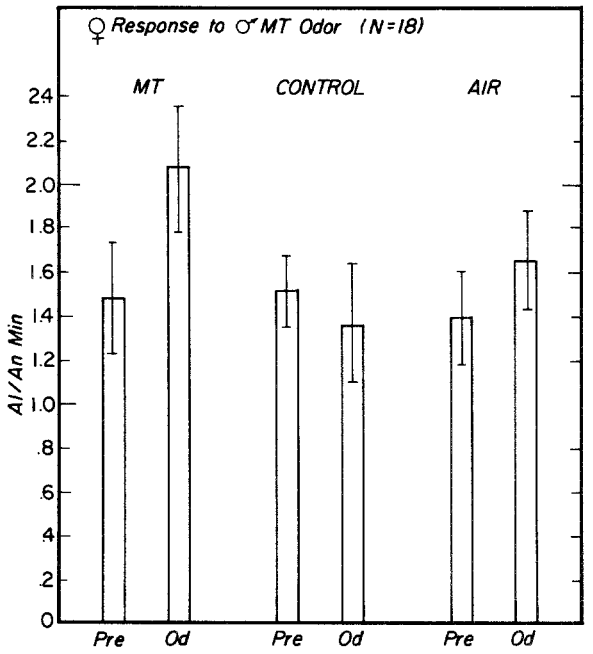

FIG. 6. Alert responses of females to male metatarsal odor; as in Figure 3.

Daylight vs. Dusk. MT tests were run at low light intensities at dusk. The light intensities varied from 10,000 to 0.1 Lux. The females' responses to male MT odor did not differ between day and "night" (Figures 7 and 8). Figures 7 and 8 plot the pre-odor values of single tests (abscissa) against the values for MT, air, and control odors, respectively (ordinate).

Seasonal Differences. The alert response of female black-tailed deer varies with the seasons. They are most alert to MT odor during the months of May/June, and least so in September/October. May/June is the time of year when fawns are born, although the experimental animals did not have fawns. However, the alert response during the first preodor period of a given day (blanks, i.e., no air flow), varies in the same fashion, with a peak in May/June and a minimum in September/October (Figure 9). The level of alert response in the presence of blanks tends to be lower than that to MT odor. However, significant differences between MT odor and blanks exist only during March/April and November/December. (In January and February the freezing temperatures do not allow MT testing.)

Response Level and Relative Humidity. Between 50 and 100% relative humidity, the alert score to MT odor increases from 1.12 to 2.83 ($t = 4.393$; $P < 0.001$), while there is no significant increase for the blank (first preodor

TABLE 2. MEAN NUMBERS OF ALERTS/ANIMAL MINUTE BY FEMALES BEFORE AND DURING ODOR DELIVERY FOR FIVE EXPERIMENTS

			MT		Control odors		Air	
Experiment	Odor	*N*	Pre	Odor	Pre	Odor	Pre	Odor
I	♂	11	1.05 ± 0.13	1.70 ± 0.14	1.07 ± 0.12	1.28 ± 0.13	1.23 ± 0.18	1.02 ± 0.13
II	♂	11	1.23 ± 0.30	2.01 ± 0.33	1.39 ± 0.20	1.29 ± 0.29	1.40 ± 0.36	1.14 ± 0.30
III	♂	18	1.49 ± 0.25	2.08 ± 0.29	1.52 ± 0.17	1.36 ± 0.27	1.39 ± 0.21	1.65 ± 0.22
IV	♀	13	1.72 ± 0.27	2.59 ± 0.24	1.70 ± 0.25	1.94 ± 0.29	2.16 ± 0.31	2.60 ± 0.24
V	♀,♂	12	2.22 ± 0.27	3.20 ± 0.60	1.94 ± 0.40	2.68 ± 0.25	2.32 ± 0.36	2.61 ± 0.45

TABLE 3. ALERT RESPONSE BY FEMALES TO MT AND CONTROL ODORS AND AIR (ANALYSIS OF COVARIANCE)

Test	Odor	N	Difference between Treatments		P values		
			F	P	MT vs. control	MT vs. air	Control vs. air
I	♂	11	89.72	0.001	0.025	0.001	0.001
II	♂	11	1.27	NS	0.025	0.05	NS
III	♂	17	3.206	0.05	0.05	NS	NS
IV	♀	13	20.718	0.001	0.05	NS	0.05
V	♂	12	1.53	NS	NS	NS	NS

period on a given day; no air flow) condition ($t = 1.632$; NS; Figure 10). Most alert scores for MT odor are higher than those for blanks, but the differences are significant only in two of seven cases (asterisks in Figure 10). This indicates that the MT odor may be responsible, and not a general readiness to be more alert in humid air. Below 60% relative humidity, the response decreased with increasing humidity, and no difference between responses to MT and blanks were observed (Figure 10).

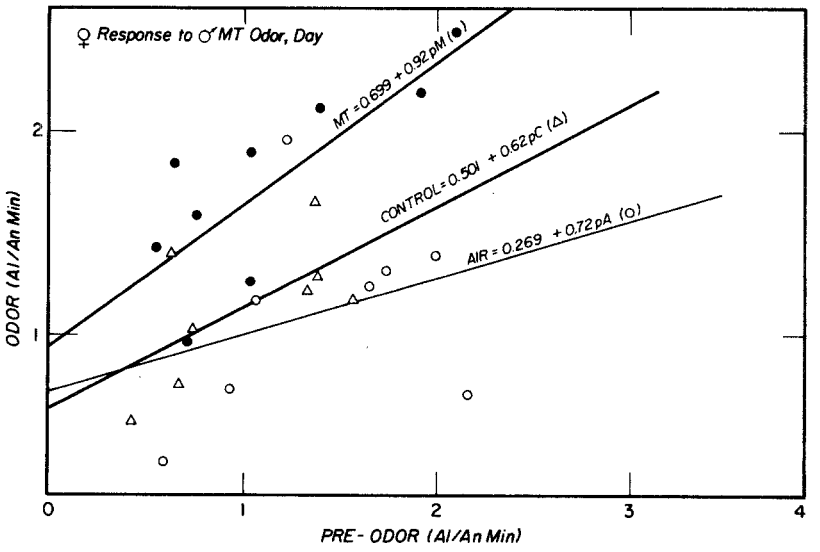

FIG. 7. Covariance between alert responses during preodor and odor periods, with regression lines, for female responses to male metatarsal odor in midday.

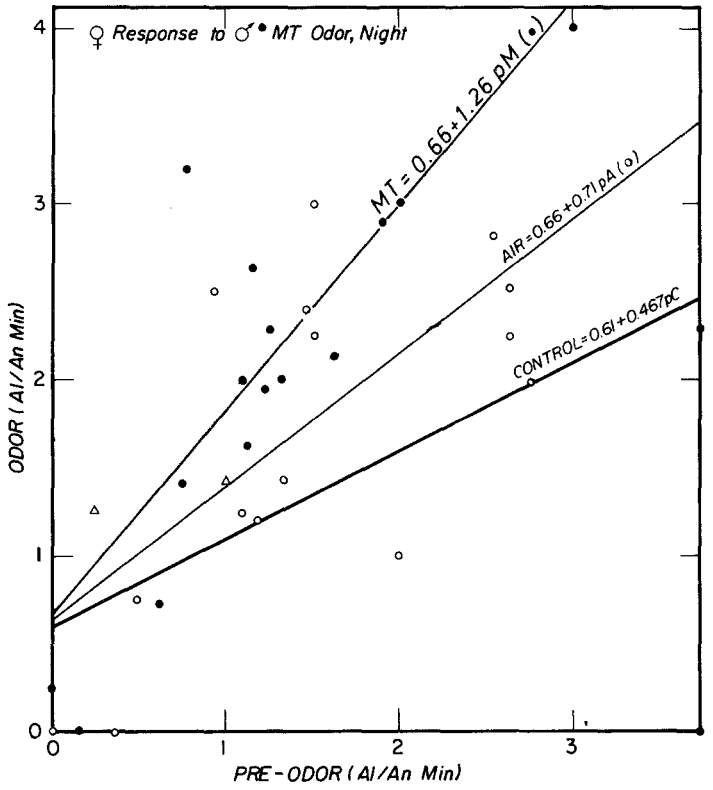

FIG. 8. Covariance between alert responses to male metatarsal odor, at dusk.

Ambient Temperature. Above the freezing point, the level of alertness increases with ambient temperature for both blanks (first preodor period) and MT odor (Figure 11). The differences between the lowest and highest response levels are significant at $P < 0.01$ and 0.05, respectively. The alert responses to MT odor are always higher than those during the first preodor period (blank). But only three differences between MT and blanks are significant (Figure 11).

Odor Specificity. Other black-tailed deer odors, such as male and female urine, did not enhance the alert response. Responses to deer urine differed significantly from those to MT odor and did not differ from those to air (Figure 12).

Leaving the Site. Females leave the feeding station most often when MT odor is present. The overall average leaving rates in all tests (I through V) are 0.497 ± 0.079 times leaving per animal minute for MT, 0.373 ± 0.047 for control odors, and 0.313 for air. The difference between MT and air is

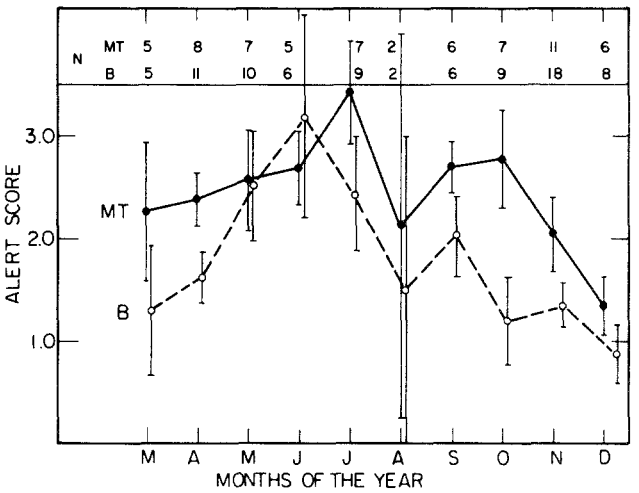

FIG. 9. Seasonal variation of the intensity of alert responses by females. Abscissa: Months of the year from March through December. Ordinate: Alerts per animal minute. Solid circles: metatarsal odor responses; open circles: responses to blanks (air only).

significant ($t = 1.77$; $P < 0.05$), while the differences between control odor and air, and between MT and control odors were not significant.

The temporal order did not influence the rate of leaving; no significant differences were found between the rates of leaving during the first, second, or third stimulus presentation in the sequence of a day's test.

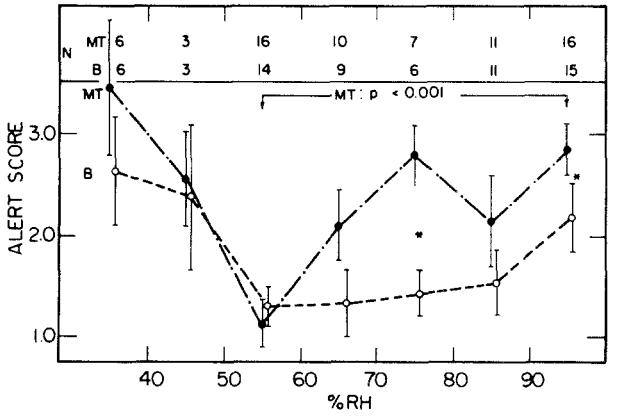

FIG. 10. Alert responses at different levels of relative humidity (% RH).

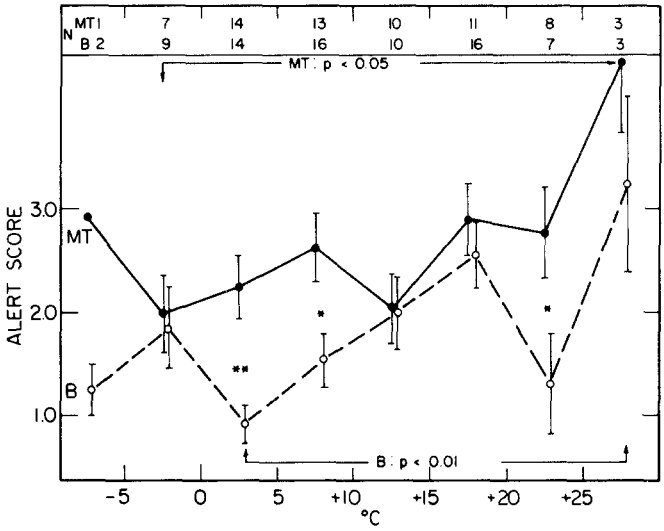

FIG. 11. Alert responses at different ambient temperatures (centigrade).

Table 4 compares the rates of leaving by females during the preodor periods with those during the odor periods. The animals are less likely to leave the station during any preodor period, since the food has just been provided, but there is a slight tendency for the rate of leaving to increase more when MT is present than when there are control odors or air.

Videotape Recordings. In test V the behavior of the deer was recorded on videotape. The following measures were evaluated: percent of time spent in full alert position, mean duration of alerts, number of alerts, and frequency of leaving the site.

The percentage of time spent in full alert by females in response to male metatarsal secretion increased by 127%. The average duration of alerts increased by 105–383% (Figure 12). For the same test series V, the coded recording on data sheets yielded only a 46% increase (not significant) (Table 3) in the alert score. Thus, as expected, the video recording reveals greater response differences than the much more conservative scores on data sheets that were used for all five experiments.

The behavior patterns recorded from the video film were full alert posture, partial alert posture, and departure from test area. The following variables were used for analysis: number of partial alerts per minute, number of full alerts per minute, frequency of leaving the site, percent of time spent in partial alert posture, percent of time spent in full alert posture, percent of time spent away from testing site, mean duration of partial alert stances, mean duration of full alert stances, mean duration of time spent

TABLE 4. RATES OF LEAVING BY FEMALES, COMPARISON OF PREODOR AND ODOR LEVELS

		MT			Control			Air		
Test	*N*	Pre	Odor	*P*	Pre	Odor	*P*	Pre	Odor	*P*
MT I	8	0.29 ± 0.07	0.74 ± 0.03	0.001	0.30 ± 0.08	0.42 ± 0.09	NS	0.15 ± 0.05	0.33 ± 0.14	0.05
MT II	13	0.19 ± 0.12	0.23 ± 0.06	NS	0.18 ± 0.12	0.19 ± 0.06	NS	0.10 ± 0.09	0.18 ± 0.06	NS
MT III	18	0.30 ± 0.07	0.37 ± 0.10	NS	0.28 ± 0.02	0.45 ± 0.09	NS	0.28 ± 0.12	0.35 ± 0.07	NS
MT IV	16	0.10 ± 0.04	0.50 ± 0.18	0.05	0.26 ± 0.10	0.38 ± 0.08	NS	0.18 ± 0.09	0.20 ± 0.07	NS
MT V	12	0.06 ± 0.04	0.16 ± 0.07	NS	0.07 ± 0.05	0.12 ± 0.05	NS	0.14 ± 0.06	0.20 ± 0.09	NS
Overall means		0.19 ± 0.04	0.40 ± 0.10	NS	0.22 ± 0.04	0.30 ± 0.066	NS	0.17 ± 0.03	0.25 ± 0.04	NS

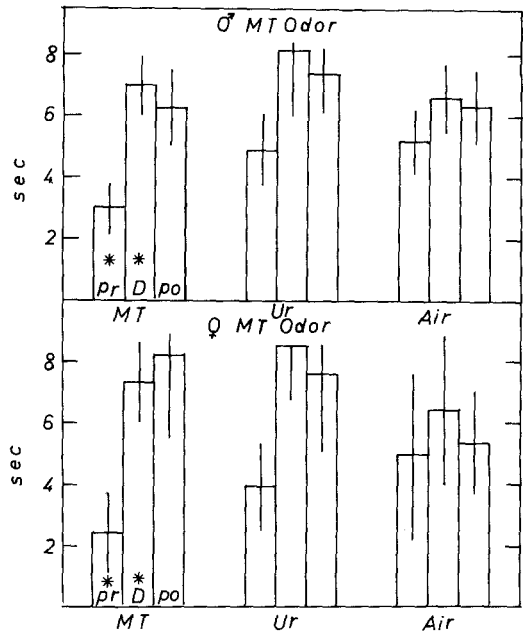

FIG. 12. Average duration in seconds (ordinate) of the full alert posture in females in the presence of metatarsal odor (MT), deer urine (Ur), and air; abscissa. For each treatment there is a preodor (pr), odor (D), and postodor (po) value. Pairs of bars with asterisks differ significantly ($P < 0.05$). Top: female responses to male MT odor. Bottom: female responses to female MT odor.

away from site, and the total number of alerts (full and partial) per minute. All these values were generated for pre-, during, and postodor flow, and for metatarsal odor, control odor (deer urine), and air. The data were further separated by sex of donor and subject. Analysis of variance led to 351

TABLE 5. FREQUENCIES OF SIGNIFICANT DIFFERENCES OF ALERT AND LEAVING RESPONSES BETWEEN PRE- AND DURING ODOR LEVELS, WITH DURING VALUE HIGHER, AS OBTAINED FROM TIME ANALYSIS OF VIDEOTAPED BEHAVIOR

	Significance level (P)			
	0.05	0.01	0.005	Total
Air	2	3	1	6
Control	0	1	1	2
MT	13	14	8	35
Total	15	18	10	43

$\chi^2 = 49.8$, $P < 0.001$

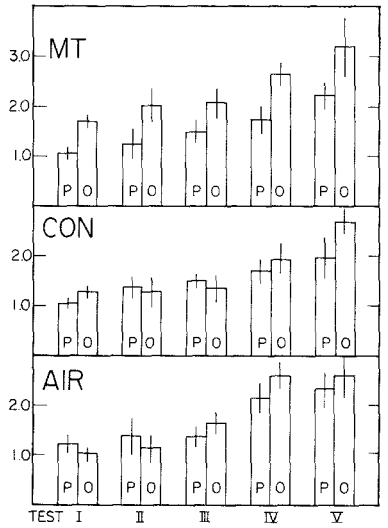

FIG. 13. The means of the preodor and odor levels of the alert responses by females for the three treatments in all five tests.

comparisons for each of the three treatments (13 tables with nine variables and three conditions: pre-, during, and postodor). Forty-three significant differences where the "during odor" value was highest, were found. They are distributed as shown in Table 5 (Porter, 1982).

Figures 13 and 14 summarize the relationship between preodor and odor alert responses in all five tests. Each data point is the mean for one of the five tests. The response increases from preodor to odor condition are clearly higher for MT odor than those for control odors or air.

If the scores for "alert" (Figure 15) and "leaving" (Figure 16) are combined into a single X score by means of discriminant analysis, the responses to air and metatarsal secretion become much better separated (Figure 17). The difference is significant ($T^2 = 19.902$; $F = 9.864$; $df = 2$; 113; $P < 0.001$).

DISCUSSION

The experiment showed that black-tailed deer respond to MT odor from conspecifics. The response consists of increased alertness. Only females show a response, and they do so to both male and female MT odor. This indicates that the main function of MT odor is social, not sexual. Females are more likely to be in social groups composed of related individuals, as yearling does stay with their mothers, and mother–yearling–fawn triads form the basic units in larger aggregations. Thus, MT

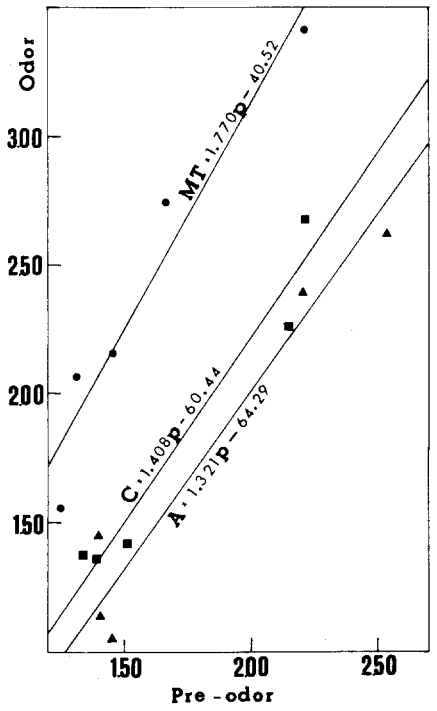

FIG. 14. Regression lines of the means of the five experiments. Solid circles: MT; squares: control odors (C); triangles: air (A); p: preodor value. Abscissa: preodor values. Ordinate: Values during odor flow.

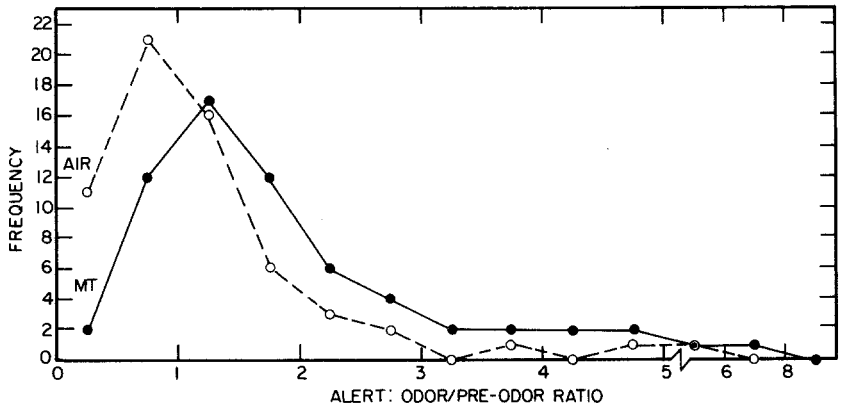

FIG. 15. Comparison of the ratios of the alert scores during and before odor flow for air and MT. Abscissa: odor/preodor ratios of alert scores.

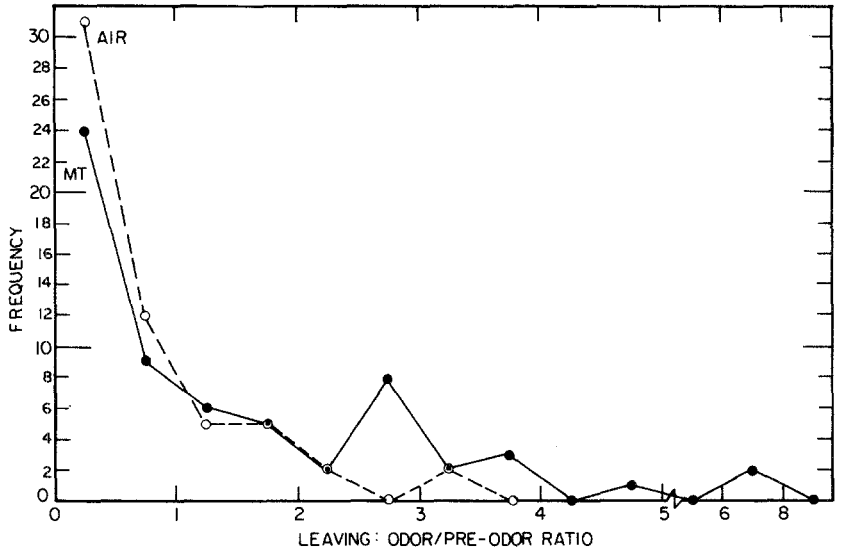

FIG. 16. Comparison of the leaving scores during and before odor flow for air and MT. Abscissa: odor/preodor ratios of leaving scores.

signals may have an important function in kin selection as the olfactory equivalent to warning calls in other mammalian and avian species. But males still have a functioning MT gland that does not differ in size from that of females.

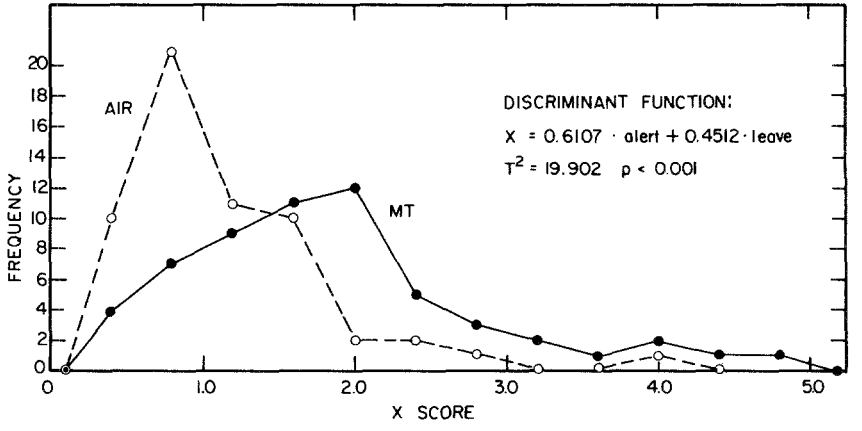

FIG. 17. Discriminant functions of MT and air. Abscissa: X score, combining alert and leaving ratios from Figures 15 and 16.

The seasonal peak of the alert response to both MT odor and blanks coincides with the fawning season, although none of our experimental does had fawns (they were penned together with vasectomized bucks).

The gradual increase of response intensity from test I to test V (Figure 13) is difficult to explain. This increase occurs for all three treatments. The most likely explanation is that the alert response increases with the age of the animals, as essentially the same herd of deer was used for this experiment, with only occasional replacements. Another possibility is increased observer efficiency in recording the responses.

The positive correlation of response level and relative humidity between 50 and 100% has considerable ecological implications. It is well known that dogs track better under humid conditions. Water molecules compete with odor molecules on surfaces, and thus more odoriferous material is released. This was demonstrated in model experiments with phenylacetic acid in high and low humidity by Regnier and Goodwin (1977). The range of the black-tailed deer and mule deer covers mostly arid areas (with the exception of the Pacific Northwest). Under arid conditions, the deer would be in a better position to control the release of odor from the gland and skin surface themselves by action of the arrector pili muscles. By contrast, in a humid climate, there would be more odor exchange with the environment beyond the control of the animal, and thus possibly continual release of odor. This may explain why the white-tailed (WT) deer (*Odocoileus virginianus*) does not possess the MT system as known in mule and black-tailed deer; WT deer MT glands are small, do not have the odor, and WT deer do not respond to their own or black-tailed deer MT odor (Müller-Schwarze and Volkman, 1977; Volkman, 1981).

Considering the small amount of material used, the lack of chemical identification of any of the active components, and our ignorance of the sensory thresholds for any of these unknown components, it is not surprising that the responses are subtle and cannot always be demonstrated. Graded concentrations of compounds known to be active would probably produce more predictable effects. Nevertheless, in the following we attempt to estimate the odor concentration present near the odor outlet.

Assuming the release of 10 ng material into 240 ml flowing air over 2 min, 8.333×10^{-8} mg/sec are delivered. Assuming further a molecular weight of 34 (H_2S) of the active compound(s)—unknown at this point—$(8.333 \times 10^{-8})/(34 \times 10^{3}) = 2.45 \times 10^{-12}$ mol/sec are delivered. Using Loschmidt's number (6.023×10^{23}), this converts to 1.476×10^{12} molecules/sec or 7.378×10^{11} molecules/cm^3 air at the outlet (since flow is 2 cm^3 air/sec). How this will be diluted into the 1.57 m^3 air around the outlet will depend on air currents, temperature, and other features. If 100 ng of active material are released, there will be 7.38×10^{12} molecules/cm^3 air. Actual

thresholds have not been measured for black-tailed deer. Thresholds in other mammals range from 9.0×10^3 molecules/cm^3 for butyric acid in dogs (Neuhaus, 1953) to 6.4×10^{13} for propionic acid in rats (Gruch, 1957). Dogs can detect α-ionone in concentrations from $4 \times 10^{4.5}$ to $4 \times 10^{6.5}$ molecules/cm^3 air (Moulton and Marshall, 1976). Thus, the estimated concentration of some metatarsal components can be assumed to be well above threshold. Man, a microsmate, detects fatty acids in concentrations of 7.0×10^9 to 5.0×10^{13} molecules/cm^3 air (Skramlik, 1948). Deer can be expected to be orders of magnitude more sensitive than that, especially to biologically significant odors, as the MT obviously is.

In conclusion, based on our experimental evidence thus far, we propose to classify the metatarsal odor as a type of alarm pheromone, more specifically an alert pheromone. An alarm pheromone is defined as "chemical substance exchanged among members of the same species that induces a state of alertness or alarm in the face of a common threat" (Wilson, 1975). "Alert" in turn, is defined as "watchful and prompt to meet danger," and "quick to perceive and act." As a verb, "alerting" is defined as "to call to a state of readiness (warn)," and as a noun "an alarm or other signal of danger" (Webster, 1969). Thus, it seems, both terms "alarm pheromone" and "alert pheromone" are applicable.

Alarm pheromones, in the widest sense, are chemical signals between conspecifics that induce, maintain, or enhance a state of alertness or alarm, which in turn may lead to (mostly interspecific) avoidance, escape, or defense behavior.

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IDENTIFICATION AND SOURCE OF A QUEEN-SPECIFIC CHEMICAL IN THE PHARAOH'S ANT, *Monomorium pharaonis* (L.)

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Abstract—Fertile queens of the ant *Monomorium pharaonis* produce a chemical substance which is not present in worker ants or in young, alate (virgin) queens. The material has been identified as (*E,E,E*)-1-isopropenyl-4,8,12-trimethylcyclotetradeca-3,7,11-triene (neocembrene). This chemical is produced in the Dufour's gland of fertile queens and may serve as a queen-recognition pheromone.

Key Words—Ants, Hymenoptera, Formicidae, *Monomorium pharaonis*, caste, diterpene, neocembrene, exocrine, Dufour's, pheromone, queen substance.

INTRODUCTION

In many social insects, and especially in ants, chemical signals play an important role in communication between colony members. These semiochemicals are known to mediate several aspects of social behavior including alarm and defence, trail following, and the recognition of individual colonies and of different castes within colonies (Wilson, 1971). The material initiating or promoting a particular behavioral response may be a single chemical species or a mixture of compounds in which each component releases part of the overall behavior pattern (Bradshaw et al., 1975).

Recent studies on the chemistry of the ant *Monomorium pharaonis* (L.) (subfamily Myrmicinae, tribe Solenopsidini) have resulted in the identification of several chemicals produced in the exocrine glands of this species. Ritter et al. (1973) identified 3-butyl-5-methyloctahydroindolizine (monomorine I) in the poison gland of worker ants and, subsequently, a series of

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dialkyl pyrrolidines were also identified as poison gland constituents (Talman et al., 1974; Ritter et al., 1975). These compounds, also named monomorines, appear to be part of an homologous series and the most abundant material (monomorine III) was identified as 2(5′-hexenyl)-5-pentyl pyrrolidine. Initially, these compounds were thought to be the active components of the trail pheromone but later, a much more active trail substance, (3*S*, 4*R*, 6*E*, 10*Z*)-3,4,7,11-tetramethyl-6,10-tridecadien-1-al (faranal) was identified from the Dufour's gland of workers (Ritter et al., 1977, 1981). By analogy with the proposed role of similar dialkyl pyrrolidines found in the venom of related species (e.g., *Monomorium,* Jones et al., 1982; and *Solenopsis* spp., Pedder et al., 1976; Jones et al., 1979; Blum et al., 1980) and on the basis of bioassay of synthetic monomorine I (Edwards and Pinniger, 1978) and III (Legakis, 1980), it now seems likely that the indolizine and pyrrolidine compounds may serve as alarm pheromones and defence substances involved in the foraging strategy of *M. pharaonis* described by Holldobler (1973). In addition, Ritter et al. (1975) reported a material which was found only in extracts of young, alate queens (virgins). Since this material, an unidentified bicyclic hydrocarbon ($C_{18}H_{30}$) apparently occurs only in virgin queens, it is possible that it is the attractant sex pheromone which was previously proposed by Holldobler and Wust (1973).

In *M. pharaonis,* as in some other ant species, the removal of queens from an established colony results in the rapid appearance of new sexual brood (Peacock et al., 1954). This suggests that extant queens are able to suppress the development of new queens. That this ability to inhibit the production of new queens might be mediated by chemical signals produced by extant queens was first suggested by Petersen-Braun (1975). Further evidence for the existence of this inhibitory queen substance was provided by Berndt (1977), who found that daily rinsing of fertile queens in organic solvent removed their ability to inhibit the production of new sexuals. Further investigation has revealed that virgin queens are unable to inhibit the rearing of new sexuals (Berndt and Nitschmann, 1979). Moreover, the ability of fertilized queens to inhibit sexual production is dependent upon their age, such that young queens and senile queens are less able to inhibit sexual production than mature queens (Petersen-Braun, 1975).

In this paper we present the results of a comparative study of the chemical constituents of the exocrine glands of workers and queens of *M. pharaonis* which was conducted as part of a detailed investigation of the mechanisms of caste regulation and determination in this species.

METHODS AND MATERIALS

GC Analysis of Solvent Rinses from Workers and Queens. All insects used were taken from laboratory colonies of *Monomorium pharaonis*

maintained and reared in the manner previously described by Edwards (1975). Workers and fertile queens were removed from thriving colonies. Virgin queens were obtained by removing all extant queens from colonies, thereby inducing the production of new male and queen larvae. Subsequently, queen pupae were removed from these colonies and isolated with worker ants such that the emerging adult queens had no contact with males.

Solvent rinses of workers or queens were made by gentle agitation of insects in excess diethyl ether (AnalaR) for 4 min. Preliminary experiments had indicated that rinsing for longer periods served only to increase the quantity of high-molecular-weight lipids in the extract. Insect rinses were reduced to dryness under a stream of dry N_2 at room temperature and the residues redissolved in a known volume of ether. Subsequently, aliquots of these samples were analyzed by gas–liquid chromatography (GC).

GC analyses were performed on a Varian instrument (model 3700) equipped with a flame ionization detector. Samples were run on a 1-m (2.0 mm ID) glass column containing 5% OV-101 on Diatomite CLQ (80–100 mesh). Helium was used as the carrier gas (30 ml/min), and each analysis was run over a programed temperature range (100–250°C at 10°C/min) with initial and final isothermal periods of 6 min. Injector and detector temperatures were maintained at 260°C throughout the analyses.

Preparative Gas Chromatography of Monomorium *"d."* A pure sample of GC peak d (*Monomorium* "d") from fertile queens was obtained by preparative GC using the Varian 3700 instrument equipped with a (nondestructive) thermal conductivity detector. Fertile queens (1500) were rinsed in diethyl ether and the solvent rinse reduced to approx 1 ml. From this sample, 100-μl aliquots were run on a 1-m (4 mm ID) glass column packed with 5% OV-101 on Diatomite CLQ (80–100 mesh) under isothermal conditions (190°C). During each run, the effluent corresponding to *Monomorium* "d" was trapped in an air-cooled glass tube inserted into the outlet port of the chromatograph. Subsequently, the tubes were rinsed with ether and the combined rinses, containing *Monomorium* "d," reduced to a smaller volume and stored at −20°C. In this way a sample (approx 680 μg) of pure *Monomorium* "d" was obtained for further analysis.

Analysis of Chemical Structures. All glassware was heated overnight at 510°C in a muffle oven. All solvents were fractionally distilled and checked for purity by GC which was generally performed on a Varian 3700 instrument with helium as the carrier gas.

Analysis by combined gas chromatography–mass spectroscopy (GC-MS) of peaks from fertile queen rinses was conducted on a VG Micromass 12B instrument by electron impact at 30 eV with source oven temperature of 190°C. Separation of peaks for MS was accomplished using a 1-m (2 mm ID) glass column packed with 5% OV-101 on Chromosorb W-HP, 100–120 mesh, 40–200°C at 2°C/min. High-resolution MS of a sample of purified

Monomorium "d" was performed on a VG ZAB-IF instrument by electron impact at 70 eV with source oven temperature at 210°C, and the nuclear magnetic resonance (NMR) spectrum of this compound was recorded on a JEOL JNM PS 100 spectrometer as a $CDCl_3$ solution with Me_4Si ($\delta = 0.00$ ppm) as internal standard. Other NMR measurements were similarly conducted on a Varian EM 360 spectrometer.

Perhydrogenation of Monomorium *"d."* A sample (100 μg) of purified *Monomorium* "d" in ethanol (1 ml; absolute, distilled from 2,4-dinitrophenylhydrazine) was stirred magnetically at 20°C with platinum (IV) oxide (1.5 mg) in a flask attached to a rubber balloon (party grade, W.H. Smith & Son Ltd) filled with hydrogen, and the reduction monitored by capillary GC (split injection, 25 m OV-101 at 190°C). After 30 min, the peak at 10.8 min due to *Monomorium* "d" had been replaced by four peaks with similar GC retention times. Filtration gave a solution which was subsequently analyzed by capillary GC-MS (splitless injection at 50°C, 22 m OV-101, rapid rise to 160°C, VG ZAB-IF, electron impact at 70 eV).

Synthesis of Neocembrene. For purposes of comparison, a synthetic sample of neocembrene was prepared in the following way from a precursor alcohol kindly supplied by Prof. T Kato, Tohoku University, Sendai 980, Japan.

A mixture of alcohol Va (118 mg), acetic anhydride (fractionally distilled bp 134–138°C; 1.6 ml) and pyridine (distilled from KOH, bp 112–114°C; 4 ml) was kept at 20°C for 16 hr, then poured onto ice, and extracted with Et_2O (4 × 12.5 ml). Washing with aqueous $CuSO_4$ (3 × 15 ml) and brine (3 × 15 ml), drying (Na_2SO_4), and removal of solvent, gave acetate Vb as a sharp colorless oil (108 mg); R_f ($CHCl_3$ on SiO_2) 0.66 (cf alcohol Va 0.45); ν_{max} (CCl_4) 2930, 1736, 1442, 1375, 1241, 1022, and 897 cm^{-1} (Perkin-Elmer 177 spectrophotometer with polystyrene as external standard); δ5.70–4.57 (m, 6H, 3 × =CH, 1 × $=CH_2$, 1 × O−CH−C=), 2.70–1.80 (m, 11H, 5 × CH_2−C=, 1 × CH−C=), 1.97 (s, 3H, 1 × CH_3OCO), 1.70 (m, 6H, 2 × CH_3−C=), 1.55 (m, 6H, 2 × CH_3−C=), and 1.80–1.30 (m, 2H, 1 × CH_2). In a repeat experiment, alcohol Va (117 mg) gave acetate Vb (103 mg).

Argon ($CaCl_2$ dried; 20 ml/min) was introduced to a 25-ml round-bottom flask through the top socket of a stillhead. The side arm of the stillhead was connected to the angled side neck of a 50-ml two-neck pear-shape flask. This was equipped with an empty angled drying tube whose outer end was closed with a bung which held the argon outlet tube. The apparatus was placed in a dry box and loaded with ethylamine (10 ml) and KOH pellets (ca. 5 g) in the round-bottom flask, acetate Vb (83 mg) in the pear-shape flask, and lithium (freshly pressed into wire of 0.5 mm diameter; 38 mg) in the drying tube. The pear-shape flask was cooled in acetone–Dry Ice and, when distillation of the ethylamine was virtually complete (30 min),

the apparatus was tilted to admit the lithium wire to the pear-shaped flask. The mixture was then stirred magnetically as vigorously as possible, and after 6 min it turned deep blue. After a further 10 min, NH_4Cl (1 g) was added, and the mixture was allowed to warm to room temperature. Addition of H_2O (15 ml), extraction into Et_2O (4 × 15 ml), washing with brine (4 × 15 ml), drying ($MgSO_4$), and removal of solvent gave a colorless oil.

Chromatography on SiO_2 (Reeve Angel type SO.TLC; 12.4 g) with hexane under a small positive pressure of N_2 gave two fractions. GC-MS (1 m × 2 mm ID, 5% OV-17 on Supelcoport 100–120 at 180° C; electron impact at 70eV) showed the first fraction (19 mg) to contain four materials, of which the two major components had M^+ 274, and the second fraction (38 mg) to contain four materials, of which the two major components had M^+ 272 and one minor component had M^+ 274. Further chromatography of the second fraction on SiO_2 (46g) with hexane gave a fraction (24mg) containing only two materials, both with M^+ 272. These were separated by preparative GC (2 m × 4 mm ID, 5% OV-101 on Diatomite CLQ 80–100 at 180° C with thermal conductivity detector). The major fraction (17 mg) was identified as neocembrene (I), GC (split injection, 25 m OV-17 at 200° C) 9.4 min, δ 5.30–4.80 (m, 3H, 3 × =CH), 4.61 (m, 2H, 1 × $=CH_2$), 2.30–1.75 (m, 13H, 6 × CH_2—C= and 1 × CH—C=), 1.64 (m, 3H, 1 × CH_3—C=), 1.55 (m, 9H, 3 × CH_3—C=), and 1.55–1.40 (m, 2H, 1 × CH_2). The minor fraction (4 mg) remained as an unidentified isomer of neocembrene, GC 8.2 min, which gave an NMR spectrum resembling that of neocembrene but with additional signals at δ 5.67 (d of d, $J = 4.5$ and 16 Hz, 1H?, 1 × C<u>H</u>=CH *trans*) and 1.02 (d, $J = 7$Hz, 6H?, 1 × ($C\underline{H}_3$)$_2$CH). GC-MS (conditions as before) of the two materials gave the data shown in Table 1. In a repeat preparation, acetate Vb (112 mg) gave neocembrene (20 mg) and the minor isomer (5 mg).

Location of Source of Neocembrene in Fertile Queens. Two hundred fertile queens were cut into three major body portions (head, thorax, and gaster) and ether rinses of each portion were analyzed by GC (1 m × 2 mm ID, OV-101, 100–250° C at 10° C/min). Subsequently, analysis of the constituents of poison glands and Dufour's glands dissected from fertile queens was conducted using GC and GC-MS. For GC, five individual glands were combined and extracted in 50 μl diethyl ether from which 10-μl aliquots were removed for analysis. For GC-MS, single dissected glands were introduced into the mass spectrometer (VG Micromass 12B) on the direct insertion probe.

Relative Abundance of Neocembrene during Queen's Lifetime. A large laboratory colony of *M. pharaonis* was induced to produce a new batch of sexuals (males and queens) by the removal of all extant queens. Following the appearance of winged, adult, sexuals in the colony, randomly selected

TABLE 1. MASS SPECTRA OF *MONOMORIUM* "d," NEOCEMBRENE-A (I), SYNTHESIZED NEOCEMBRENE (I), AND A MINOR ISOMER

m/z	Monomorium "d" (%)	Neocembrene-A (I, %) (Birch et al., 1972)	Synthesized neocembrene (%)	Synthesized minor isomer (%)
272	15	38	11	14
257	13	24	12	14
189	14	15	8	20
161	13	18	10	38
147	17	20	13	28
135	15	26	17	26
133	19	23	17	44
121	49	55	44	48
119	20	23	23	55
107	46	53	46	79
105	23	27	17	46
93	60	73	67	100
81	51	64	63	89
79	32	25	33	56
69	6	27	32	39
68	100	100	100	26
55	36	33	40	73

batches of 10 dealate queens were removed at intervals during the sexual brood cycle, spanning the development of queens from fertilization through maturity to senility. Each batch of 10 queens was rinsed in 4 ml redistilled methylene chloride for exactly 4 min, and the solvent reduced to dryness (N_2 at 25° C). Subsequently, the residue was stored under nitrogen in a sealed glass ampoule at −20° C until all samples were obtained. For analysis, residues were redissolved in 50 μl diethyl ether from which 5-μl samples were analyzed by GC as described above. Relative quantities of neocembrene and monomorine III in these samples were calculated by cutting out and weighing the peaks corresponding to the two materials. Peaks obtained from a standard solution of eicosane, $C_{20}H_{42}$, (100 ng/μl) were used to obtain an approximate indication of the absolute quantity of neocembrene present in each sample.

RESULTS AND DISCUSSION

GC Analysis of Ether Rinses From Workers and Queens. Figure 1 shows the chromatograms obtained from GC analysis of the various female types. A pattern of five peaks was found to occur consistently in all samples (peaks a, a^1, a^2, b, and c). In addition, an extra peak (c^1) occurred only in

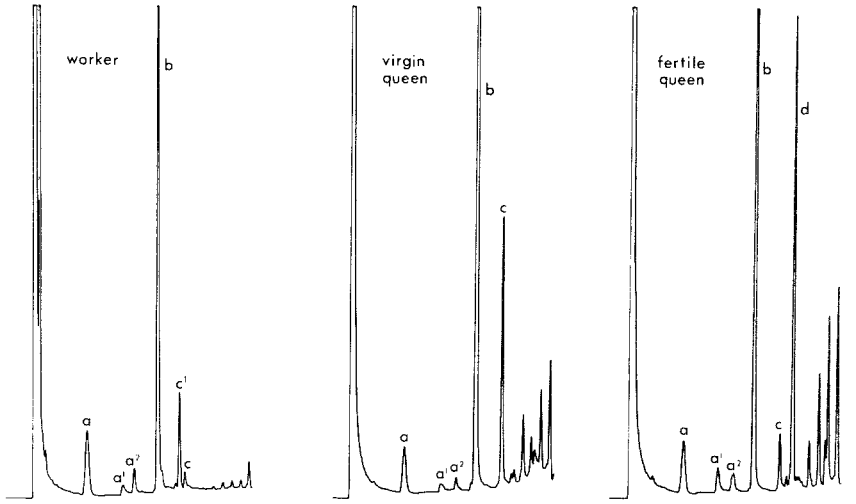

FIG. 1. Gas–liquid chromatograms of ether rinses of fertile queens, young alate (virgin) queens, and workers of *Monomorium pharaonis*.

worker rinses, and another (*Monomorium* "d") was found only in rinses of fertile queens (Figure 1).

Comparison of the retention times of GC peaks a and b with those of synthetic monomorine I and monomorine III (kindly supplied by Dr. P. Sonnet, USDA, Gainesville, Florida, and Dr. J. Newton, Rentokil Ltd., U.K., respectively) suggested that these materials were identical to peaks a and b. This inference was confirmed by mass spectrometric analysis of peaks in fertile queen rinses, peak a giving ions at m/z 138 (base peak), 180, and 195 (molecular ion) and peak b giving ions at m/z 140 (base peak), 152, and 223 (molecular ion). In both cases, the patterns and intensities of the fragmentation ions were identical with those obtained from synthetic samples of monomorine I and III respectively.

Comparison of the retention time of peak c^1 from worker rinses with that of synthetic faranal (kindly supplied by Prof. R. Baker, Southampton, U.K.) also suggested that these materials may have been identical. Mass spectrometric analysis of GC peak a^1 from fertile queen rinses gave ions at m/z 126 (base peak), 140, and 197 which were consistent with the structure of monomorine II, 2-butyl-5-hexenyl pyrrolidine (Talman et al., 1974). Similarly, MS analysis of peak c from fertile queen rinses gave ions at m/z 152 (base peak), 168, and 251 (molecular ion) consistent with the expected fragmentation pattern of monomorine IV, 2-hexenyl-5-heptyl pyrrolidine (Ritter et al., 1975). Thus, on the basis of these data, we were able to assign chemical structures of previously identified materials to the majority of

peaks present in rinses. Peak a[2] (present in all rinses) gave ions at m/z 220 and 205. These data suggested that this material was not one of the previously identified monomorines, but we did not characterize it further. One major GC peak (*Monomorium* "d," found only in fertile queen rinses) gave a retention time and mass spectrometric fragmentation pattern which was not consistent with the parameters indicating indolizine or pyrrolidine compounds and did not correspond to any previously identified compound in this species.

Identification of Chemical Structure of Monomorium *"d" from Fertile Queen Rinses.* The mass spectrum (30 eV) of *Monomorium* "d" in fertile queen rinses gave ions at m/z 68 (100), 81 (64), 93 (94), 107(76), 121 (78), 257 (38), and 272 (52). MS by chemical ionization confirmed m/z 272 as the molecular ion. The fragmentation pattern (Figure 2) suggested that the compound might be a terpenoid, although it had a relatively unusual base peak at m/z 68. GC-MS (70 eV) of a purified sample of *Monomorium* "d" at high resolution (10,000) gave m/z 272.2513 (15%) for the molecular ion, confirming the elemental composition as $C_{20}H_{32}$ (272.2504) and showing that the sum of the rings and double bonds is 5. Other peaks were as given in Table 1.

The Fourier transformed NMR spectrum of *Monomorium* "d" gave signals at δ 5.29–4.89 (m, 3H, 3 × =CH), 4.68 (m, 2H, 1 × $=CH_2$), 2.25–1.75 (m, 13H, 6 × CH_2–C= and 1 × CH–C=), 1.66 (m, 3H, 1 × CH_3–C=), 1.56 (m, 9H, 3 × CH_3–C=), and 1.55–1.40 (m, 2H, 1 × CH_2), and was consistent

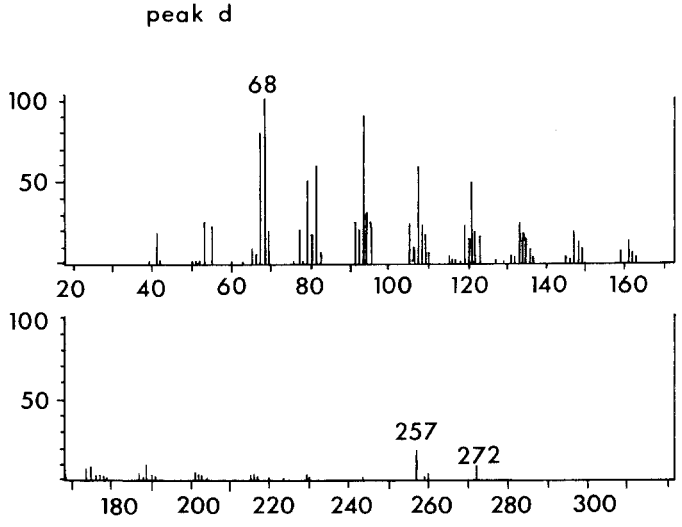

FIG. 2. Mass spectrum of *Monomorium* "d" from fertile queen rinses.

with a diterpene hydrocarbon. Signals were attributable to five olefinic protons (the two at δ 4.68 being geminal), six methylene groups, and one methine proton, each being singly allylic, and four allylic methyl groups, which together implied that *Monomorium* "d" has four double bonds and therefore one ring. To complete the assignments, there was a single nonallylic methylene group. The NMR evidence accounts for all 20 carbon atoms and means that, apart from any branching which there might be at the eight olefinic carbon atoms, there is only one other branching point, the carbon atom with the single methine proton.

Perhydrogenation of *Monomorium* "d" gave a mixture of four materials (f, g, h, and j), with similar GC retention times and similar MS data (Table 2). Although the molecular ion of one minor material, f, was not obvious, the other three materials all had M^+ 280, and there was no evidence for any unsaturation remaining. This confirmed that *Monomorium* "d" had four double bonds. Moreover, all four materials gave large peaks at m/z 236 and 237, suggesting that loss of a C_3 fragment is significant. In all probability this would have occurred by loss of a side chain from the ring through cleavage at the branching point. This would have to be the carbon atom with the single methine proton since this could not be substituted with any other length of side chain, for which there was no mass spectral evidence, nor even a methyl group, because in *Monomorium* "d" they are all allylic. This point also requires that the C_3 side chain in *Monomorium* "d" is

TABLE 2. MASS SPECTRA OF PERHYDROGENATION PRODUCTS OF *MONOMORIUM* "d" (f, g, h, and j), OCTAHYDROCEMBRENE, AND AN ANALOG WITH A SIX-MEMBERED RING

	Perhydrogenated products of *Monomorium* "d" (%)				Octahydrocembrene (%) (Birch et al., 1972)	Six-membered ring analog (Birch et al., 1972)
m/z	f (minor)	g (shoulder)	h (major)	j (minor)		
280		2	3	2	6	9
237	49	51	57	52	55	
236	88	86	89	71	78	
125	30	32	34	32	25	19
111	55	58	61	57	49	
97	78	81	86	82	73	100
96	8	9	10	8	10	83
85	28	29	32	32	27	10
83	67	72	75	71	69	14
71	44	48	50	53	47	21
69	91	92	95	92	88	33
57	80	82	83	88	84	40
55	100	100	100	100	100	62

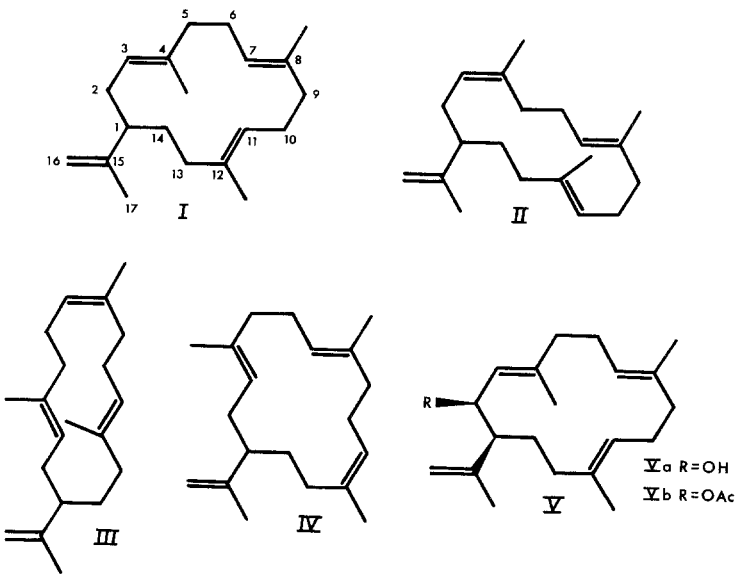

FIG. 3. Chemical structures. I: (*E,E,E*)-1-isopropenyl-4,8,12-trimethylcyclotetradeca-3,7,11-triene, neocembrene (Shmidt et al., 1970), cembrene-A (Patil et al., 1973), neocembrene-S (Birch et al., 1973). II: (*Z,Z,Z*)—cembrene-A (Wiemer et al., 1979). III: (*E,Z,E*)-neocembrene (Kato et al., 1980). IV: (*E,E,Z*)-1-isopropenyl-4,8,12-trimethylcyclotetradeca-3,3,11-triene. Vb: 2-acetoxy-(*E,E,E*)-1-isopropenyl-4,8,12-trimethylcyclotetradeca-3,7,11-triene.

unsaturated. The two propenyl possibilities are excluded because there is no NMR evidence for a coupling between vicinal olefinic protons, therefore the side chain could only be isopropenyl. The remaining C_{17} fragment of *Monomorium* "d" could then consist only of three isopentylidene units, the nonallylic methylene group and the carbon atom with the single methine proton which together would form a 14-membered ring.

A search of the literature for monocyclic compounds of molecular formula $C_{20}H_{32}$ with MS and NMR characteristics similar to those of *Monomorium* "d" revealed a single suitable structure (Figure 3, I). This structure had been assigned almost simultaneously by three independent groups to natural products from four sources, although none of the groups determined the stereochemistry about the double bonds in the ring. Shmidt et al. (1970) identified it as an oleoresin component of *Picea obovata* (Ledb.) (Siberian spruce) and *Pinus koraensis* (Sieb Zucc.) (Korean pine) and called it neocembrene. Patil et al. (1973) found it in the gum resin of *Commiphora mukul* (Hook, ex Stocks) Engl. (syn. *Balsamodendron mukul* Hook, ex Stocks), named it cembrene-A, and added that it was the same as neocembrene. Birch et al. (1972) assigned the structure to a material which had been

identified by Moore (1966) as the trail pheromone of *Nasutitermes exitiosus* (Hill). They called it neocembrene-A but mentioned that it was identical with cembrene-A. The geometrical isomerism in the ring was established by Kodama et al. (1975), who showed, by comparison of the natural product with synthetic materials, that all three double bonds have the *trans* or *E* configuration. Subsequently, Wiemer et al. (1979) found cembrene-A and its 3*Z* isomer (II) in the termite *Cubitermes umbratus* (Williams). Interestingly, neocembrene is also the active principal of guggulu, an ayurvedic drug, reputed to have aphrodisiac properties and to cure a range of ailments including ulcers, leprosy, and hemorrhoids (Dash and Kashyap, 1980).

For comparison with *Monomorium* "d," the MS data reported by Birch et al. (1972) for neocembrene-A are given in Table 1. Despite the inevitable differences which arise from the use of different mass spectrometers, it will be seen that there is close agreement between the spectra and that the two materials share the same uncommon base peak at m/z 68. Moreover, the same authors reported that the perhydro derivative of neocembrene-A can exist in four diastereoisomeric forms. This would account for the four materials, observed on perhydrogenation of *Monomorium* "d." The MS data for the four hydrogenation products closely resemble those reported for what was called octahydrocembrene, whereas there is little similarity with a six-membered ring analog, 2,6-dimethyl-10-(4-methylcyclohexyl)undecane, which had been synthesized (Table 2). As expected, this latter compound had no reported peaks at m/z 236 and 237, but the base peak was at 97, corresponding to cleavage of the side chain at the branching point. It is not clear why, for all the other compounds in Table 2, the peak at m/z 236 should be more intense than that at 237. However, it seems not to be a specific property of this molecule alone since the base peak of the sesterterpene analog with a 14-membered ring but a longer side chain occurs a m/z 236 while there is no reported peak at 237 (Miyamoto et al., 1979).

The NMR data for *Monomorium* "d" agree reasonably well with those reported by Wiemer et al. (1979) for cembrene-A. Moreover, both have one allylic methyl group at about 1.67δ and three at about 1.57δ, whereas the 3*Z* isomer (II) (Wiemer et al., 1979) and the other two isomers with one double bond in the *Z* configuration (III and IV) (Kato et al., 1980) have two near 1.67δ and two near 1.57δ. This strongly suggests that *Monomorium* "d," like cembrene-A, is the *E,E,E* isomer (I).

In view of the limited amount available, it was not possible to establish the optical purity of *Monomorium* "d," although it is not yet known how important this would be for any biological activity in *M. pharaonis*. For example Kato et al. (1980) reported that for trail-laying activity in *N. exitiosus*, the resolved optical isomers showed similar high activity and that optical was less important than geometrical isomeric purity. On the other

hand, worker *M. pharaonis* do discriminate between enantiomers of faranal (Kobayashi et al., 1980).

For comparison with *Monomorium* "d," neocembrene (I) was synthesized from alcohol Va via acetate Vb. The route adopted was similar to that outlined by Kato et al. (1980) for the isomers III and IV. After gaining experience with model compounds, modifications were made to the conditions for the reduction step with acetate Vb which were as described in Methods and Materials. Nevertheless the resultant solution of required product contained several other materials and a two-stage purification was necessary because one material with M^+ 274 was not separable from the neocembrene by preparative GC while another, with M^+ 272, was not separable by column chromatography alone. A pure sample of this minor isomer was isolated, and the MS data obtained are shown in Table 1, but it was not characterized fully.

Comparison of *Monomorium* "d" with the synthesized neocembrene by packed column GC (2 m × 2 mm ID, 15% Carbowax 20 M on Chromosorb W-HP 100–120 at 200°C) gave: *Monomorium* "d" 7.12 min, neocembrene 7.10 min, and 1:1 mixture 7.10 min. Comparison by capillary GC (splitless injection, 25 m OV-17, 40° at 20°/min to 220°C) gave: *Monomorium* "d" 13.73 min, neocembrene 13.75 min and 1:1 mixture 13.70 min, suggesting that these materials were identical.

Comparison of *Monomorium* "d" with the synthesized neocembrene by MS (Table 1) and by NMR confirmed that *Monomorium* "d" has structure I.

Source of Neocembrene in Fertile Queens. Figure 4 shows the chromatograms obtained from GLC analysis of ether rinses of the major body portions of fertile queens. These results indicated that the gaster was the primary source of all compounds previously identified in whole body rinses. GLC analysis (Figure 5) of extracts from the two major exocrine glands associated with this portion of the body (poison gland and Dufour's gland) indicated that neocembrene was present only in the Dufour's gland. Mass spectroscopy of a single poison gland by direct insertion gave ions at m/z 168, 152, 140, 97, 85, 71, and 57, indicating the presence of monomorines. Subsequent insertion of a single Dufour's gland gave all the signals observed with the poison gland at low intensity, and further peaks at m/z 272, 257, 121, 93, 81, and 68, indicating the presence of neocembrene. From these data we conclude that the Dufour's gland is the source of neocembrene in fertile queens.

Relative Abundance of Neocembrene during the Queen's Lifetime. Table 3 presents the results of GLC analysis of the quantity of neocembrene present in random samples of fertile queens at different periods during their lifetimes. The results show that the quantity of extractable neocembrene per

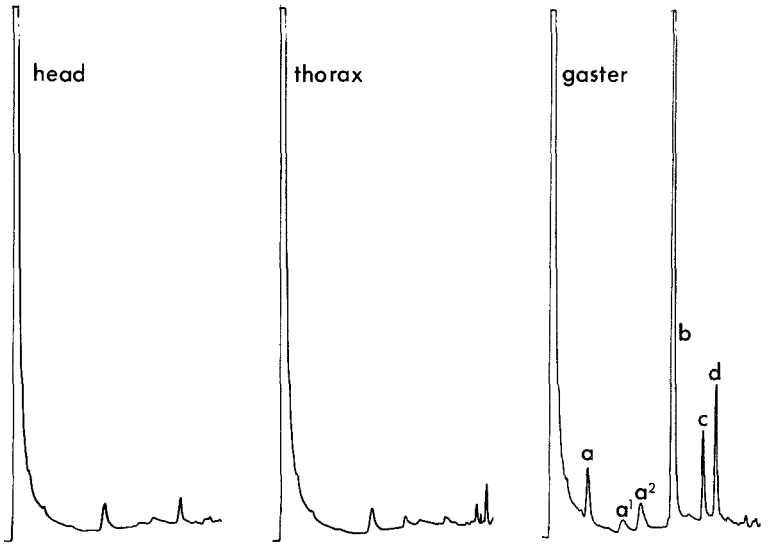

FIG. 4. Gas–liquid chromatograms of ether rinses of head, thorax, and gaster of fertile queen *Monomorium pharaonis*.

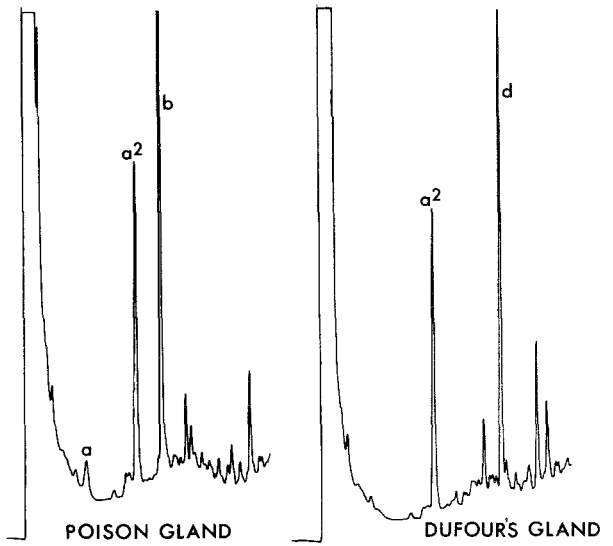

FIG. 5. Gas–liquid chromatograms of ether extracts of poison glands and Dufour's glands from fertile queens of *M. pharaonis*.

TABLE 3. RELATIVE QUANTITIES (GC PEAK WEIGHT) OF MONOMORINE III AND NEOCEMBRENE PRESENT IN QUEENS OF DIFFERENT AGES

Queen age (weeks after sexual production)	Weight (mg) of GLC peaks		Estimated quantity of neocembrene/queen (ng/insect)
	peak b (monomorine III)	peak d (neocembrene)	
1[a]	38.74	3.52	78.2
3[a]	54.22	11.81	262.4
6[a]	63.45	25.46	565.7
10	49.61	24.42	542.6
15	58.52	34.36	763.5
20	48.50	31.42	698.2
30	29.00	24.69	548.6
50	45.68	30.94	687.5
60	40.90	24.80	551.1
70[b]	43.09	28.67	637.1

[a]Winged queens present in nest.
[b]New sexual brood in nest.

queen increases during the first six weeks of adult life, but thereafter remains relatively constant. By contrast, no age-related changes in the relative amounts of monomorine III present in queens were apparent (Table 3). From a comparison of the neocembrene peak weights with those obtained with a standard solution of eicosane ($C_{20}H_{42}$), we have estimated the absolute quantities of neocembrene in mature queens (Table 3) to be approximately 600 ng/insect.

General Discussion. The existence of neocembrene in a specific caste of the ant *Monomorium pharaonis,* and its association with a specific exocrine organ, suggests that this material has a specific role as a semiochemical within the social organization of the species. Furthermore, the apparent absence of the material in young, alate (virgin) queens, indicates that the role of neocembrene may be associated with the differences between virgin queens and fertile queens. Since mature, fertile queens are able to suppress the development of new sexual forms (males and queens), and since this ability is lacking in virgin queens (Berndt and Nitschmann, 1979), it is possible that neocembrene, which is present in fertile queens and absent in young unfertilized queens, might be responsible for the inhibitory effect of fertile queens. Thus, neocembrene might act in a way analagous to that of the queen substance of the honeybee *Apis mellifera,* which has been identified as 9-oxodec-*trans*-2-enoic acid (Butler et al., 1962) and which inhibits the rearing of new queens (Butler, 1961). However, changes in the quantities of neocembrene per queen during her lifetime are inconsistent

with the possibility that the material acts as an "inhibitory pheromone." This is because, although both juvenile and senile fertile queens are much less able than mature fertile queens to inhibit the development of sexuals in the nest, only juvenile queens appear to have a low level of neocembrene. Moreover, the inhibitory pheromone proposed by Petersen-Braun (1975) is thought to be evenly distributed over the entire body surface of queens (Berndt, 1977). However, as we have demonstrated, neocembrene is found only in or on the gaster and, because the material is present in the Dufour's gland, is probably dispensed via the sting.

At present we are conducting more detailed investigations into the role of neocembrene in the social biology of pharaoh's ant. However, preliminary investigations have revealed that neocembrene is highly attractive to worker ants (Edwards, unpublished results). Queens themselves are highly attractive to workers, and fertile queens are more attractive to workers than are virgin queens. The latter spend much less time in the vicinity of the nest and are sometimes attacked by workers and by fertile queens (Edwards, unpublished results). On the basis of these preliminary studies, we suggest that neocembrene may serve as a queen-recognition substance which enables workers to identify fertile (i.e., reproductively active) queens from other female colony members. Such queen-recognition pheromones have been found in other ant species (e.g., *Myrmica rubra* L., Brian, 1973; *Camponotus pennsylvanicus* De Geer, Fowler and Roberts, 1982; *Lasius alienus* Foerster and *Pheidole pallidula* Nylander, Stumper, 1956) and recently have been reported (Jouvenaz et al., 1974) and identified (Rocca et al., 1983a, b) as poison gland constituents of queens of the related Myrmicine *Solenopsis invicta* Buren (Van der Meer et al., 1980).

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CHARACTERIZATION OF AND MALE ADAPTATION TO PHEROMONE OF FEMALE *Trichostrongylus colubriformis* (NEMATODA)[1]

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Abstract—Males of the ruminant nematode *Trichostrongylus colubriformis* were significantly attracted to an incubate from their females during in vitro assay. Exposure of males to sera from uninfected and infected animals or selected neurotransmitters had no effect on their subsequent responsiveness to the female's pheromone. In contrast, exposure to 1500 female-hours of pheromone decreased male responsiveness after moderate rinsing prior to bioassay. Rinsing of the males with larger volumes of Tyrode's solution prior to in vitro assay increased their subsequent response to the female's pheromone. High-performance liquid chromatography yielded a presumptive pheromone peak with a fivefold increase in biological activity. This peak was soluble in alcohols and tetrahydrofuran, based on elution from reverse-phase Sep-Pak cartridges. Stability of the peak was increased by EDTA or ascorbic acid (10 mM). Storage for six weeks in ascorbic acid at 4°C allowed recovery of 47.3% of the original material.

Key Words—Pheromone communication, nematode reproduction, Nematoda, *Trichostrongylus colubriformis*, biological control, pheromone solubility, pheromone stability.

INTRODUCTION

Sexual communication by pheromones has been reported for five species of zooparasitic nematodes through in vivo or in vitro assays (Bone, 1982).

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Based on the available literature, the behavior of *Nippostrongylus brasiliensis* and *Trichinella spiralis* is the most studied area. However, some information about pheromone chemistry is available. The pheromone from females of mouse-adapted *N. brasiliensis* was composed of at least two compounds of different molecular weight and solubility (Bone et al., 1979; Ward and Bone, 1983). Only limited stability was reported. Behaviorally, exposure of males of *N. brasiliensis* to the female's pheromone disrupted their subsequent ability to locate females during in vivo and in vitro tests (Bone and Shorey, 1977; Glassburg et al., 1981). Thus, the conceptual use of a pheromone-based scheme of biological control seems feasible with appropriate advances in our understanding of the chemistry of the active compounds and mechanism of pheromone reception.

In contrast, no information is available regarding communication between the sexes of nematodes that infect livestock since most reports have concerned more readily studied laboratory models. Various trichostrongylid helminths are economically important parasites of several ruminants. The taxonomic position of *N. brasiliensis* suggests that our knowledge of its pheromone system may prove applicable to these trichostrongylids. Continued progress in the investigations of the pheromones of *N. brasiliensis* may rapidly expand our understanding of more important parasites, such as *T. colubriformis*. Accordingly, studies were initiated on selected behavioral and chemical aspects of the pheromone system of the ruminant nematode *Trichostrongylus colubriformis*.

METHODS AND MATERIALS

Trichostrongylus colubriformis was maintained in mixed-breed, male goats. Animals were held in a pen with a concrete floor that was disinfected daily. Infected and worm-free animals were housed separately. Hosts weighed about 20 kg at infection and were sacrificed at 21 days postinfection for recovery of adult helminths.

The responses of male nematodes to dosages of pheromone from the female were determined by in vitro assay. Procedures for bioassay were similar to previous methods (Papademetriou and Bone, 1983). Females were collected and held in Tyrode's solution at 37°C. Biological activity of this incubate was expressed as female-hours/unit volume (Bone et al., 1978). Various dosages of female-hours were placed on filter paper circles in an agar-coated Petri dish. A volume of 20 μl was used. Then, three males were placed equidistantly in the dish and 3 cm from the pheromone source. Attraction was determined as the percentage of males that contacted the

filter paper circles after 24 hr at 37°C. Sixty replicates of the male's response were done for each dosage of pheromone.

Males were exposed to selected chemical stimuli for 2 hr at 37°C to evaluate their subsequent response to female-hours of pheromone during in vitro assay. Males were held in normal or immune sera that was taken from uninfected or 90-day postinfection animals, respectively, to determine effects of host antibody on the male's response to pheromone. Concentrations of tested sera were 50 and 100%. Similarly, the biogenic amines, octopamine and serotonin, were examined at dosages from 10^{-7} to 10^{-2} M, based on the attraction of male *N. brasiliensis* to these compounds (Ward et al., 1984). Males of *T. colubriformis* were maintained also in a pheromone solution of 1500 female-hours prior to assay of their subsequent response to female pheromone. Males in the above studies were rinsed in three 10-ml volumes of Tyrode's solution prior to bioassay. Additionally, other males that were incubated in female pheromone were rinsed in three volumes of 100 ml to ascertain the effect of excessive rinsing on their response.

After determination of dose–response data for the crude incubate from females, this solution was separated by high-performance liquid chromatography (HPLC). The incubate was fractionated by reverse-phase chromatography with an automated high-performance liquid chromatograph system (Waters Associates). The mobile phase was water–methanol (95:5 v/v). Separation procedures were similar to those of Ward and Bone (1983). A peak(s) with an average retention time of 4.5 min, depending on column conditions, was collected manually for subsequent bioassay. Various dosages were assayed to determine the male's response. Sixty replicates were done for each dosage.

The peak from HPLC was tested also for solubility and stability after storage. Solubility was examined by retention on a C-18 Sep-Pak cartridge (Waters Associates), according to Ward and Bone (1983), and subsequent elution with selected solutions. Recovery of the peak was determined by the resultant absorbance value during HPLC and bioassay of the recovered material after drying by nitrogen evaporation and addition of Tyrode's solution. Bioassays were conducted when the absorbancy indicated sufficient recovery of material. Stability was investigated by storage for 48 hr at 4°C in water, methanol, and dried under nitrogen. Additionally, aqueous samples were held for 48 hr at 20°C with mercaptoethanol (1 mM), dithiothreitol (1 mM), ascorbic acid (10 mM), EDTA (10 mM), and sucrose (0.1 M). HPLC absorbance and bioassay were used to evaluate remaining activity.

Data were analyzed by linear regression, and Student's t test. The 0.05 probability level was considered significant.

RESULTS

The responses of male *T. colubriformis* to dosages of female-hours of incubate are shown in Figure 1. Male responses increased linearly ($r = 0.84$) as the pheromone dose was raised from 58 to 543 female-hours. Dosages above 100 female-hours elicited responsiveness by males that was signifi-

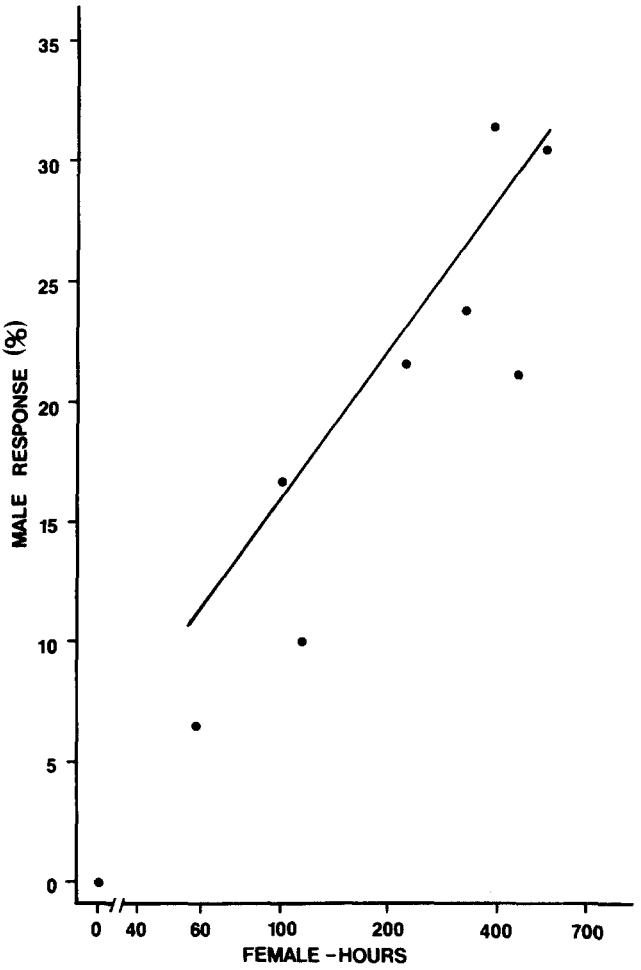

FIG. 1. Response of male *T. colubriformis* by in vitro assay to dosages of female pheromone, expressed as female-hours ($r = 0.84$, MSE = 3.36).

cantly different from zero. The mean response for all dosages was 20.3% in comparison to zero for the Tyrode's solution.

Table 1 gives the responses of male *T. colubriformis* to female-hours of pheromone after preexposure of the males to the indicated chemical stimuli. Sera from infected and uninfected animals had no effect on the male's response. The tested biogenic amines also caused no change. In contrast, exposure of the males to 1500 female-hours of incubate significantly reduced their responsiveness after moderate rinsing ($p \leq 0.05$). However, their response was restored to an enhanced level after rinsing with 300 ml of Tyrode's solution ($P \leq 0.05$).

HPLC methodology enabled separation of incubate that was derived from females of *T. colubriformis* (Figure 2). Based on previous data from another helminth, *Nippostrongylus brasiliensis,* a peak(s) with a 4- to 5-min retention time (4.05 as shown in Figure 2) was selected for bioassay.

Male responses to this HPLC peak are given in Figure 3. The responsiveness of the males increased linearly ($r = 0.88$) as the dose of female-hours was elevated from 10 to 114. Comparison of the male responses to crude versus HPLC pheromone indicated that a five-fold increase in biological activity was obtained by HPLC. Bioassay of absorbance units of pheromone rather than female-hours yielded responses by males that were more linear ($r = 0.94$).

Solubility of the pheromone peak from HPLC is given in Table 2, based on recovery of absorbance units. Methanol and, to a lesser extent, other alcohols recovered more of the absorbant material than did the other solvents. Low recoveries from some organic solvents prohibited bioassay.

TABLE 1. EFFECT OF PREEXPOSURE TO VARIOUS CHEMICAL STIMULI ON SUBSEQUENT RESPONSE OF MALE *T. colubriformis* TO FEMALE PHEROMONE

Preexposure treatment of males	Response (%)	Change (%)[a]
None	39.2 (± 3.4)	
Uninfected goat sera	40.6	+ 3.7
Infected goat sera	33.3	− 15.1
Serotonin	43.3	+ 10.4
Octopamine	36.6	− 6.6
Pheromone (1500 female-hours) (rinsed 3× 10 ml)	3.0	− 92.3
Pheromone (1500 female-hours) (rinsed 3× 100 ml)	91.7	+ 133.9

[a]Percent change in response versus the expected response of male, predicted by regression analysis, to dosages of female pheromone.

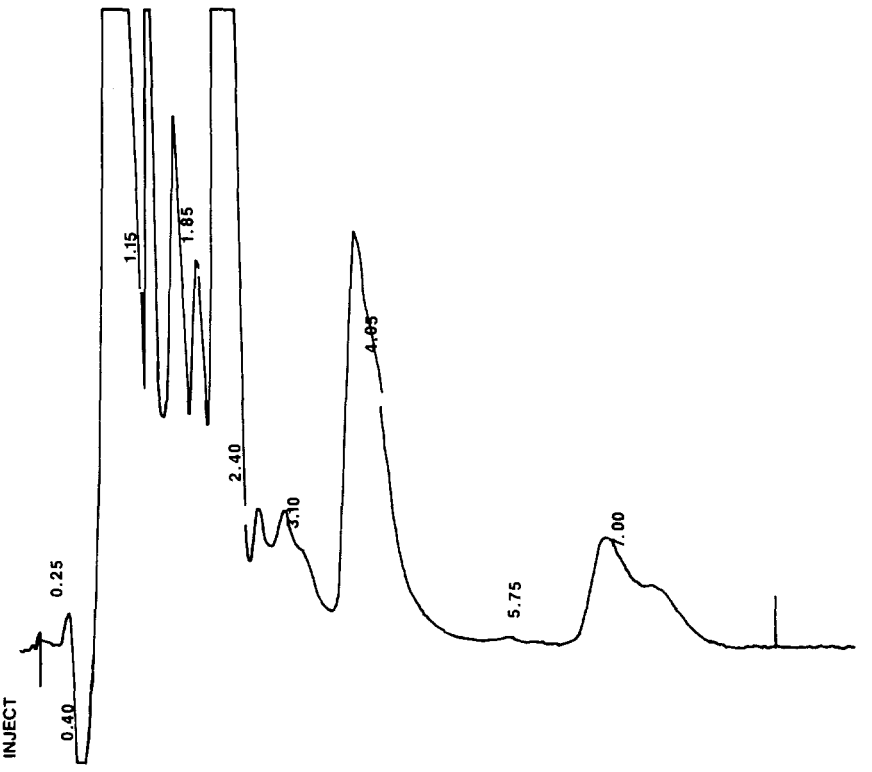

FIG. 2. Representative HPLC separation of incubate from female *T. colubriformis* on a reverse-phase column that yielded peaks with the indicated retention times at 3 ml/min flow rate.

However, the biological activity that was recovered by elution with methanol, ethanol, butanol, and tetrahydrofuran was not significantly different from that predicted by regression analysis for calculated dosages of absorbance units ($P \geq 0.05$).

Table 3 shows the recovery of the pheromone peak after various treatments for stability. More material was found after aqueous storage for 48 hr at 4°C than after methanol or dry storage. However, the antioxidants ascorbic acid and EDTA yielded more stability at 20°C. Based on these results, a sample of the pheromone peak was stored in 10 mM ascorbic acid at 4°C for five weeks; recovered absorbance indicated that 47% of the material remained. Male responses were 53% of those expected, based on prediction from regression analysis for a calculated dose of absorbance

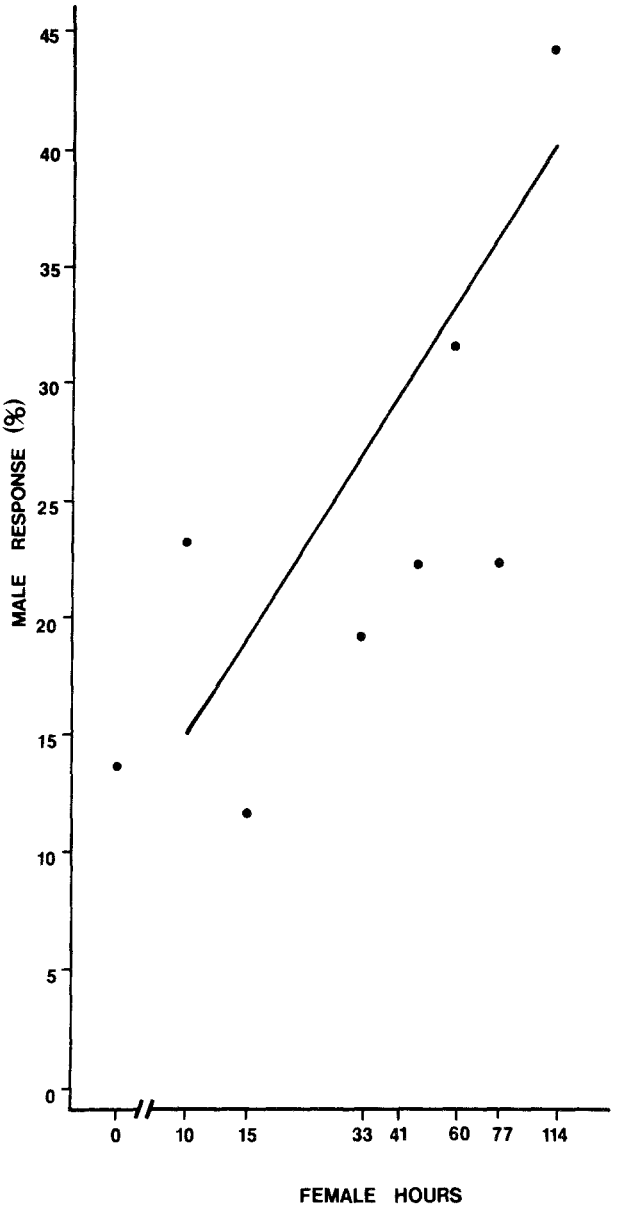

FIG. 3. Response of male *T. colubriformis* by in vitro assay to dosages of an HPLC peak(s) expressed as female-hours that had a 4.05 min retention time as shown in Figure 2 ($r = 0.88$, MSE = 3.44).

TABLE 2. RECOVERY OF HPLC PEAK(S) FROM *T. colubriformis* AFTER ELUTION FROM C-18 SEP-PAK CARTRIDGES

Solvent	Relative recovery (%)[a]
Dimethylsulfoxide	5.1
Acetonitrile	20.4
Acetone	16.1
Methanol	94.7
Ethylacetate	1.9
Ethanol	45.8
Chloroform	2.6
Tetrahydrofuran	39.6
n-Butanol	41.3
Methylene tetrachloride	10.1
Ethyl ether	1.1
Carbon disulfide	1.3

[a]Percent of absorbance units (210 nm) recovered versus maximum recovery of 88.1% for water.

TABLE 3. RECOVERY OF HPLC PEAK(S) FROM *T. colubriformis* AFTER INDICATED TREATMENTS FOR 48 HOURS

Treatment	Recovery (%)[a]
Aqueous, 20°C	14
Mercaptoethanol (1 mM), 20°C	37
Dithiothreitol (1 mM), 20°C	18
Ascorbic acid (10 mM), 20°C	98
EDTA (10 mM), 20°C	100
Sucrose (100 mM), 20°C	0
Aqueous, 4°C	89
Methanol, 4°C	58
Dried, 4°C	68

[a]Percent of absorbance units (210 nM) recovered versus original absorbance of HPLC peak.

units. Thus, cold storage with the addition of an antioxidant offers partial stabilization of the pheromone peak.

DISCUSSION

Males of *T. colubriformis* are attracted to the secretory–excretory products of their females by in vitro assay. However, the male's responsive-

ness may be reduced or eliminated for a period by exposure to the female's pheromone. Bone and Shorey (1977) proposed pheromone disruption for control of zooparasitic nematodes, while Zuckerman (1983) suggested that eradication of chemoreception may prevent location of a host plant by phytoparasites. Thus, based on this and previous studies, reduced responses of nematodes to concentrated chemical solutions may be common events.

Sensory adaptation or habituation were suggested previously as a cause of reduced responsiveness to pheromone by males (Bone and Shorey, 1977). However, the male's recovery after repeated rinsing in this study indicates that responsiveness was blocked peripherally. Thus, sensory adaptation rather than habituation of the nervous system may have occurred. The enhanced responsiveness of males after exposure to pheromone and adequate rinsing is interesting, but no mechanism is apparant. A related effect was observed in the human blood fluke *Schistosoma mansoni* (Shirazian and Schiller, 1982). Mating between the flukes in vitro was greater when the original sexual partner was included rather than excluded. Perhaps these increased sensitivities allow the subsequent location of another receptive, but more distant, female. Thus, maximal fecundity in the population may be facilitated.

The biological activity of the female's incubate may result from multiple components as in *Nippostrongylus brasiliensis* (Ward and Bone, 1983). The retention time of the peak from incubation of *T. colubriformis* suggests that the compound(s) may be similar or identical to that from *N. brasiliensis.*

The degradation of this material is apparently oxidative, based on the increased stability afforded by antioxidants. Addition of greater or repeated quantities of antioxidants to the pheromone may allow increased stabilization and an increased accumulation of the pheromone compound for spectral analyses.

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OLFACTION IN THE BOLL WEEVIL, *Anthonomus grandis* BOH. (COLEOPTERA: CURCULIONIDAE): Electroantennogram Studies[1,2]

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Abstract—Electroantennogram (EAG) techniques were utilized to measure the antennal olfactory responsiveness of adult boll weevils, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae), to 38 odorants, including both insect and host plant (*Gossypium hirsutum* L.) volatiles. EAGs of both sexes were indicative of at least two receptor populations: one receptor population primarily responsive to pheromone components and related compounds, the other receptor population primarily responsive to plant odors. Similar responses to male aggregation pheromone components (i.e., compounds I, II, and III + IV) were obtained from both sexes, but females were slightly more sensitive to I. Both sexes were highly responsive to components of the "green leaf volatile complex," especially the six-carbon saturated and monounsaturated primary alcohols. Heptanal was the most active aldehyde tested. More acceptors responded to oxygenated monoterpenes than to monoterpene hydrocarbons. β-Bisabolol, the major volatile of cotton, was the most active sesquiterpene. In general, males, which are responsible for host selection and pheromone production, were more sensitive to plant odors than were females. In fact, males were as sensitive to β-bisabolol and heptanal as to aggregation pheromone components. Electrophysiological data are discussed with regard to the role of insect and host plant volatiles in host selection and aggregation behavior of the boll weevil.

[1]This manuscript is dedicated to Dr. W.H. Cross, who died recently on an expedition near Durango, Mexico. Dr. Cross was an expert on the natural history of both the boll weevil and its host plants. He looked forward to the publication of this manuscript and our future collaboration on field experiments. He will be sorely missed.

[2]Mention of a trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

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Key Words—Cotton boll weevil, *Anthonomus grandis,* Coleoptera, Curculionidae, pheromone, kairomone, plant odor, olfaction, electroantennogram, attractant, host plant, green leaf volatiles.

INTRODUCTION

The cotton boll weevil, *Anthonomus grandis* Boh. (Coleoptera: Curculionidae) is a serious pest of cotton, *Gossypium hirsutum* L., in the United States, Central and South America (Cross, 1973, 1983; Ridgway and Lloyd, 1983). It is narrowly oligophagous and develops primarily on several genera within the tribe Gossypieae of the family Malvaceae (Cross et al., 1975). Both visual and chemical stimuli have been shown to play a role in the orientation of the weevil to its host and conspecifics (Cross et al., 1976; Tumlinson et al., 1969; Parencia et al., 1964).

Overwintered males are the first to emerge in the spring and consistently represent 60–65% of the emerging population (Cross, 1983). It may be these males which are responsible for locating a suitable host. Two theories have been proposed for host location by adult weevils. One theory proposes that emerging boll weevils do not recognize the cotton plant at distances greater than a few inches and that, upon locating the cotton plant by random movement, adult weevils are elicited to feed by a chemical arrestant (Hunter and Pierce, 1912; Hardee et al., 1969; Cross 1973, 1983). The second theory asserts that adult *A. grandis* are attracted to cotton plants from some distance by volatiles emanating from the plant (Parencia et al., 1964; Smith et al., 1965; Mistric and Mitchell, 1966; Mitchell and Taft, 1966).

Once the male weevil locates the host plant, he begins to feed and release a pheromonal attractant (Cross and Mitchell, 1966; Keller et al., 1964). This pheromone, isolated from male frass, was shown to consist of four oxygenated monoterpenoid components: I [(+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol]; II (*cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol); III (*cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde); and IV (*trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde)(Tumlinson et al. 1969), and it aggregated both males and females in field tests (Hardee et al., 1969, 1972). Although both sexes were caught in pheromone traps placed in the field during the spring and fall, during the interim period mainly females were captured (Mitchell et al., 1972). Thus the pheromone was thought to function either as an aggregating pheromone or as a sex attractant depending on the time of year.

Although much is known about the attraction of boll weevils to odorous stimuli in laboratory bioassays and field tests (Cross, 1973; Hedin et al., 1973), little is known about the actual perception of these compounds

by the insect. Using ablation techniques, Hardee et al. (1966) demonstrated that receptors responsible for the orientation of adult *A. grandis* to host-plant volatiles were located on the antennal club. More recently, electrophysiological techniques were used to show that receptors for the pheromone components were also located on the antennal club (Gutmann et al., 1980).

The purpose of this study was to investigate the peripheral olfactory responsiveness of adult *A. grandis* to components of the aggregation pheromone in relation to the responsiveness to host-plant odors. This research was further intended to elucidate the receptor system for various classes of volatiles previously identified from the cotton plant and to provide a basis for single-cell studies.

METHODS AND MATERIALS

Insects. Adult *A. grandis* used in this study were obtained from a small laboratory colony annually infused with feral insects maintained at the USDA Boll Weevil Research Laboratory. Newly emerged to 24-hr-old weevils were sexed and maintained on moist filter paper in Petri dishes at room temperature (24–26° C) until use within a few hours.

Electrophysiological Methodology. Electroantennogram (EAG) techniques utilized in these studies were a modification of a previous technique (Schneider, 1957) and are described in detail elsewhere (Payne, 1970; Dickens and Payne, 1977; Dickens, 1979). In general, Ag–AgCl capillary electrodes filled with insect physiological saline (Pantin, 1948; Oakley and Schafer, 1978) were used. Following prepuncture with a sharpened tungsten needle, the recording electrode was inserted into the distal end of the antennal club. The indifferent electrode was implanted in the distal end of the scape. The signal was amplified 10× by a Grass P-16 AC/DC preamplifier prior to viewing on a Tektronix 5223 digitizing oscilloscope. For subsequent analyses and storage, EAGs were recorded on graph paper by an *x–y* plotter.

Odorous Stimuli. Chemicals used as olfactory stimuli are listed in Table 1. Test compounds were chosen based primarily on their presence in the cotton plant and/or in the green leaf volatile complex (Visser et al., 1979) Components of the male boll weevil aggregation pheromone were also tested (Tumlinson et al., 1969). Several isomers of these compounds were also included.

Stimulus compounds diluted in nanograde pentane were delivered as 10 μl samples placed on filter paper (8 × 18 mm) inserted into glass cartridges (80 mm l. × 5 mm ID) and oriented toward the preparation from ca. 1 cm. Hydrocarbon-free air (filtered and dried) carried odor molecules evaporating from the filter paper over the antennal preparation. Since the compounds

TABLE 1. SOURCE AND PURITY OF ODOROUS STIMULI USED IN ELECTROPHYSIOLOGICAL STUDIES AND THEIR PRESENCE IN COTTON PLANT, *Gossypium* spp.

Compound	Chemical purity (%)	Source of supply[a]	Presence in cotton volatile emission[b,c] :essential oil[d]
Aggregation pheromone			
Compound I, (+)-*cis*-2-isopropenyl-1-methylcyclobutane-ethanol	85–90	A	
Compound II, *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclo-hexaneethanol	85–90	A	
Compound III + IV, 50:50, mixture of *cis*:*trans* -3,3-di-methyl-$\Delta^{1,\alpha}$-cyclo-hexane acetalde-hyde	85–90	A	
Aliphatic alcohols			
1-Propanol	>99	B	
1-Butanol	>99	B	
1-Pentanol	>99	C	:+
1-Hexanol	98	D	+:+
trans-2-Hexen-1-ol	97	D	:+
cis-2-Hexen-1-ol	98	D	
cis-3-Hexen-1-ol	98	D	:+
1-Heptanol	98	D	
1-Octanol	99+	D	+:
1-Nananol	97	D	+:
1-Decanol	99+	D	
Aliphatic aldehydes			
Hexanal	99+	D	:+
trans-2-Hexenal	99	D	:+
Heptanal	95	D	:+
Octanal	99	D	
Nonanal	98	D	+:+
Decanal	98	D	+:
Monoterpene hydrocarbons			
(+)-α-Pinene	98	D	
(−)-α-Pinene	98	D	+:+
(+)-Limonene	97	D	
(−)-Limonene	97	D	+:+
Myrcene	85	D	+:+
Oxygenated monoterpenes			
d-carvone	96	D	
l-carvone	98	D	
Geraniol	>90	E	+:+
Nerol	>65	E	:+
Linalool	99	D	+:+

TABLE 1. Continued

Compound	Chemical purity (%)	Source of supply[a]	Presence in cotton volatile emission[b,c] :essential oil[d]
Sesquiterpenes			
α-Bisabolol	92	F	:+
β-Bisabolol	83	F	:+
Caryophyllene oxide	97	D	:+
β-Caryophylene	90.2	G	:+
Other compounds			
Acetophenone	99	D	+:
Benzaldehyde	98+	D	+:+
Decane	99+	D	+:
Undecane	99	D	+:

[a]A, Frank Enterprises Inc., Columbus, Ohio; B, Fisher Scientific Co., Pittsburgh, Pennsylvania; C, J.T. Baker Chem. Co., Phillipsburg, New Jersey; D, Aldrich Chem. Co., Milwaukee, Wisconsin; E, Pfaltz & Bauer, Inc., Stamford, Connecticut; F, P.A. Hedin, USDA, ARS, Mississippi State, Mississippi; G, ICN—K&K Laboratories, Inc., Plainview, New York.
[b]Hedin (1976).
[c]Hedin et al. (1975).
[d]Hedin et al. 1973).

tested did not have the same volatility, comparisons made between odorous stimuli are relative.

Stimulus duration was 1 sec. Air flow was 1 m/sec as measured by a thermistor. The atmosphere around the preparation was continuously exhausted. Intermittent puffs of odorant were chosen as the method of stimulus delivery since injection of the odorant into a flowing air stream could dry the preparation during long-term experiments.

Experimental Protocol. Two series of experiments were performed. In the first series of experiments, the general responsiveness of the antennal receptors to the individual odorants was measured by recording EAGs to a 100 μg dose of each. Presentation of each odorant was randomly ordered for each preparation.

In the second series of experiments, several odorants were selected for closer examination based on data obtained in initial experiments and/or previous knowledge of their role in boll weevil behavior. Dosage–response curves were constructed from EAGs elicited by serial dilutions of each pheromone (0.001–100 μg) and selected host-plant odor (0.01–100 μg). In every instance, serial dilutions were presented in order from the lowest to the highest dosage.

In both experimental series, EAGs from three weevils of each sex were recorded for each stimulus. At least 4 min were allowed between each

stimulation. This time was found to be more than adequate for complete recovery of the EAG.

1-Hexanol at the 100 μg dosage was used as a standard for normalizing all responses so that responses from different preparations could be compared. Stimulation with the standard either preceded or followed each stimulus by 4 min. Responses to intervening test stimuli were represented as a percent of the mean of the two nearest responses to the standard (Dickens, 1978, 1981). Based on responses of 42 weevils tested (19 males and 23 females), no significant differences were found in responses of the sexes to the standard.

The size of the EAG depolarization was considered to be a measure of the relative number of acceptors responding (Payne, 1975; Dickens and Payne, 1977). The threshold of response 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. Saturation level was considered to be that dosage at which the standard error of the response elicited overlapped with the standard error of the response at the highest dosage. Responses were compared for significant differences using the t test for two means (Ostle, 1969).

RESULTS

The mean responses of male and female *A. grandis* to the 1-hexanol standard (100 μg) were −0.97 mV (SE = 0.05 mV) and −1.06 mV (SE = 0.06 mV), respectively. The difference in responses of the two sexes to the standard was not significant ($P < 0.05$).

Selectivity

In general, results from the first series of experiments indicated significant differences in the size of acceptor populations for each of the various odorants examined. Although slight differences between the sexes in EAGs to each of the odorants were noted, only a few significant differences occurred.

Pheromone Components. Both male and female weevils were significantly more responsive to the mixture of III and IV than to I ($P < 0.05$) (Figure 1). Although both male and female response to II exceeded that to I, only male response to II was significantly greater ($P < 0.05$). Responses of either sex to II and the mixture of III + IV were not significantly different. No sexual dimorphism was apparent in responses to the pheromone components at this dosage. EAGs evoked by each pheromone component were always less than those evoked by the standard.

Aliphatic Alcohols and Aldehydes. EAGs elicited by terminal aliphatic alcohols of chain lengths of 3–10 carbons were maximal to the six-carbon alcohol for both male and female weevils (Figure 2A). No significant

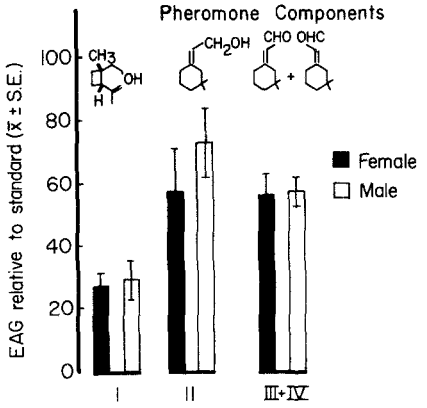

FIG. 1. Mean EAGs of male and female *A. grandis* to 100 μg dosage of male aggregation pheromone components.

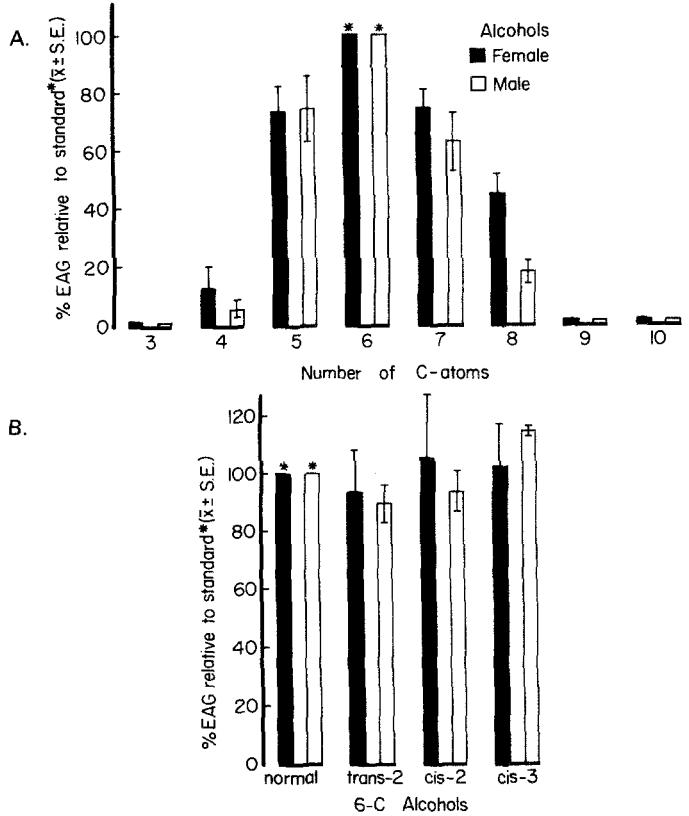

FIG. 2. Mean EAGs of male and female *A. grandis* to 100 μg dosage of terminal saturated alcohols of various carbon chain lengths (A). Mean EAGs of male and female *A. grandis* to 100 μg dosage of terminal saturated and monounsaturated 6-carbon alcohols (B).

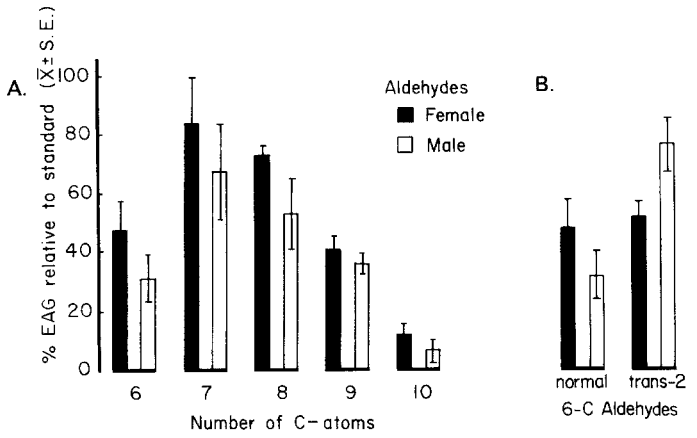

FIG. 3. Mean EAGs of male and female *A. grandis* to 100 μg dosage of saturated aldehydes of various carbon chain lengths (A). Mean EAGs of male and female *A. grandis* to 100 μg dosage of saturated and monounsaturated six-carbon aldehydes (B).

differences were found between responses of either sex to the series of alcohols, with the exception of the eight-carbon alcohol to which the female antenna was significantly more responsive than the male ($P < 0.05$). EAGs evoked by several monounsaturated six-carbon alcohols showed no intersexual differences in responsiveness at this dosage (Figure 2B). However, response of the male to *cis*-3-hexen-1-ol was significantly greater than responses elicited by any other six-carbon alcohol ($P < 0.05$).

In contrast to the responses to the aliphatic alcohols, EAGs elicited by a series of aliphatic aldehydes of various chain lengths were maximal to the seven-carbon aldehyde (Figure 3A). Although no significant differences in responses of the sexes to the aldehyde series were apparent, male response to the monounsaturated aldehyde, *trans*-2-hexenal, was significantly greater than response of the female ($P < 0.05$) (Figure 3B). EAGs evoked by *trans*-2-hexenal in the male were also significantly greater than those evoked by the saturated six-carbon aldehyde ($P > 0.05$).

Monoterpenes. Significant EAGs were elicited by each oxygenated monoterpene tested (Figure 4A). However, among the monoterpene hydrocarbons tested, significant EAGs were evoked only by (+)-limonene in the females and (+)- and (−)-limonene in the males, and none of these responses exceeded 6% of the standard (Figure 4B). No sexual differences were noted for EAGs elicited by any monoterpene. However, responses of both male and female weevils to nerol were significantly greater than to its geometric isomer, geraniol ($P < 0.10$). Chiral specificity was not evident for any optical isomer examined.

Sesquiterpenes. EAGs elicited by β-bisabolol were larger than those

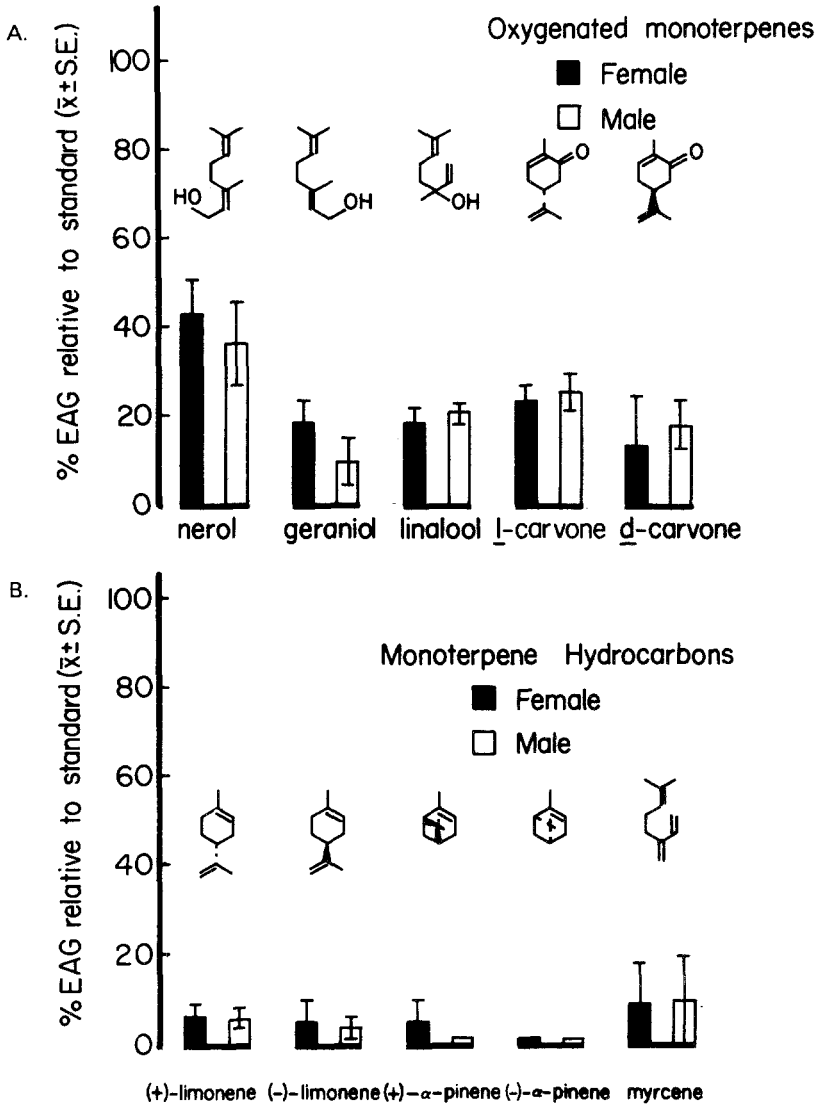

FIG. 4. Mean EAGs of male and female *A. grandis* to 100 μg dosage of oxygenated monoterpenes (A) and monoterpene hydrocarbons (B).

elicited by any other sesquiterpene (Figure 5). Responses of both male and female weevils to α-bisabolol were significantly less than those to its positional isomer, β-bisabolol ($P < 0.01$ and <0.10 for males and females, respectively). Although EAGs evoked by β-caryophyllene were larger than those evoked by caryophyllene oxide, only in the females was this difference significant ($P < 0.05$).

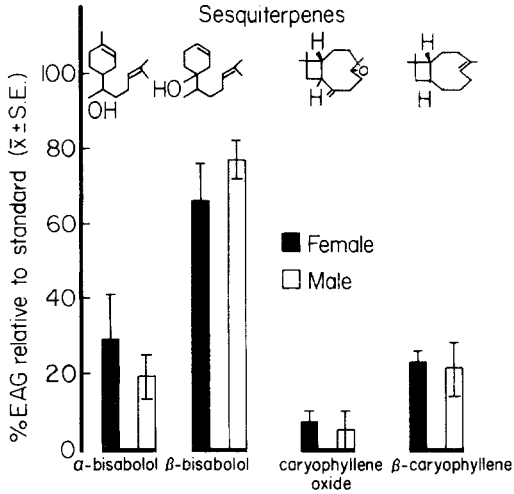

FIG. 5. Mean EAGs of male and female *A. grandis* to 100 μg dosage of sesquiterpenes.

Other Compounds. Of the two aromatic compounds tested, acetophenone elicited larger EAGs than did benzaldehyde (Figure 6). Indeed, male weevils were significantly more responsive to acetophenone than to benzaldehyde at this dosage ($P < 0.10$). EAGs elicited by benzaldehyde in females were significantly larger than in males ($P < 0.10$). Two alkanes, decane and undecane, failed to elicit a response at this dosage (Figure 6).

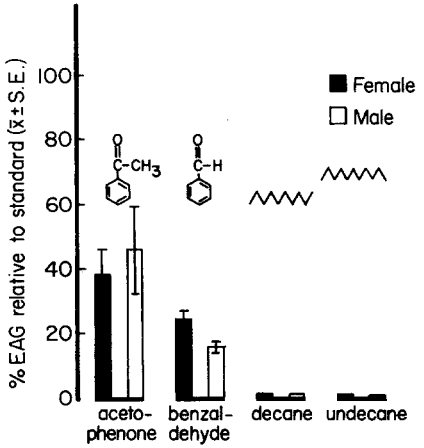

FIG 6. Mean EAGs of male and female *A. grandis* to 100 μg dosage of benzenoid and alkane compounds.

TABLE 2. COMPARISON OF THRESHOLD AND SATURATION DOSAGES FOR PHEROMONES AND SELECTED HOST COMPOUNDS

Compound	Threshold (μg)	Saturation dosage (μg)	EAG at saturation dosage (%) ($X \pm SE$)
Pheromones			
I	♂ 10	10	13.23 ± 3.09
	♀ 1.0	10	10.07 ± 4.59
II	♂ 0.1	10	41.90 ± 7.41
	♀ 0.1	10	30.37 ± 4.01
III + IV	♂ 0.1	10	67.37 ± 15.47
	♀ 0.1	10	55.40 ± 9.96
Host compounds			
1-Hexanol	♂ 1.0	100	100
	♀ 1.0	1000	115.73 ± 13.88
Hexanal	♂ 1.0	1000	113.47 ± 17.10[a]
	♀ 10	1000	100.67 ± 12.67[a]
Heptaldehyde	♂ ≤0.1	1000	132.87 ± 12.87[a]
	♀ 1.0	1000	100.67 ± 22.67[a]
Nerol	♂ 1.0	100	49.07 ± 15.54
	♀ 10	100	61.67 ± 7.05
β-Bisabolol	♂ ≤0.1	≥1000	106.13 ± 3.55
	♀ 1.0	100	102.97 ± 4.66[a]
β-Caryophyllene	♂ 1.0	10	18.70 ± 1.28
	♀ 1.0	10	16.77 ± 1.03

[a]Saturation not apparent at highest dosage tested.

Sensitivity

In general, after reaching threshold, responses to the compounds tested increased with increasing dosages until saturation occurred. For most compounds examined, both the threshold for a significant EAG and the dosage at which saturation occurred differed (Table 2).

Pheromones. The shapes of dosage–response curves for EAGs for each pheromone component were similar for both sexes (Figure 7). Female weevils had a 10× lower threshold for I than did males; however, saturation was reached at 10 μg for both sexes (Table 2). The thresholds of response to II and to the mixture of III and IV were 0.1 μg for both sexes. Similar to I, the saturation dosage for II and the mixture of III and IV was 10 μg.

Aliphatic Alcohols and Aldehydes. Dosage–response curves for EAGs of males and females to 1-hexanol were almost identical (Figure 8). Both sexes had a threshold of 1.0 μg and gave increasing responses through saturation at 100 μg and 1000 μg for males and females, respectively (Table 2).

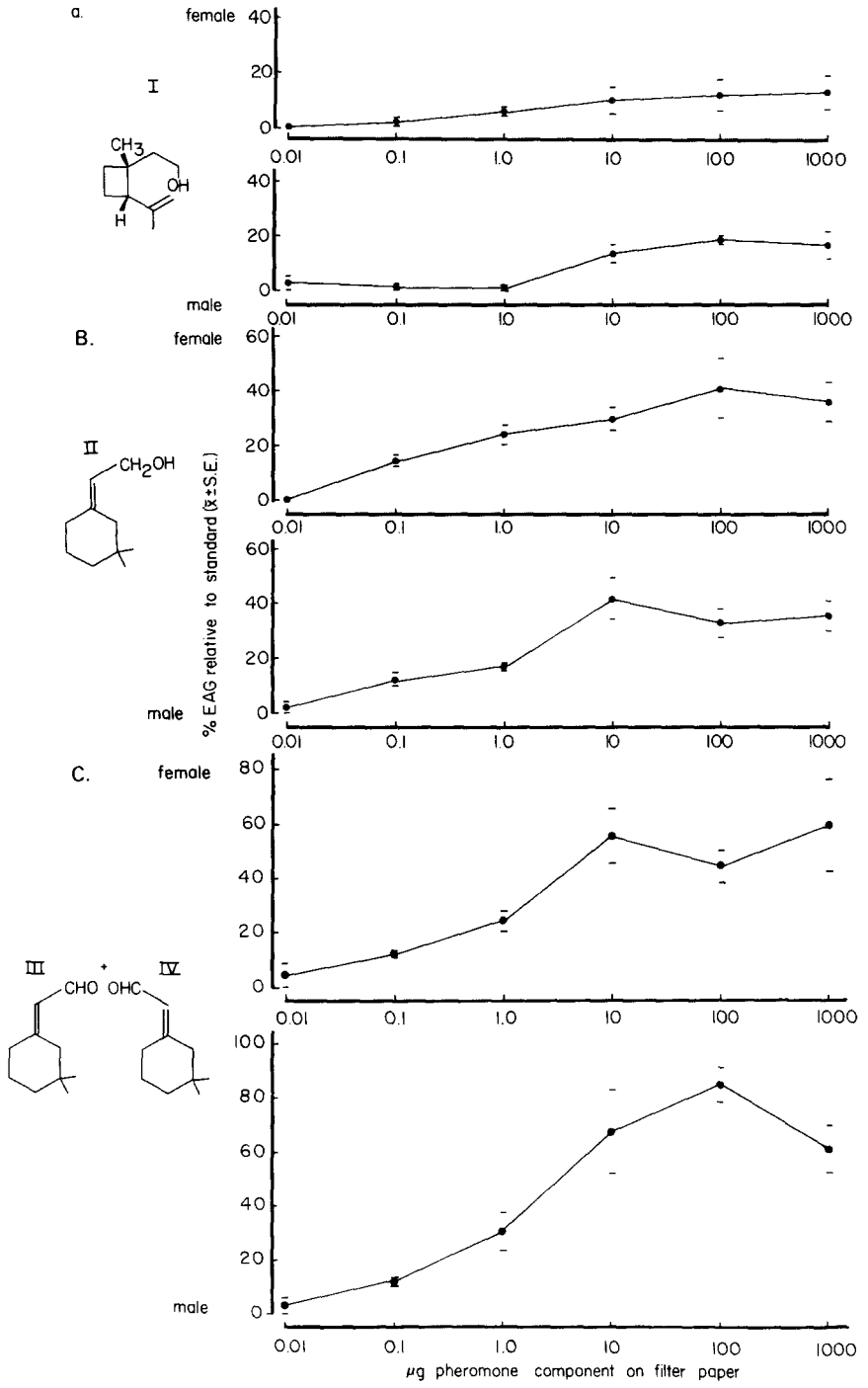

FIG. 7. Dosage–response curves constructed from EAGs of male and female *A. grandis* to serial dilutions of male aggregation pheromone components I (A), II (B), and a 50:50 mixture of III + IV (C).

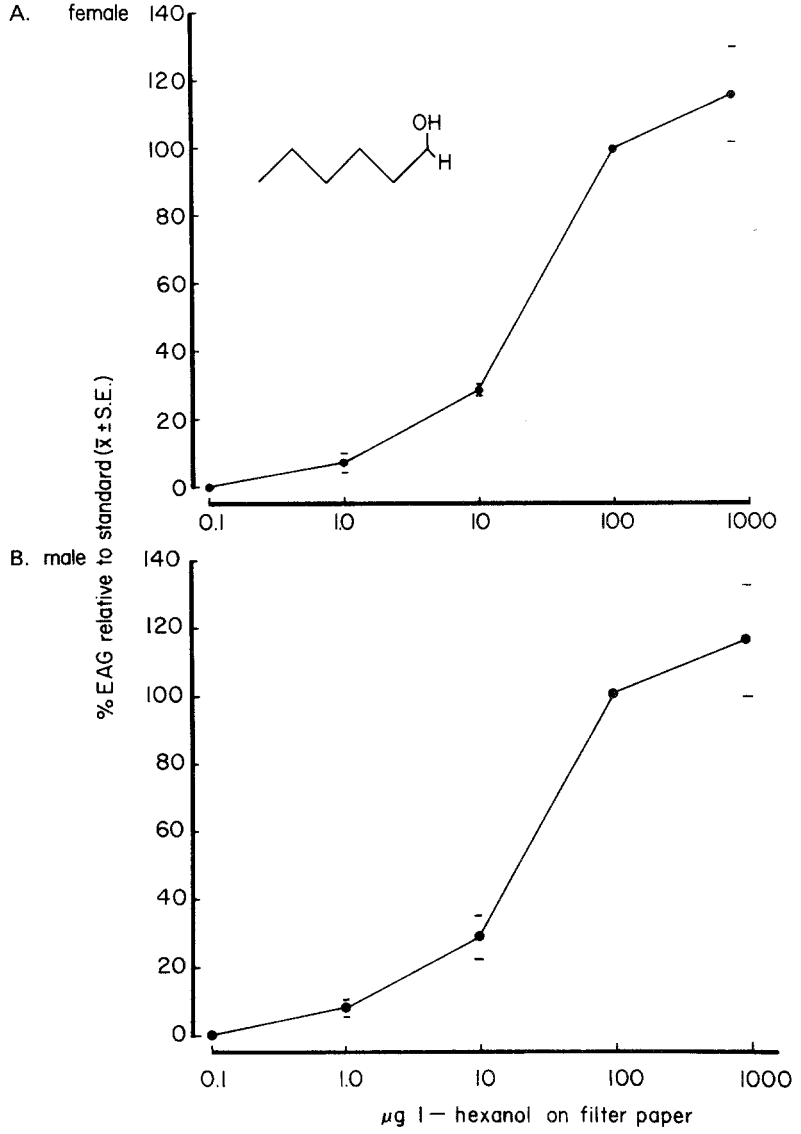

FIG. 8. Dosage–response curves constructed from EAGs of female (A) and male (B) *A. grandis* to serial dilutions of 1-hexanol.

The shapes of dosage–response curves for both the six- and seven-carbon aldehydes were similar, although the curve for the six-carbon aldehyde was shifted closer to the abscissa (Figures 9 and 10). Male weevils had at least a 10× lower threshold for both aldehydes than that observed for females, while responses elicited at the highest dosage tested were not significantly different (Table 2).

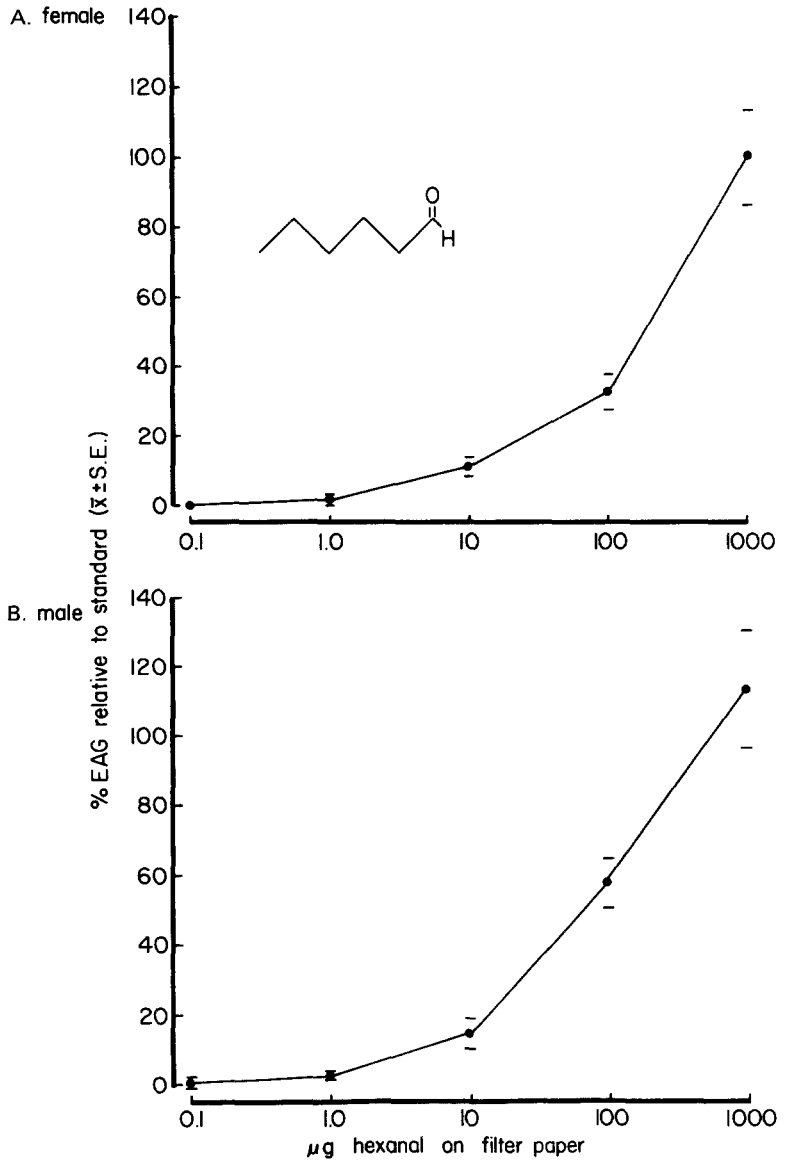

FIG. 9. Dosage-response curves constructed from EAGs of female (A) and male (B) *A. grandis* to serial dilutions of hexanal.

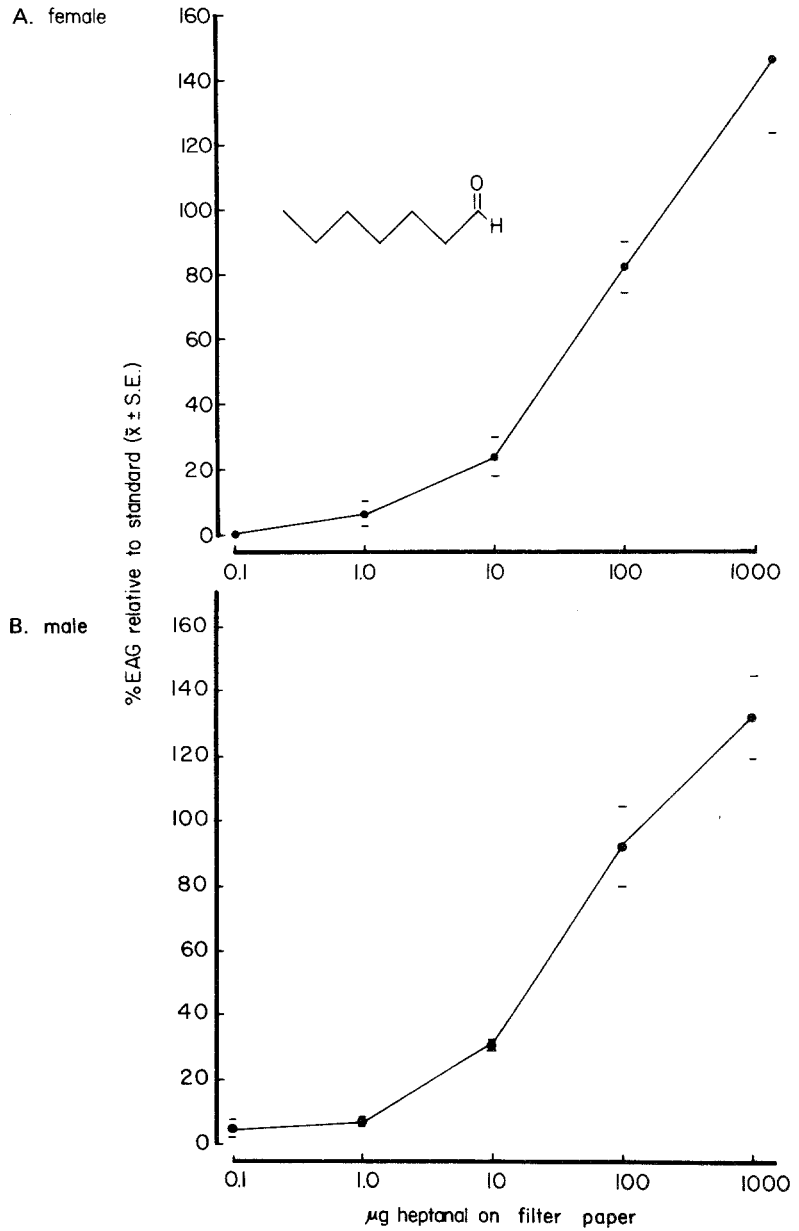

FIG. 10. Dosage–response curves constructed from EAGs of female (A) and male (B) *A. grandis* to serial dilutions of heptanal.

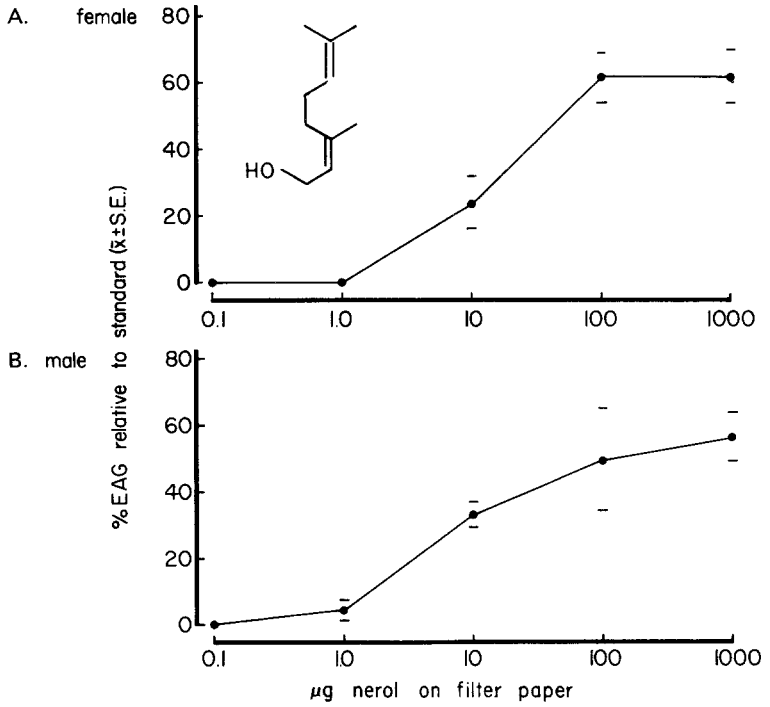

FIG. 11. Dosage–response curves constructed from EAGs of female (A) and male (B) *A. grandis* to serial dilutions of nerol.

Monoterpenes. Both the dosage–response curves and dosages necessary for acceptor saturation were similar in EAGs elicited from male and female weevils by nerol (Figure 11; Table 2). The threshold of response of male and female weevils was 1.0 μg and 10 μg, respectively.

Sesquiterpenes. Although the dosage–response curves for EAGs of both sexes to β-bisabolol were similar, males had at least a 10× lower threshold at <0.1 μg than did females (Figure 12; Table 2). EAGs of female weevils saturated at 100 μg, while male response did not saturate even at 1000 μg.

Dosage–response curves of both male and female weevils for β-caryophyllene were almost identical (Figure 13). Threshold was reached at the 1.0 μg dosage, with saturation occurring at 10 μg (Table 2).

DISCUSSION

General. The olfactory receptor system in both male and female *A. grandis* consisted of at least two types of receptors. One receptor type was characterized by a threshold dosage of 0.1–10 μg with saturation of the EAG occurring quickly at ≤100 μg at a level <70% of the standard.

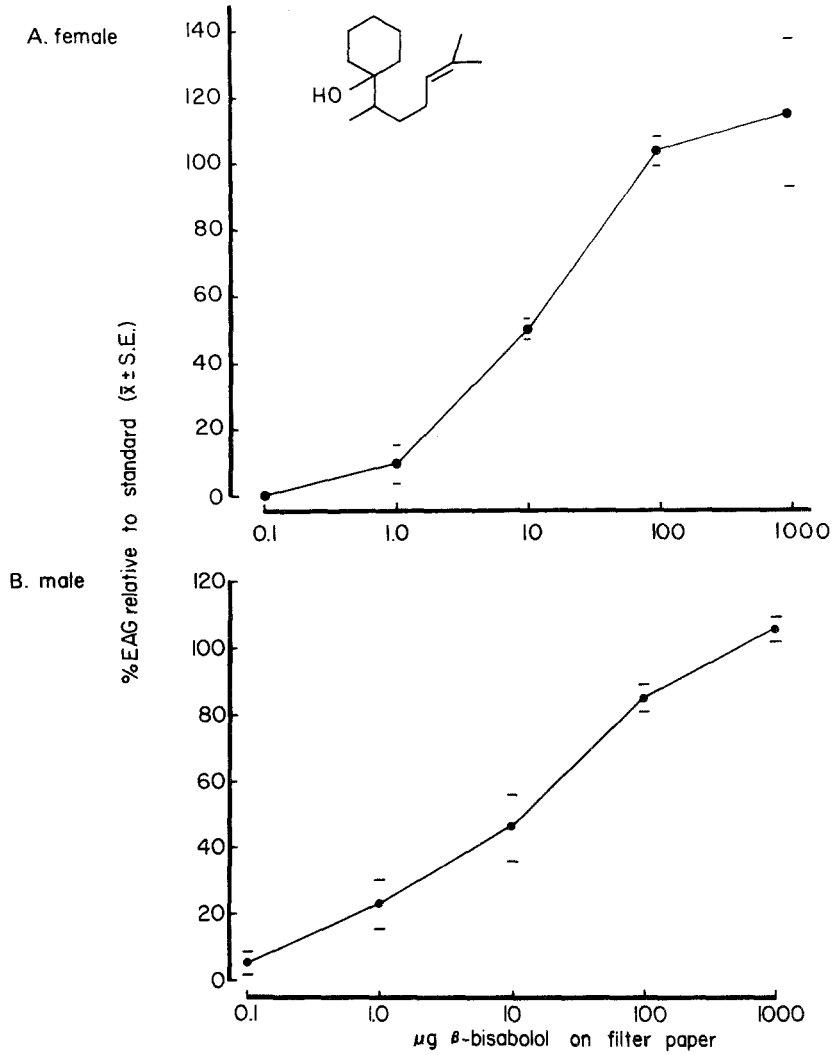

FIG. 12. Dosage–response curves constructed from EAGs of female (A) and male (B) *A. grandis* to serial dilustions of β-bisabolol.

Receptors for the various pheromonal components and possibly nerol and β-caryophyllene were of this type (Figures 7, 11, 13; Table 2).

A second receptor type had a threshold of ≤0.1–10 μg with saturation only at dosages ≥100 μg at a level ≥100% of the standard. Receptors of this type were responsive to components of the green leaf volatile complex (see Visser, 1983), heptanal, and β-bisabolol (Figures 8, 9, 10, 12; Table 2).

Mustaparta (1973) grouped the olfactory sensilla on the antennal club of the pine weevil, *Hylobius abietus* L. (Coleoptera: Curculionidae), into two types: the sensilla basiconica and sensilla trichodea. The sensilla

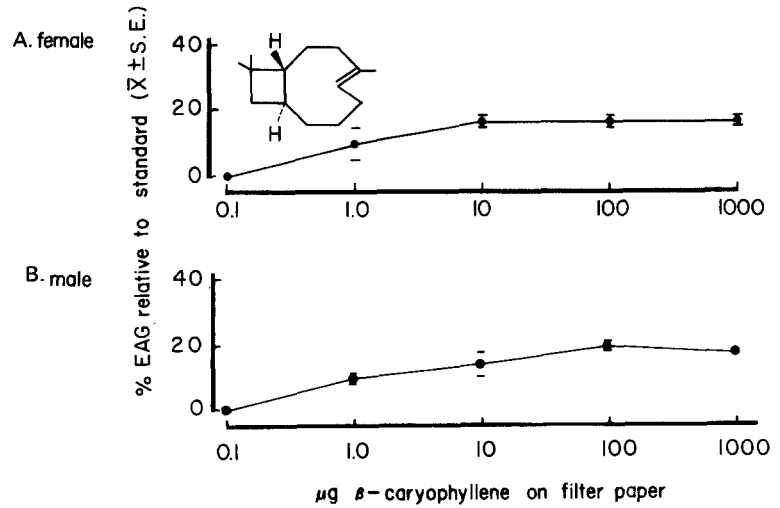

FIG. 13. Dosage–response curves constructed from EAGs of female (A) and male (B) *A. grandis* to serial dilutions of β-caryophyllene.

basiconica were innervated by one to two cells and occurred in much larger numbers than the sensilla trichodea which were innervated by a single cell. Electrophysiological studies revealed that cells associated with the trichoid sensilla were generally most responsive to pheromonal compounds, whereas sensilla basiconica cells responded to plant odors (Mustaparta, 1975a). Similarly, the boll weevil antennal club has two types of olfactory sensilla which occur in a similar proportion to the pine weevil (Dickens, unpublished). Thus the two types of receptors indicated by EAG studies may correspond to the sensilla trichodea, which occur in low numbers and respond to pheromone components, and the sensilla basiconica, which occur in higher numbers and are primarily sensitive to plant odors.

Whether the boll weevil perceives and is attracted to its host plant over any distance, or whether it locates its host by random flights has been debated for many years (Hunter and Pierce, 1912; Mistric and Mitchell, 1966; Mitchell and Taft, 1966; Hardee et al., 1969; Cross, 1983). In support of the former theory, field experiments have shown attraction of the insect to fruiting cotton plants (Parencia et al., 1964; Smith et al., 1965; Mistric and Mitchell, 1966; Mitchell and Taft, 1966) and laboratory bioassays have indicated attraction of the weevil to various plant extracts (see Hedin et al., 1973). The random flight theory is substantiated by field tests in which more overwintered and migrating boll weevils were attracted to caged males feeding on buds or squares, and males on whole plants, than to caged cotton plants which differed little from unbaited controls (Hardee et al., 1969).

Both the high sensitivity and wide range of responses of antennal receptors for plant odors in adult boll weevils indicate an important role for plant odors in the orientation of the insect to its host (Table 2). This is especially true for male weevils which were as sensitive to β-bisabolol, the major volatile of cotton and thus far reported only in malvaceous plants (Thompson et al., 1971; Thompson, personal communication), as to components of their aggregation pheromone.

That adult weevils responded strongly in favor of the pheromone source as compared to the host plant in field tests (Hardee et al., 1969) might be explained by selective activation of different groups of receptor cells by each odorant. Mustaparta (1975b) found that in the pine weevil both *trans*-verbenol, a bark beetle pheromone which activated cells associated with sensilla trichodea, and α-pinene, a host odor which primarily activated cells innervating sensilla basiconica, were attractive when presented separately in laboratory bioassays. However, when the weevils were allowed a choice between the two odorants the attractive effect of the host odor was dominated by the pheromone. A similar situation may exist with the boll weevil.

Pheromone Receptor System. The olfactory receptor systems for components of the male-produced aggregation pheromone in both male and female *A. grandis* are very similar. Initial EAG experiments indicated no significant differences in responses of males and females to the 100 μg dosage of I, II, or the mixture of III + IV (Figure 1). Furthermore, except for a slightly lower threshold for I for female weevils, dosage–response curves for both sexes showed no significant differences in threshold dosage, saturation dosage and EAGs at saturation dosage for either I, II, or the mixture of III + IV (Figure 7; Table 2). In a previous study, Gutmann et al. (1981) also found a slightly lower threshold for I in females than in males.

The similarity in receptor systems of male and female *A. grandis* for components of the male pheromone is indicative of its role in the aggregation of the sexes for mating and host colonization. Hardee et al. (1972) observed in field tests that both males and females were attracted to males fed squares (presumably releasing pheromone) and to synthetic grandlure (mixture of I, II, III, and IV) with similar sex ratios as obtained from control traps. Other reports which indicate seasonal variation in sex ratios of boll weevils responding to pheromone traps (Mitchell et al., 1972; Mitchell and Hardee, 1974; Rummel and Bottrell, 1976) must represent changes in responses of the insects' central nervous system to peripheral stimuli and/or modulation of receptor sensitivity due to changes in hormonal levels (Palaniswamy et al., 1979; Davis and Takahashi, 1980; Dickens, unpublished).

The greater sensitivity of *A. grandis* females for I observed in EAG studies correlates well with an hypothesis put forth by Gueldner and Wiygul

(1978). They observed a circadian rhythm in the ratio of I:II which was low when the amount of pheromone was high. They suggested that this ratio might signal the female as to the age of pheromone-releasing frass excreted by the male, thus facilitating orientation to fresh frass and increasing the likelihood of mate location.

Two components of the male-produced aggregation pheromone, i.e., I and II, along with a sesquiterpene from the cotton plant, β-caryophyllene, were isolated in low concentrations from the frass of *A. grandis* females and were shown at high dosages to be attractive to males in laboratory bioassays (McKibben et al., 1977; Hedin et al., 1979). This attractive response was enhanced by the addition of α-pinene, myrcene, and *l*-limonene. Although males were more responsive in laboratory bioassays than females, no significant difference was found in responses of the sexes in field tests. The greater response of the male to the tripartite mixture must be explained by differences in higher-order neural mechanisms, since both males and females have receptors sensitive to each compound. Females are more sensitive to I than males, however, and it has been hypothesized that I may play a role in female behavior (Gueldner and Wiygul, 1978). Therefore the observed response of groups of females in the laboratory bioassay could be explained simply by their rejection of the incomplete aggregation pheromone.

Dosage–response curves for the individual pheromone components in this study differ slightly from those obtained in a previous study (Gutmann et al., 1981). Gutmann et al. (1981) found more acceptors responded to III + IV than to either alcohol component. Similar results were found in this study. However, they suggested that the low EAG threshold for grandlure (ca. 0.1 μg dosage) observed in both male and female *A. grandis* could be accounted for by the presence of I, with its purported low threshold, in the mixture. Close examination of dosage–response curves to I in their study reveal, in fact, that EAGs greater than the pentane control were obtained at <0.01 μg for the females but only at 1.0 μg (10$\times$ the grandlure threshold) for males. Males had a lower threshold (0.1 μg) for II than for the other pheromone components. In the current study, the threshold of the antennal receptors of the female for I was only 10$\times$ lower than that observed for the male, and both sexes had a lower threshold for II and the mixture of III + IV than for I. These differences between the 2 studies might be accounted for by the fact that the two studies were conducted using different populations of *A. grandis* (Texas vs. Mississippi), insects of differing ages and physiological condition (insects of unknown age obtained from pheromone traps vs. newly emerged weevils), and slightly different stimulation and recording techniques.

Host-Odor Receptor System. The antennal olfactory receptor system is

well-adapted for the perception of odors emanating from the cotton plant. Not only is the receptor system attuned to general green leaf volatiles (see Visser, 1983), but also it detects compounds more specific to the cotton plant.

Visser and his coworkers (Visser et al., 1979; Visser and Avé, 1978; Visser, 1983) hypothesized that six-carbon alcohols and aldehydes, i.e., "green leaf volatiles," might play an important role in the orientation of phytophagous insects to their hosts. This volatile complex consists mainly of straight-chain saturated and monounsaturated six-carbon aldehydes and primary alcohols, which are formed by oxidative degradation of plant lipids. EAGs recorded from both sexes of *A. grandis* to a series of primary alcohols of varying chain lengths revealed peak responsiveness of antennal receptors to the six-carbon alcohol. When various monounsaturated stereoisomers of 1-hexanol were tested, responses of females did not differ significantly from the standard; however, males were significantly more responsive to the *cis*-3 isomer (Figure 2). Females were more responsive to 1-octanol at this dosage than were males.

EAGs to the corresponding six-carbon aldehydes were significantly less than to the alcohols, with the exception of *trans*-2-hexenal, which elicited a significantly greater response in males than females and was equivalent to its corresponding alcohol at this dosage (Figures 2 and 3). Greater responsiveness to leaf alcohols relative to their aldehyde analogs was also found for the Colorado beetle, *Leptinotarsa decemlineata* Say (Visser, 1979). Contrasting results, in which the insect herbivore was less responsive to the leaf alcohols than to the aldehydes, were found in the carrot rust fly, *Psila rosae* (F.) (Guerin and Visser, 1980) and the cereal aphid, *Sitobion avenae* (F.) (Yan and Visser, 1982).

Although the six-carbon aldehydes elicited significant responses from both male and female *A. grandis,* maximal EAGs were elicited by the seven-carbon aldehyde (Figure 3). This result is comparable to findings for EAGs for a series of aldehydes of varying chain lengths for several lepidopterous (Van der Pers, 1981) and dipterous species (Guerin and Städler, 1982). These results contrast with those obtained for several other insect species (see Visser, 1983), including the oak leaf weevil, *Rhychaenus quercus* L. (Kozlowski and Visser, 1981), which were most responsive to both the six-carbon alcohols and aldehydes.

In general, both male and female *A. grandis* had more acceptors responsive to the oxygenated monoterpenes than to the monoterpene hydrocarbons at the dosage tested (Figure 4). Of particular interest were the significantly greater EAGs elicited by nerol than those elicited by its geometric isomer, geraniol. The significance of this result must be guarded, however, since the nerol used in this study was only >65% pure. Greater

responsiveness at the EAG level for oxygenated monoterpenes vs. monoterpene hydrocarbons has also been found for several other phytophagous insects including *L. decemlineata* (Visser, 1979), *R. quercus* (Kozlowski and Visser, 1981), and *P. rosae* (Guerin and Visser, 1980).

The acceptor population for β-bisabolol was significantly greater than for any other sesquiterpene and equal to or greater than that for any other odorant tested, except for heptanal (Figures 1–6; Table 1). β-Bisabolol is the major component of the volatile complex emitted by the growing cotton plant, and composes almost 14% of cotton essential oil (Thompson et al., 1971). Its presence has been reported only in the essential oil of several malvaceous plants. Previous studies have shown β-bisabolol to be attractive to *A. grandis* adults in laboratory bioassays (Minyard et al., 1969). Both the fact that β-bisabolol has been reported only from cotton and its close relatives and its attractiveness to boll weevils in laboratory bioassays indicate its possible role in the orientation of the insect to its host plant. Furthermore, the presence of higher concentrations of β-bisabolol in the essential oils of *Hibiscus militaris* Cav. and *H. lasiocarpus* Cav. relative to cotton might explain the greater response of *A. grandis* to these extracts in laboratory bioassays (Thompson et al., 1970, 1971).

In general, dosage–response curves constructed from EAGs of male and female *A. grandis* to selected plant odors revealed the male to be more sensitive than the female to each odor tested, except for 1-hexanol and β-caryophyllene, to which responses of both sexes were almost identical (Figures 8–13). In fact, the male antenna was as sensitive to heptanal and β-bisabolol (Figures 10 and 12; Table 2) as to components of its aggregation pheromone (Figure 7; Table 2). Additionally, the male was 10× more sensitive than was the female to nerol (Figure 11), which has been implicated as a possible pheromone precursor (Thompson and Mitlin, 1979).

Recently Cross (1983) reported that boll weevil populations emerging in the spring were mostly males and that the first few emerging weevils were males. The greater sensitivity of the male to plant odors, in general and specifically to the major volatile component of its host plant, i.e., β-bisabolol, correlates well with the role of the male in host plant selection and production of the aggregation pheromone.

Previous studies utilizing greenhouse-grown early-fruiting cotton plants placed in the field at the time of emergence of overwintered weevils showed that weevils could find isolated groups of cotton plants (Parencia et al., 1964; Smith et al., 1965). However, no weevils were detected on fruiting cotton plants placed in hibernation sites (Smith et al., 1965). This lack of response of the overwintered weevils to these plants was attributed to the lack of perception of plant attractants by the insects in the surface trash (i.e., hibernation site). Another explanation for this phenomenon, since it is likely that the plant was perceived by the overwintered adult (Dickens, unpublished), is that the behavioral threshold for response to odorous

stimuli might be modified by flight exercise as shown for other insects (Atkins, 1966, 1969; Bennett and Bordon, 1971; Andryszak et al., 1982).

Synthesis. The olfactory receptor systems of male and female *A. grandis* are well adapted for the perception of odorous chemical messengers from their environment. The different thresholds and saturation ranges observed for the various odorants in electrophysiological studies might be indicative of the role of each compound in host selection and aggregation. A low threshold for a given compound might indicate the ability of the insect to perceive the compound in low concentrations at greater distances from its source. Perception of a compound over a wide range of concentrations would indicate the capability of the insect to perceive changes in concentration of a compound as it diffused from its source and might also aid in the orientation of the insect over distance.

Sensitivity of receptors for aggregation pheromone component II and the mixture of III + IV was equal to or greater than the sensitivity for any other odorant tested (Figure 14). High sensitivity to these odorants would indicate their role in orientation of the insect over distance. The higher threshold for I for both sexes could be indicative of its activity over shorter distances.

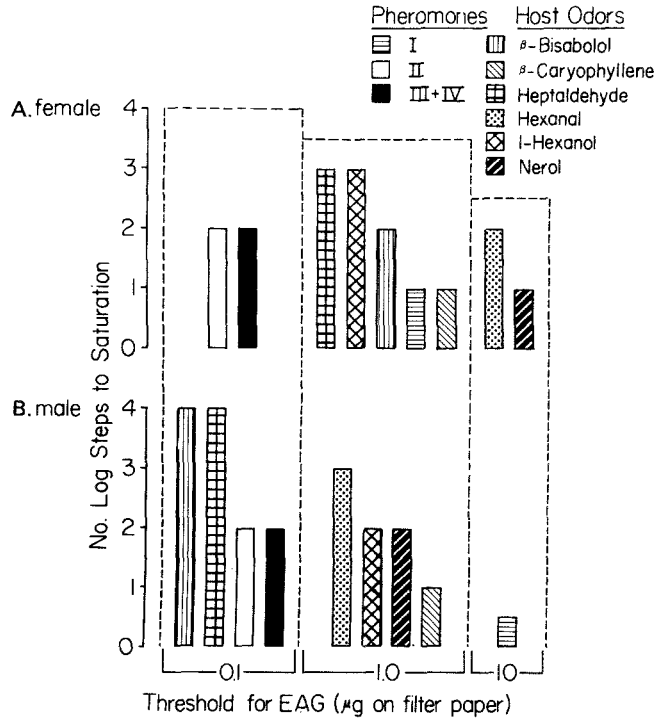

FIG. 14. Graph showing relationship between EAG threshold and number of log steps to acceptor saturation for *A. grandis* pheromones and host-plant odors.

Males are probably the first to emerge in the spring and are possibly responsible for host selection and production of the aggregation pheromone (Cross, 1983). In this regard, males have a low threshold for β-bisabolol and heptanal, both volatile components of the cotton plant (Figure 14) (see Hedin, 1973). Both the low threshold and wide dosage range over which these compounds are perceived might indicate their role in distance orientation by the insect. Also, the greater sensitivity of males relative to females to nerol, a possible pheromone precursor (Thompson and Mitlin, 1979), would facilitate selection by the male of a host suitable for pheromone production. The lower sensitivity of the female to host odors relative to certain pheromone components could coincide with the activity of these chemicals over shorter distances than in the male. In other insects, plant odors have been shown to function as attractants, arrestants, and sexual and ovipositional stimulants (see Städler, 1977).

Even though males were equally sensitive to several components of their aggregation pheromone and certain plant odors, saturation of the EAG to pheromone components occurred over only two log steps for the pheromone as compared to four log steps for the plant odor (Figure 14). It seems likely that the concentration of plant odors in the atmosphere far exceeds that of insect-produced volatiles. Thus, the small but highly sensitive population of acceptors responsive to pheromone (i.e., relative to the larger acceptor population responsive to plant odors) is well adapted to facilitate orientation of the weevil to conspecifics.

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ROLE OF GLANDULAR SCALES OF LEPIDOTE RHODODENDRONS IN INSECT RESISTANCE[1]

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Abstract—Glandular scales on selected lepidote rhododendron species varied in density from 109 ± 13 to 4180 ± 60/cm^2 of leaf surface. Globules contained within the scales stained with Sudan IV, a lipophilic dye. Essential oil contents of the scales varied with species from 24 ± 8 to 151 ± 35 ng/scale. Black vine weevil [(*Otiorhynchus sulcatus* (F.)] feeding on leaves from a sample of rhododendron species was inversely related to leaf essential oil content, and weevil feeding on membrane filters was inhibited by application of essential oil extracts from leaves of most lepidote rhododendrons tested. Results suggest that the glandular scales of the lepidote rhododendrons function, at least in part, in plant defense against insects.

Key Words—Coleoptera, Curculionidae, *Otiorhynchus sulcatus* (F.), black vine weevil, Ericaceae, *Rhododendron,* trichomes, glandular scales, essential oils, volatiles, plant resistance.

INTRODUCTION

Glandular scales of the lepidote rhododendrons were described by DeBary (1884). In 1950, Cowan (p. 107) speculated that in the genus *Rhododendron* ". . .an efficient protection against desiccation is furnished by dendroid, ramiform, rosulate, funnel-shaped, loriform, long rayed, and radiate hairs and the most complete protection by a dense covering of scales." Moreover, Cowan believed that scales could absorb moisture from the atmosphere and

[1]Mention of a trade name or proprietary product does not constitute an endorsement by the U.S. Department of Agriculture, Agriculture Research Service.

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could secrete excess water under conditions of reduced transpiration. Despite the fact that there is no direct evidence to support the contention that rhododendron leaf scales function primarily in regulating water passage into and out of the leaf, this idea has generally been accepted without question (e.g., Leach, 1961, pp. 25–28), although Seithe (1980) stated that scales do not absorb water.

Bell and Clarke (1978) recently published data showing that rhododendron species differed in their resistance to foliar feeding by adult obscure root weevils (*Sciopithes obscurus* Horn), a serious pest of rhododendrons in the Pacific Northwest. Analysis of these data showed that lepidote species (those with leaf scales) were more resistant than elepidote species (Doss, 1980). Hexane-soluble materials from resistant lepidote species inhibited obscure root weevil feeding (Doss, 1980) and, in the case of *R. edgeworthii* Hook., a particulary resistant species, a steam volatile fraction containing, among other compounds, the sesquiterpene germacrone, acted as a weevil feeding deterrent (Doss et al., 1980).

These findings suggest that glandular scales of lepidote rhododendrons may function in plant defense by containing or secreting volatile materials that discourage insect feeding. The study described below was carried out to test this hypothesis.

METHODS AND MATERIALS

Table 1 lists the rhododendrons used in this study. These comprise a geographically and taxonomically diverse sample, including 11 lepidotes, all of which are reported to be resistant to obscure root weevil feeding (Bell and Clarke, 1978; Antonelli and Campbell, 1980), two elepidotes, and an elepidote hybrid. Among the elepidotes, *R. smirnowii* has been reported as resistant to black vine weevil [*Otiorhynchus sulcatus* (F.)] feeding (Valla, 1980; Nielsen and Dunlap, 1981). *R.* "Cynthia" was found to be susceptible to feeding by obscure root weevil (Doss, 1980), and *R. catawbiense,* to feeding by black vine weevil (Nielsen and Dunlap, 1981).

Leaves used in this study, with the exception of those from *R.* "Cynthia," were obtained from the Rhododendron Species Foundation, Federal Way, Washington. "Cynthia" leaves were obtained from a hedge planting at Western Washington Research and Extension Center, Puyallup, Washington.

Glandular scales from a measured area of leaf were removed with a fine-tipped scalpel and placed into a small amount of diethyl either (Harborne, 1973, p. 103). After 30 min, the ether was evaporated under nitrogen and the extracted materials were redissolved in hexane. Leaf volatiles were extracted from small leaf disks into diethyl ether (see above)

TABLE 1. RHODODENDRON SPECIES (AND CULTIVAR) USED FOR ESSENTIAL OIL STUDY

Species (Cultivar)	Clone[a]	Series[b] (subsection)[c]	Geographical distrubution[b,c]	Scale type[d]
1. *R. campylogynum* Franch.	74.76	Campylogynum (Campylogyna)	Western Yunnan, Tibet, Burma	vesicular (L)
2. *R. carolinianum* Rehd.	75.133	Carolinianum (Caroliniana)	Southeastern U.S.	entire (U, L)
3. *R. catawbiense* Michx.	76.12	Ponticum (Pontica)	Southeastern U.S.	
4. *R. chryseum*[e] Balf. f. et Wand	75.28	Lapponicum (Lapponica)	Yunnan to Tibet	undulate (U, L)
5. *R.* "Cynthia"[f]				
6. *R. dauricum* Linn.	66.590	Dauricum (Rhodorasta)	Altai, Korea, Japan	entire (U, L)
7. *R. edgeworthii* Hook. f.	65.383	Edgeworthii (Edgeworthia)	Sikkim, Bhutan	entire (L)[g]
8. *R. ferrugineum* Linn.	76.381	Ferrugineum (Rhododendron)	Pyrenees east to Austrian Alps	entire (L)
9. *R. hanceanum* Hemsl.	76.34	Triflorum (Tephropepla)	Szechwan	entire (U, L)
10. *R. heliolepis* Franch.	65.374	Heliolepis (Heliolepida)	Yunnan	entire (U, L)
11. *R. lepidotum* G. Don	79.53	Lepidotum (Lepidota)	India, Nepal, Bhutan Burma, China	entire to undulate (U, L)
12. *R. rigidum* Franch.	73.353	Triflorum (Triflora)	Yunnan	entire (L)
13. *R. smirnowii* Trautv.	77.319	Ponticum (Pontica)	Caucasus Mountains	entire (U, L)
14. *R. xanthocodon*[h] Hutch.	73.305	Cinnabrinum (Cinnabarina)	Sikkim	

[a]Clone number of Rhododendron Species Foundation, Federal Way, Washington.
[b]See Leach (1961).
[c]See Cullen and Chamberlain (1978) and Cullen (1980).
[d]See Cowan (1960) and Seithe (1980). U = scales on upper surface of leaf, L = scales on lower surface of leaf.
[e]Considered by some as a subspecies of *R. rupicola* W.W. Sm. (Cullen and Chamberlain, 1978).
[f]R. "Cynthia" is a hybrid of *R. catawbiense* Michx. and *R. griffithianum* Wight. *R. griffithianum* is a member of the Fortunei series (Fortunea subsection).
[g]*R. edgeworthii* possesses a few vestigal scales on the upper leaf surface.
[h]Considered by some as a subspecies of *R. cinnabarinum* Hook (Cullen 1980).

or obtained by steam distillation from leaves using a modified Nielsen-Kryger apparatus with hexane as the organic solvent (Veith and Kiwus, 1977). Leaf disks or leaves of lepidote rhododendrons used for extraction bore scales. Scale densities were determined by counting them on 5.5-mm-diameter leaf disks cut from four separate leaves.

The amount of essential oils present in the scales was estimated by gas chromatography from ether extracts made using small leaf disks. A 1.9-m × 2-mm (ID) glass column packed with 3% (w/w) SP2100® on 100–120 mesh

Supelcoport® was used. Nitrogen was employed as the carrier gas (30 cm^3/min). The flame ionization detector and the injector were maintained at 260°C. Column temperature was increased from 100 to 200°C at a rate of 5°C/min with initial and final temperatures held for 2 min.

A nerolidol standard allowed estimation of the amount of essential oils present in the leaf extract. Gas chromatographic estimates were checked against estimates obtained by weighing materials obtained by steam distillation. Gas chromatographic traces obtained with extracts prepared from detached scales were compared with traces obtained with extracts of leaf disks.

Leaf cross-sections (50 μm) cut using a vibrating microtome (Vibratome®) were stained using the lipophilic dye Sudan IV (Jensen, 1962, pp. 264–265) and mounted in glycerine jelly (Sass, 1958, pp. 102–103). Detached scales were mounted without staining.

Black vine weevil colonies were maintained at 20°C under either continous darkness or photoperiodic cycles consisting of 16 hr of fluorescent light and 8 hr of darkness. Weevil colonies were fed leaves from either "Alpine" strawberry (*Fragaria vesca* L.) or "Totem" strawberry (*F.* × *ananassa* Duch.).

To determine the feeding response of black vine weevil adults to the rhododendron species tested, leaf disks 14.4 mm in diameter were cut using a notched cork borer that left the petiole attached to the leaf disk. Petioles were inserted through a serum stopper into vials containing water. A moist dental wick, four black vine weevils, and a vial with a leaf disk were placed into each of the cylindrical carboard containers (about 250 cm^3 in volume) used as bioassay arenas (Bristow et al., 1979). Bioassays were run for 24 hr at 20°C, after which time the areas eaten from the leaves were measured. With *R. chryseum* (leaf area = 93 ± 6 mm^2, X ± SE for four leaves), *R ferrugineum* (leaf area = 146 ± 10 mm^2, for five leaves), and *R. lepidotum* (leaf area = 188 ±7 mm^2, for five leaves), intact leaves, instead of leaf disks, were used because leaves of these species were small. With other species, leaf disks rather than intact leaves were used to eliminate any effect of leaf size on weevil feeding.

The influence of essential oils on black vine weevil feeding was determined by using a membrane filter bioassay procedure (Doss, 1980). Membrane filters were first pretreated with 50 mg-equivalents of an ethanolic extract of "Alpine" strawberry leaves that stimulated black vine weevil feeding (Doss and Shanks, unpublished). Then 1 disk treated with the strawberry extract and 1 disk treated with the extract plus a rhododendron essential oil extract (from a portion of leaf equivalent in area to a 13-mm-diameter membrane filter) were placed into a bioassay arena along with a moist dental wick and four adult black vine weevils. Bioassays (choice tests)

were run at 20° C for 24 hr. Weevil feeding on the filters was compared using the paired *t*-test.

RESULTS

Histological examination indicated that rhododendron leaf scales contained lipophilic globules (Figure 1). Gas–liquid chromatographic traces obtained using extracts made from scales and from leaf disks were qualitatively identical. Figure 2 shows such a trace obtained with an extract of *R. dauricum* leaves. Extracts made using detached scales contained less volatile material (on an area basis) than leaf disks. Some oil was contained in the scale stalk which remained attached to the leaf upon scale removal (Figure 1). Of course, the leaves, exclusive of the scales, also contained volatiles. The 11 lepidotes yielded much larger volatile fractions, averaging about 100,000 ng/cm^2 of leaf, than the elepidotes which averaged about 12,000 ng/cm^2. *R. smirnowii,* a heavily indumented elepidote, yielded more volatiles than three of the lepidote species.

Essential oil contents varied with species from about 25 to 150 ng/scale for the lepidotes examined (Table 2). The values reported here were estimated gas chromatographically and are, on average 60 ± 10% (X ± SE) of the values obtained by weighing steam distillate, except for *R.* "Cynthia," The estimate obtained for this cultivar by weighing steam distillate was 1.3 mg/g fresh wt. In an earlier study (Doss et al., 1980), a value of 0.64 mg/g fresh wt was obtained for *R.* "Cynthia," suggesting that the gas chromatographic value reported in Table 2 (equivalent to 0.008 mg/g) could be a serious underestimate. Note that values were calculated assuming that all essential oils were contained within the scales even though some volatiles are present on nonscale-bearing leaves.

There was a significant negative correlation between the areas eaten from leaf disks (or small leaves) and the volatile oil content (Figure 3). This was also true when only the lepidote species were considered ($r = -0.76$ with 8 df). The estimates of essential oil contents used in this analysis were obtained by weighing steam distillate, but using estimates obtained gas chromatographically resulted in a similar relationship and correlation coefficient ($r = -0.70$). The species *R. heliolepis* was omitted from this study through oversight.

In choice tests with membrane filters, nine of the 11 lepidote species yielded extracts that inhibited weevil feeding (Table 3). None of the elepidote species yielded inhibitory extracts and, with the lepidotes, noninhibitory extracts came fron species with relatively small amounts of essential oils. In no-choice tests (not discussed in Materials and Methods), using only 1 membrane filter disk per arena, there was large variation in feeding. The

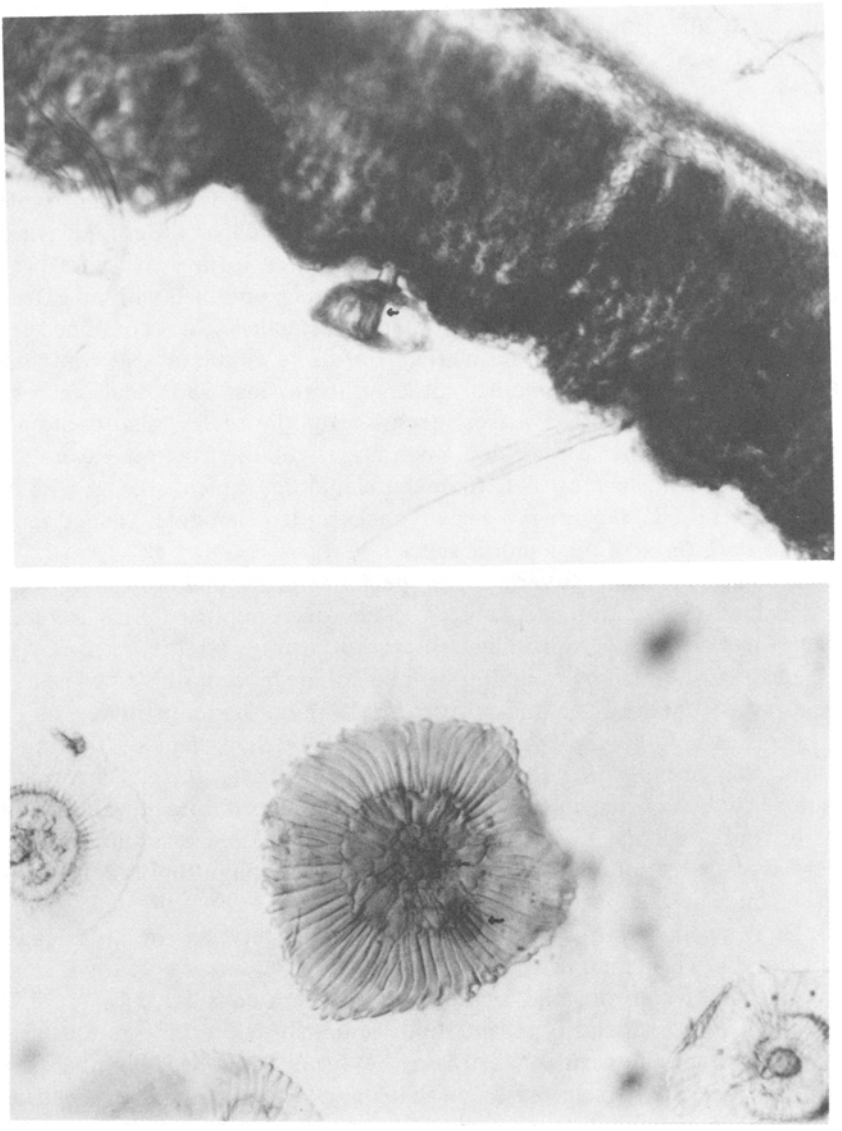

FIG. 1. (top) Cross-section through a *Rhododendron edgeworthii* leaf showing glandular scale. Scales are about 110 μm in diameter. (bottom) Detached scale (300 μm in diameter) from *R. chryseum.* Oil droplets are indicated by arrows.

correlation between areas eaten by black vine weevils from phagostimulant-bearing membrane filters treated with essential oil extracts and the essential oil contents of the leaves was not significant with the no-choice tests (data not shown).

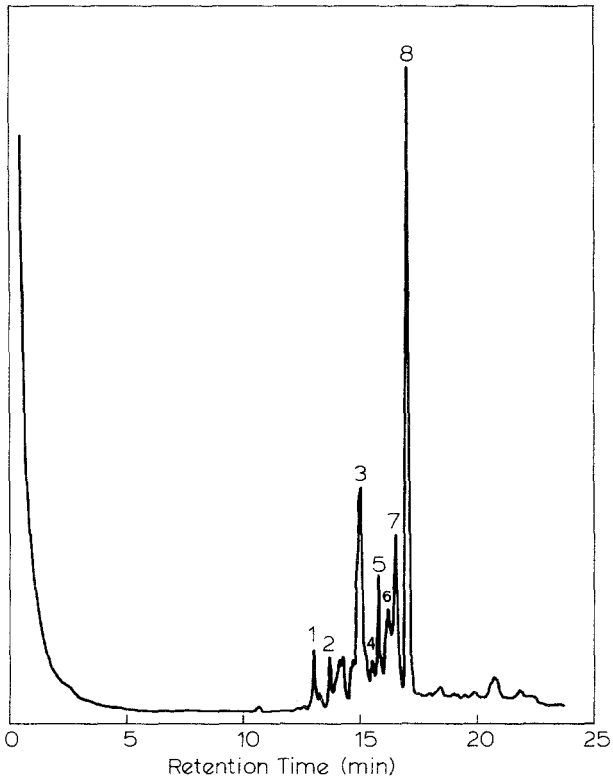

FIG. 2. Gas chromatographic trace of *Rhododendron dauricum* essential oil extract. The probable identities of the materials giving rise to the number peaks are as follows: 1. β-caryopheyllene, 2. α-humulene, 3. *cis*-nerolidol, 4. *trans*-nerolidol, 5. β-elemenone, 6. γ-eudesmol, 7. α/β-eudesmol, 8. germacrone.

DISCUSSION

The role of various types of trichomes in conferring insect resistance upon plants is well documented (Levin, 1973; Webster, 1975; Stipanovic, 1983). In fact, it has been suggested that the presence of trichomes is more often associated with defense against insect attack than with reduction of water loss, a commonly suggested function of plant trichomes (Fitter and Hay, 1981, p. 283).

With *Rhododendron,* Valla (1980) noted that indumented and lepidote types suffered less adult black vine weevil feeding damage than did glabrous leaf types. Data presented by Bell and Clarke (1978) suggested that the lepidote rhododendrons were more ofter resistant to obscure root weevil feeding than elepidotes (Doss, 1980).

With respect to the lepidote rhododendrons, results presented here

TABLE 2. ESSENTIAL OIL CONTENTS OF 11 LEPIDOTE AND 3 ELEPIDOTE RHODODENDRON SPECIES (AND CULTIVAR) MEASURED FROM EXTRACTS OF LEAF DISKS

Species (cultivar)	Leafweight (mg/cm²)[a]		Scales per cm²		Essential oils[c]	
	Fresh	Dry	Upper surface	Lower surface	ng/scale	ng/cm²
R. campylogynum	21.8 ± 0.8	12.6 ± 0.4	0	315 ± 21	65 ± 8	20600 ± 2100
R. carolinianum	22.7 ± 0.8	10.9 ± 0.8	172 ± 29	1206 ± 59	86 ± 12	118000 ± 15000
R. catawbiense	27.3 ± 1.3	13.9 ± 0.4	no scales			1656 ± 221
R. chryseum	21.4 ± 0.8	11.4 ± 0.4	1916 ± 39	2273 ± 46	43 ± 9	180000 ± 39000
R. 'Cynthia'	44.1 ± 3.4	22.7 ± 1.7	no scales			357 ± 72
R. dauricum	22.3 ± 0.4	11.4 ± 0.4	122 ± 29	798 ± 63	128 ± 30	118000 ± 25000
R. edgeworthii	34.5 ± 1.3	18.9 ± 0.4	0	2168 ± 25	151 ± 35	328150 ± 75000
R. ferrugineum	29.0 ± 0.8	13.9 ± 0.8	0	1409 ± 101	70 ± 7	98900 ± 5900
R. hanceanum	34.9 ± 0.4	15.6 ± 0.4	59 ± 4	219 ± 4	128 ± 25	35600 ± 7000
R. heliolepis	26.9 ± 0.8	10.9 ± 0.4	399 ± 29	412 ± 17	26 ± 3	21400 ± 2000
R. lepidotum	16.0 ± 0.8	6.7 ± 0.4	614 ± 17	672 ± 25	25 ± 8	311000 ± 10100
R. rigidum	21.7 ± 1.7	11.4 ± 0.8	0	109 ± 13	32 ± 5	3430 ± 400
R. smirnowii	32.4 ± 0.4	16.8 ± 0.4	no scales			27400 ± 5000
R. xanthocodon	22.7 ± 1.3	11.4 ± 1.3	538 ± 118	1370 ± 50	71 ± 8	136000 ± 12000

[a]Means ± standard errors for five samples.
[b]Means ± standard for four samples.
[c]Means ±standard errors for four extracts. Note that ng/scale values ignore volatiles present in the leaf lamina and are thus overestimates (see Results).

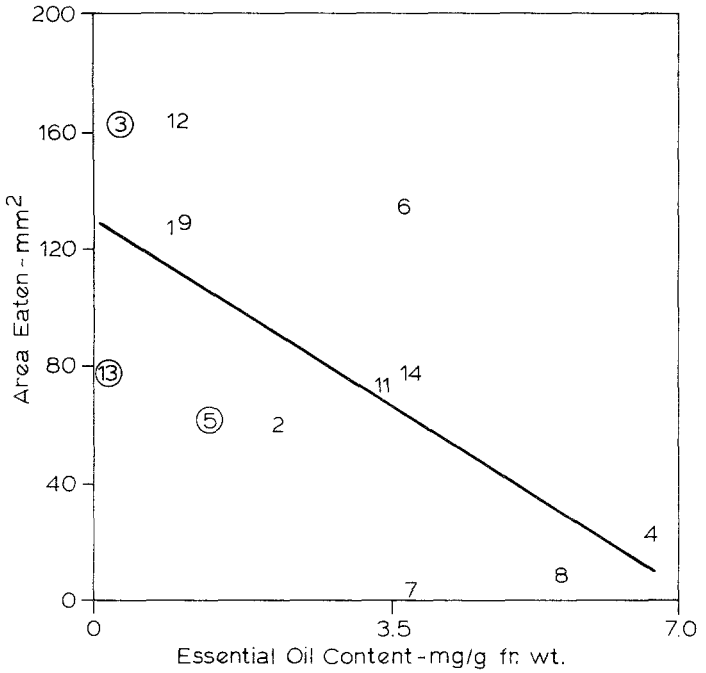

FIG. 3. Areas eaten from *Rhododendron* leaves and essential oil contents. Numbers indicate species as listed in Table 1. Circled numbers represent elepidotes. The line represents the best fit linear estimate of the relationship and is given by the formula: area eaten = −17.9 (essential oil content) + 131.3 ($r = -0.69$ with 11 df).

show that the scales contain essential oils which can inhibit black vine weevil feeding. The compositions of these oils are complex (Figure 2). Sesquiterpenes usually comprised the most prominent components of the oils so far examined, with some compounds common to many species (Figure 2; Doss, unpublished). Work to characterize the essential oils from a number of lepidote rhododendrons is in progress.

Among the elepidotes studied, *R. smirnowii* contained substantial amounts of volatile materials (Table 2). The source of these compounds is unknown. The lower surfaces of leaves of this species are tomentose, and it is possible that oils are present within or upon the hairs. It is interesting that *R. smirnowii* has been reported by some to be resistant to black vine weevil feeding (Valla, 1980; Nielsen and Dunlap, 1981).

Although compounds present in the essential oils of several lepidote species have been indentified (Puzulevskiĭ and Belova, 1959, 1974; Hsu and Yu, 1976; Lab Resources, Quinghai Sheng Inst. Biol. Lab Phytochem. Inst. Biol., 1978; Doss et al., 1980), in none of these studies were the scales shown to be the source of the oils. Seithe (1960) recognized the glandular nature of

TABLE 3. BLACK VINE WEEVIL FEEDING BIOASSAY OF ESSENTIAL OIL EXTRACTS

Species	Area eaten (mm^2)[a]		Paired *t*-statistic[b]
	+Essential oil	−Essential oil	
R. campylogynum	43.2	56.5	−2.81
R. carolinianum	5.1	38.1	−2.96
R. catawbiense (E)[c]	42.0	51.1	−1.59
R. chryseum	0.7	38.6	−5.48
R. "Cynthia" (E)[c]	28.0	26.2	0.43
R. dauricum	5.6	53.1	−4.10
R. edgeworthii	29.7	68.0	−3.29
R. ferrugineum	4.7	29.1	−2.97
R. hanceanum	20.6	56.9	−3.15
R. heliolepis	8.6	21.8	−1.97
R. lepidotum	5.7	58.4	−5.92
R. rigidum	28.8	42.1	−0.85
R. smirnowii (E)[c]	25.8	30.0	−0.77
R. xanthocodon	14.9	45.5	−3.24

[a]Means for five arenas each containing one 1.3-cm-diameter filter disk bearing 50 mg-equivalents of a phagostimulatory extract made from *Fragaria vesca* leaves and a filter disk bearing the *F. vesca* extract plus essential oils extracted from 1.3 cm^2 of rhododendron leaf.
[b]$t_{0.05}$ with 4 df = 2.78; $t_{0.01}$ with 4 df = 3.75.
[c]E = elepidote.

scales and characterized them as producing nonsticky (*nicht klebig*) secretions. Doss et al. (1980) speculated, without evidence, that the scales were the source of volatile terpenes that could inhibit obscure root weevil feeding. Nevertheless, even though DeBary (1884) noted that rhododendron scales contained "ethereal" oils, subsequent authors have not attempted to relate the pungent leaf odors of some of the lepidote rhododendrons to the presence of scales.

A specific volatile component from *R. edgeworthii* leaves inhibited obscure root weevil feeding (Doss et al., 1980). However, considering the complexity of the essential oils and the fact that black vine weevil feeding is inversely related to total essential oil content, it seems likely that a number of volatile components can inhibit feeding. Unpublished results suggest that there are differences in the potency of various volatile compounds in inhibiting black vine weevil feeding.

Rhododendron scales contain secretion pores (Seithe, 1980) through which volatile compounds could (presumably) escape. This raises the question as to whether inhibition of black vine weevil feeding results from

action of the oils as feeding deterrents or repellents. Attempts to clarify this matter through studies with an olfactometer yielded equivocal results (Doss, unpublished). Weevils fed on scaled leaves (Figure 3) and on membrane filters bearing essential oil extracts (Table 3), suggesting that if repellency was involved it was not absolute.

Black vine weevil was chosen for the study because of its ready availability and because resistance to feeding by this polyphagous leaf eater should be a good indicator that resistance is of a "general type" (Levin, 1976), affecting a number of potential pest insects. Hence, although the black vine weevil is native to Europe (Smith, 1932) and may not be found in southeastern Asia where many of the *Rhododendron* species are indigenous (Leach, 1961), resistance to this insect, and to the obscure root weevil (Bell and Clarke, 1978; Doss, 1980), suggests that terpene compounds in rhododendron scales responsible for resistence could function to deter feeding by other leaf-eating insects.

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IDENTIFICATION OF ANT REPELLENT ALLOMONE PRODUCED BY SOCIAL WASP *Polistes fuscatus* (HYMENOPTERA: VESPIDAE)

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Abstract—Two active components were isolated from the ant repellent secretion produced by glandular cells on the sixth (terminal) gastral sternite of *Polistes fuscatus* (F.) females. One of the components was identified as methyl palmitate. Field bioassays with synthetic methyl palmitate showed it had repellent activity against *Solenopsis geminata* (F.) *Forelius pruinosus* (Roger), and *Pheidole* sp. The effectiveness of the repellent was greater against *F. pruinosus* and *Pheidole* sp. than against *S. geminata.* Four methyl ester homologs of methyl palmitate also were field tested for repellent activity: methyl myristate had repellent activity against all three ant species, whereas methyl stearate, methyl linoleate, and methyl linolenate exhibited no repellency.

Key Words—Social wasp, *Polistes fuscatus,* Hymenoptera, Vespidae, Formicidae, ant repellent, defensive allomone, fatty acid esters, methyl myristate, methyl palmitate

INTRODUCTION

Defensive allomones are synthesized by many arthropods (Blum, 1981). In the Hymenoptera exocrine products ranging from hydrocarbons to acids have defensive functions in sawflies, ants, and bees. In contrast, little is known of the defensive chemistry of wasps, except for the venom. Female social wasps of the genera *Mischocyttarus, Polistes,* and *Ropalidia* rub a secretion onto their nest which repels foraging ants (Jeanne, 1970; Turillazzi and Ugolini, 1979; Post and Jeanne, 1981; Kojima, 1983). The repellent is produced by glandular cells associated with an external brush of hairs at the base of the sixth (terminal) gastral sternite (Turillazzi, 1979; Post and

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Jeanne, 1980; Jeanne et al., 1983). Its chemical nature, rather than its physical properties, appears to cause the repellency (Jeanne 1970). In this paper we report on the isolation and identification of one of two active components of the repellent produced by *Polistes fuscatus* (F.).

METHODS AND MATERIALS

Collection and Extraction of Crude Secretions. Crude secretions were collected using two methods. For the first method, colonies of *P. fuscatus,* collected in Dane County, Wisconsin, from July to September were housed in Plexiglas® cages under conditions similar to those in the field (20°C scotophase, 25–30°C photophase; with a photoperiod changing from 15:9 to 12:12). At 3- to 4-day intervals the females were immobilized on ice and the secretions were collected by rubbing filter paper disks (1.27-cm-diameter antibiotic disks, Schleicher and Schuell Inc.) gently over the exposed glandular area. These disks (1850 secretions from 250 wasps) were immersed in a 50:50 mixture of hexane and benzene in a 250-ml Erlenmeyer flask and agitated on a rotary shaker for 24 hr. For the second method, at the end of the nesting season in October 300 females were killed by freezing and their terminal sternites (with the glandular cells) excised. These were refluxed in a 50:50 mixture of hexane and benzene. Extracts from each source were filtered over anhydrous sodium sulfate, concentrated using a Büchi evaporator, and then diluted to yield either one female equivalent secretion per microliter or one glandular extract per microliter.

Chromatography and Analysis of Crude Material. Extracts from each source were subjected to adsorption column chromatography (column size 45 × 2 cm) using a slurry of silica gel 100 in chloroform. Hexane (55 ml), benzene (100 ml), and methanol (100 ml) were used sequentially to elute the fractions. The fractions were compared to the crude secretions using thin-layer chromatography (TLC) with self-charring silica gel plates impregnated with 5% ammonium sulfate (Analtech Uniplates) and developed in hexane. Repellent activity was quantified for each fraction (see Procedures for Bioassays).

The active fraction was fractionated further by gas–liquid chromatography (GLC) (Spectra Physics 7100) equipped with a flame ionization detector and a variable effluent splitter (SGE Inc.). Columns used were as follows: (1) 3% OV-1 on 80–100 mesh Gas Chrom Q (5 ft × $\frac{1}{8}$ in., stainless steel), N_2 at 30 ml/min; (2) 3% OV-101 on 100–120 mesh Chromosorb WHP (2 ft × $\frac{1}{8}$ in., stainless steel), N_2 at 30 ml/min; (3) 12-m × 0.25-mm FSOT OV1-SE30 capillary column with a head pressure of 12 psi and a N_2 flow of 5 ml/min. All columns were operated as follows: 40°C (5 min); 10°C/min to 145°C (hold for 10 min); and 10°C/min to 315°C (hold for 60 min).

Preliminary qualitative tests for repellent activity were conducted with the effluents from column 1. For further quantitative tests we used the effluents from column 2. We used column 3 to determine the resolution of each component. Effluents were collected in Pasteur pipets encased in Dry Ice held in place by a conical glass jacket. Each effluent was bioassayed within its pipet (see Procedures for Bioassays).

Active components were desorbed with several washes of 1 ml hexane. The components were concentrated under argon and subjected to electron ionization mass spectrometry (Finnigan 4150 GC-MS) at 70 eV. A 15-m × 0.25-mm Durabond-5 capillary column (J&W Scientific Inc.) with a 10 psi head pressure and a flow of N_2 of 5 ml/min with a temperature program of 50°C (1 min) to 270°C at 20°/min was used in the analyses. The identification of one of two active components was based on the mass spectral data and a comparison of the retention times (using an OV-101 column and an OV-SE30 capillary column) with that of known compounds.

Procedures for Bioassays. Bioassays of the effectiveness of chromatographic fractions in repelling ants were conducted in the laboratory using colonies of *Lasius neoniger* Emery collected in Dane County, Wisconsin, and *Forelius pruinosus* (Roger) collected in Cleveland County, North Carolina. The colonies were housed in glass tubes (20 cm long × 10 mm internal diameter) plugged at one end with moistened cotton, and placed in open plastic boxes (25 × 38 × 15 cm).

Prior to conducting the bioassays, the ant colonies were starved for 4–5 days. Honey and a macerated wax moth larva [*Galleria mellonella* (L.)] were placed on the bottom of an inverted dish (2.8 cm deep × 6.0 cm in diameter) in a Petri plate flooded with water so as to form a moat. Ants were allowed to discover the food and to recruit nestmates to it via a capillary tube or Pasteur pipet (both 10 cm in length) bridging the moat to another similar dish placed upside down, without a moat. Once a large number of ants were foraging, the clean tube was replaced with one containing test material. Test material from adsorption column chromatographic fractions was placed in a band (1 cm wide) on the external surface in the middle of the capillary tube and allowed to dry for 15 min. Gas chromatographic fractions, collected directly into the Pasteur pipets (see Chromatography and Analysis of Crude Material), were bioassayed within the pipets. To force the ants to walk through the inside of the tube, we broke off the narrow tip of the Pasteur pipet and applied a ring of Tanglefoot® to the outer surface. All bioassays were conducted under a heat lamp at 30–32°C.

The response of the ants to the test material was quantified by recording for each replicate the number of ants that turned around on reaching the test material. Each replicate continued until 10 ants crossed the test material or for 30 min, whichever occurred first.

Field bioassays were conducted using synthetic methyl palmitate

($C_{17}H_{34}O_2$) (the identified active component) and the following methyl ester homologs of methyl palmitate: methyl stearate ($C_{19}H_{38}O_2$), methyl linoleate ($C_{19}H_{34}O_2$), methyl linolenate ($C_{19}H_{32}O_2$) and methyl myristate ($C_{15}H_{30}O_2$) (Sigma Chemical Co.). An apparatus similar to that described previously for the laboratory bioassays was used. The synthetic materials were dissolved in hexane and placed in a 1-cm band around the inside walls of Pasteur pipets. The chemicals were allowed to air dry for 15 min prior to each bioassay. Repellency of these compounds was tested against three species of ants in the southeastern United States: *Solenopsis geminata* (F.), in Manchester State Forest, Sumter County, South Carolina, and *F. pruinosus* and *Pheidole* sp., 10 km NE of Shelby, Cleveland County, North Carolina.

RESULTS

In the bioassay of column chromatographic fractions, significantly more ants were repelled by the benzene fraction ($R_f = 0.14$, TLC) than by the control (Table 1). Linear regression of the number of ants repelled (logarithmic values) against concentration for the benzene fraction showed increasing activity with concentration ($Y = 0.814 + 0.035x$; $r^2 = 0.69$; df = 22; $P < 0.001$). No significant activity was observed with the hexane and methanol fractions ($R_f = 0.41$ and $R_f = 0$, respectively).

Fractionation of the benzene fraction (20 equivalent secretions) by

TABLE 1. BIOASSAY OF REPELLENCY OF ADSORPTION COLUMN CHROMATOGRAPHIC FRACTIONS OF CRUDE *P. fuscatus* SECRETIONS AGAINST ANT *Lasius neoniger* IN THE LABORATORY[a]

No. of female equivalent secretions	No. of ants repelled	
	Benzene	Control[b]
0.5	2.7 ± 0.9	0.0
2.5	8.3 ± 3.0	0.6 ± 0.7
5.0	9.0 ± 1.6	0.3 ± 0.3
7.5	11.3 ± 4.0	1.3 ± 0.3
10.0	38.0 ± 13.9	0.6 ± 0.4
12.5	24.0 ± 3.3	0.3 ± 0.3
25.0	155.3 ± 29.6	0.3 ± 0.3
50.0	185.0 ± 46.0	0.0

[a]Data are the mean (± SE) numbers of ants repelled before ants crossed each fraction at each female equivalent.
[b]Controls consisted of a mixture of equal parts of benzene, methanol, and hexane.

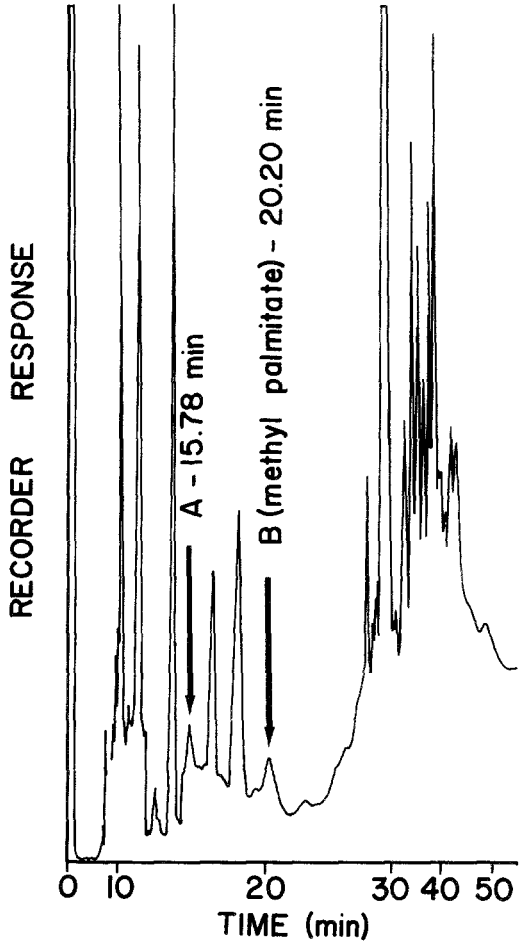

FIG. 1. Gas chromatogram of the benzene fraction of 10 equivalent secretions produced by glandular cells on the terminal gastral sternite of *Polistes fuscatus* females. The two active peaks are labeled A and B. Column used was 2 ft $\times \frac{1}{8}$ in. 3% OV-101 (100–120 mesh Chromosorb WHP). Chromatograph conditions were as follows: 40°C (5 min), 10°C/min to 145°C (10 min), and 10°C/min to 315°C (60 min). The sample was run at a sensitivity of 32×10^{-10} AFS for 5 min, 2×10^{-11} AFS to 12 min, and 16×10^{-11} AFS for the remainder of the run. Chart speed was as follows: 0.1 cm/min to 10 min, 0.25 cm/min to 25 min, and 0.1 cm/min to 60 min.

GLC on an OV-1 column yielded an active area between 10 and 20 min. Individual components in this region were further fractionated on an OV-101 column yielding one active peak at 15.78 min and another at 20.20 min (Figure 1). These peaks were present in approximately 5 and 8 ng/female

equivalent secretions, respectively. A mean of 23.3 ± 6.0 ($N = 15$ replicates) and 20.3 ± 6.2 ($N = 15$ replicates) ants were repelled by 11.3 female equivalent secretions (final yield from fractionation sequence) of the former and latter peaks, respectively. Bioassays of other peaks, either singly or in combinations, yielded no repellency. The material collected from the excised sternites of females at the end of the nesting season (October) yielded identical active peaks, although the quantity of each component was much less than in wasps collected in July and August.

Gas chromatography–mass spectrometry (electron impact) revealed that the two active components had peak retention times of 8.92 min and 10.36 min on a DB-1 column. Diagnostic fragments indicated that the latter component was a saturated fatty acid methyl ester: m/z (relative percent), 55 (35), 74 (100), 87 (60), 227 (6), 239 (4), 270 (5). Given the molecular ion at 270 and the ion at M−31 indicating a methyl ester, the molecule was identified as methyl hexadecanoate, $C_{17}H_{34}O_2$ (methyl palmitate). The spectral details for this component were identical to those obtained from an authentic specimen (Sigma Chemical Co.) and those reported (Stenhagen et al., 1974). Also, this ester corresponds identically to that observed in the natural product at 20.2 min on the OV-101 column.

Synthetic methyl palmitate had a repellent effect in the field against *F.*

TABLE 2. FIELD BIOASSAY OF METHYL PALMITATE FOR REPELLENCY AGAINST ANTS, *Forelius pruinosus, Pheidole* sp., AND *Solenopsis geminata*[a,b]

Concentration of methyl palmitate (ng/100 μl solvent)[c]	Species of ant		
	Forelius pruinosus	*Pheidole* sp.	*Solenopsis geminata*
4	1.0 ± 1.0 (10)	2.7 ± 0.9 (10)	2.7 ± 0.7 (10)
8	2.0 ± 1.0 (10)	0.7 ± 0.6 (10)	4.0 ± 2.1 (10)
12	3.7 ± 3.7 (10)	3.7 ± 3.7 (10)	0 (10)
24	2.7 ± 2.2 (10)	0 (10)	0.3 ± 0.3 (10)
48	1.7 ± 1.2 (10)	3.7 ± 1.8 (10)	1.7 ± 0.7 (10)
96	25.3 ± 22.9 (1.3 ± 1.8)a	1.3 ± 0.7 (10)b	2.0 ± 0.6 (10)b
192	44.0 ± 21.5 (1.3 ± 1.8)a	8.7 ± 3.8 (10)b	9.0 ± 2.0 (10)b
384	20.0 ± 10.7 (0)a	15.3 ± 7.7 (8.0 ± 2.0)b	7.7 ± 3.2 (10)b
768	12.3 ± 2.4 (0)a	16.9 ± 9.1 (0)a	16.3 ± 8.4 (10)b
1000	7.0 ± 2.3 (0)a	10.0 ± 3.2 (0)a	19.0 ± 11.0 (10)b

[a]Data are the mean (± SE) numbers of ants repelled before 10 ants crossed the methyl palmitate barrier or for 30 min, whichever occurred first. Numbers in parentheses are the mean numbers (±SE) of ants crossing the barrier. $N = 3$ replicates for each concentration for each species.

[b]At each concentration means in parentheses with different letters are significantly different from each other (LSD test: $df = 8$; $P < 0.05$).

[c]During some bioassays at concentrations >100 ng/100 μl solvent, few or no ants entered the pipet, even though many walked around the entrance of the pipet, and therefore few ants were recorded as repelled.

TABLE 3. FIELD BIOASSAYS OF FOUR METHYL ESTER HOMOLOGS OF METHYL PALMITATE AGAINST THREE SPECIES OF ANTS[a,b]

Concentration of methyl ester homologs (ng/100 μl solvent)	Species of ant		
	Forelius pruinosus	*Pheidole* sp.	*Solenopsis geminata*
Myristate			
50	8.0 ± 5.3a (6.3 ± 1.9)d	10.3 ± 4.4a (3.7 ± 1.2)d	6.0 ± 3.1a (10)e
100	9.7 ± 5.3a (0.7 ± 0.7)d	26.0 ± 9.5a (1.0 ± 0.6)d	48.0 ± 9.1b (2.7 ± 0.3)e
200	13.3 ± 5.3a (0)	4.7 ± 1.2a	43.3 ± 8.7b (0)
Stearate			
Linoleate			
Linoleneate			
50	0 (10)	0 (10)	0 (10)
100	0 (10)	0 (10)	0 (10)
200	0 (10)	0 (10)	0 (10)

[a]Data are the mean (± SE) number of ants repelled before 10 ants crossed each methyl ester barrier or for 30 min, whichever occurred first. Numbers in parentheses are the mean numbers (±SE) of ants crossing the barrier. $N = 3$ replicates for each species tested against each concentration per homolog.
[b]At each concentration means followed by the same letter are not significantly different from each other (LSD test: df = 6: $P > 0.05$).

pruinosus, S. geminata, and *Pheidole* sp. Its effectiveness as a repellent was a function of concentration, being most active at or above 12 female equivalent secretions, or 100 ng/100 μl solvent (Table 2). Although there was no difference between the three species of ants in the mean numbers repelled at each concentration tested (ANOVA: $P > 0.05$), there were species differences in the number crossing the methyl palmitate band (Table 2). At concentrations greater than 100 ng/100 μl solvent, few or no ants of *F. pruinosus* and *Pheidole* sp. entered the pipet, even though numerous ants were active near the open end of the pipet. Moreover, during these trials some ants carried small grains of sand and placed them on the Tanglefoot® barrier on the outside of the pipet. On the other hand, 10 *S. geminata* crossed the band within 30 min at all concentrations. The higher homologs of methyl palmitate (methyl stearate, methyl linoleate, and methyl linolenate) did not show repellent activity, whereas the lower homolog, methyl myristate, was active against all three species (Table 3).

DISCUSSION

This is the first demonstration of methyl palmitate as a defensive chemical in insects. Methyl palmitate also has been found in the Dufour's gland secretions of the ant *Lasius alienus* Emery (Bergström and Löfqvist,

1970) and the carpenter bee *Xylocopa virginica* (L.) (Vinson et al., 1978). During defense and attack behavior, *L. alienus* sprays Dufour's gland secretion, as well as secretion from the poison gland, on the bodies of enemies (Regnier and Wilson, 1969). Although formic acid is the major defensive component in *L. alienus,* results of our study suggest that methyl palmitate may also play an active role. In *X. virginica* a secretion containing methyl palmitate is deposited on flowers by foraging bees, where it causes foraging conspecifics to avoid recently marked flowers (Frankie and Vinson, 1977; Vinson et al., 1978). However, the possibility that it may be a defensive compound in *X. virginica* has not been investigated.

As in previous tests with the crude secretion (Post and Jeanne, 1981), the effectiveness of methyl palmitate in repelling ants varied with the ant species. Clearly, methyl palmitate was not as effective in repelling *S. geminata* as it was in repelling *Pheidole* sp. and *F. pruinosus.* There are at least two possible reasons for this difference: (1) The field bioassays with *S. geminata* were conducted under higher ambient temperatures (28–33° C) than were those with *Pheidole* sp. (23–26° C) and *F. pruinosus* (25–30° C). Thus the test substance may have evaporated faster during tests with *S. geminata* than with the other species. However, this does not explain why methyl palmitate was more active against *F. pruinosus* than against *Pheidole* sp. or why methyl myristate was more active against *S. geminata* than against the other two species. (2) There may be physiological and/or behavioral differences among the species that make *S. geminata* less responsive to methyl palmitate.

The chemical barrier on the nest petiole of *P. fuscatus* is maintained throughout the colony cycle, with females continually producing secretion and adding it to the petiole on average once every 1–3 hours (Post and Jeanne, 1981; Downing and Jeanne, 1983). It is most important early in the nesting season, prior to the emergence of workers, both because the nest frequently must be left unattended while the founding females forage (Post and Jeanne, 1981) and because this is when ant foraging rates are highest (Post and Jeanne, 1982). At daytime temperatures of 25–30° C, when the adults are foraging, a molecule of relatively low volatility, such as methyl palmitate (mp 29.5° C), would be effective in maintaining a relatively long-lasting chemical barrier. During cool days and nights, when females are on the nest and actively defend it against ants, methyl palmitate would evaporate even less rapidly, thereby conserving the material. In addition, other components in the secretion may act to reduce the rate of evaporation, and thus enhance the effectiveness of methyl palmitate.

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INFLUENCE OF MUSTELID SCENT-GLAND COMPOUNDS ON SUPPRESSION OF FEEDING BY SNOWSHOE HARES (*Lepus americanus*)

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Abstract—This study investigated the influence of mustelid anal-gland compounds in suppressing feeding by snowshoe hares on coniferous tree seedlings. Pen and field bioassays indicated that 3-propyl-1,2-dithiolane from the stoat (*Mustela erminea*), and secondarily, 2,2-dimethylthietane from the mink (*M. vison*) had a very negative effect on feeding behavior of hares. The major component of stoat anal gland secretions, 2-propylthietane, and the related compounds, thietane and 2-methylthietane, were not effective. 3,3-Dimethyl-1,2-dithiolane from the least weasel (*M. nivalis*) and ferret (*M. putorius*) and di-*n*-propyldisulfide (acyclic analog of 3-propyl-1,2-dithiolane) similarly did not affect hare feeding. 3-Propyl-1,2-dithiolane and 2,2-dimethylthietane (also found in *M. erminea*) may act as interspecific chemical signals which induce a fear or avoidance response in hares. Such compounds have outstanding potential as area repellents to reduce crop and livestock depredations. Our study reports one of the first practical utilizations of mammalian semiochemicals in crop protection and wildlife management.

Key Words—Scent-gland compounds, thietanes, dithiolanes, sulfur-containing compounds, mustelids, snowshoe hare, lodgepole pine, forestry, crop protection, feeding suppression, interspecific communication, kairomones, *Lepus americanus, Pinus contorta.*

INTRODUCTION

The impact of predator odors on the behavior of mammalian prey has been outlined by several authors (Muller-Schwarze, 1972, 1983; Henessey and

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Owings, 1978; Cattarelli and Chanel, 1979; Stoddart, 1980a; Boonstra et al., 1982; Sullivan et al., 1984a,b). In general, these studies have been short-term in duration and have used crude formulations of predator odors such as feces, urine, or scent-gland secretions. Sullivan et al. (1984a) hypothesized that the negative but transient response of snowshoe hares (*Lepus americanus*) to predator odors, particularly those of the family Mustelidae, was due to evaporative loss of the active repellent compound(s) rather than habituation by hares. If this is the case, then testing (after the appropriate identification and synthesis) of one or more of these compounds should alter hare feeding behavior for a considerable period.

The chemical composition of some mustelid anal gland secretions has been reported for the mink (*Mustela vison*) (Schildknecht et al., 1976; Brinck et al., 1978; Andersen and Bernstein, 1980; Sokolov et al., 1980), ermine or stoat (*M. erminea*) (Crump, 1978, 1980a), and ferret (*M. putorius*) (Crump 1980b). A comparison of anal-sac secretion in eight mustelid species was conducted by Brinck et al. (1983). The major sulfur-containing compounds identified in these studies were 2,2-dimethylthietane, 2-propylthietane, 3-propyl-1,2-dithiolane, and 3,3-dimethyl-1,2-dithiolane. The importance of sulfur compounds as mammalian secondary compounds and their potential influence on animal behavior and related biological functions has been outlined by Kjaer (1977), Andersen and Bernstein (1980), and Brinck et al. (1983).

This paper reports on the influence of the major anal-gland compounds from mink and stoat in suppressing snowshoe hare feeding on coniferous tree seedlings. The importance of these lagomorph herbivores in adversely affecting forest renewal in North America was discussed by Sullivan and Sullivan (1982). This study was also designed to test the hypothesis that certain mustelid scent compounds will elicit a "fear response," thereby initiating and maintaining suppression of snowshoe hare feeding.

METHODS and MATERIALS

Pen Bioassays. Bioassays were conducted at the Applied Mammal Research Institute, Langley, B. C., Canada. The bioassay enclosure (9.15 × 18.3 m) was composed of three sections or pens (Figure 1) set in a natural environment. The entire enclosure was covered by a fiberglass roof to eliminate effects of adverse weather. The control and experimental pens (A and B) were enclosed by polyethylene on the walls to keep odors within the respective treatment section. These pens had all natural vegetation cleared prior to start of trials. Some grass persisted and was kept to a minimum height by mowing. Each pen had three experimental units, each composed of 10 styrofoam blocks to anchor coniferous seedlings. One-year-

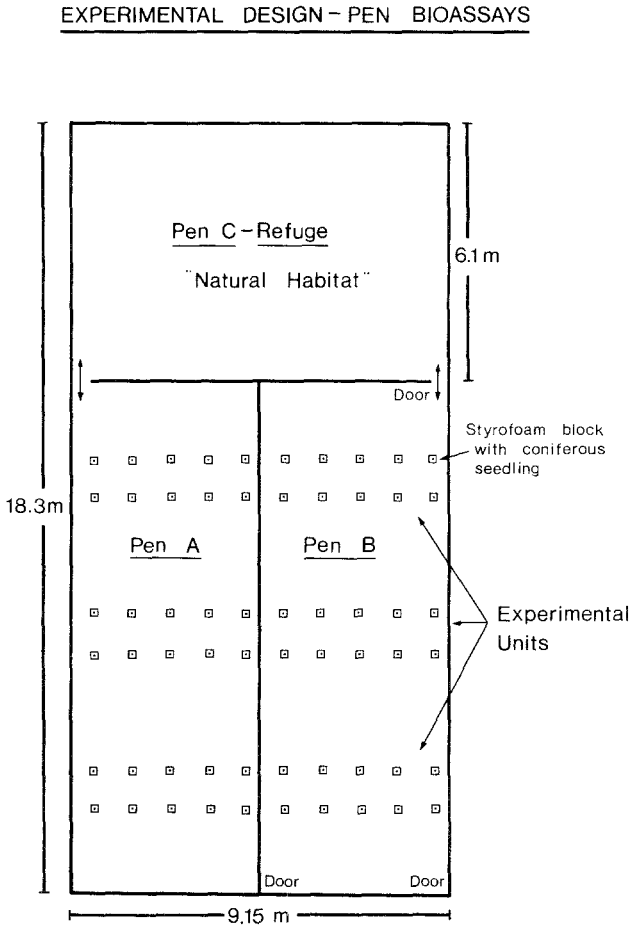

FIG. 1. Snowshoe hare enclosure used in the pen bioassay trials. Pens A and B served as control and experimental sections and pen C was the "natural habitat" or refuge area.

old (nursery stock) lodgepole pine (*Pinus contorta*) seedlings were used in all trials with one seedling per block. Thus, there was a maximum of 30 seedlings in each of the control and treatment pens (A or B) at the start of a given trial. Lodgepole pine is preferred by snowshoe hares over all other coniferous species (see Sullivan et al., 1984a).

The natural habitat (pen C) was not cleared of native vegetation and provided cover and some grasses and forbs for hares. Thus, this pen served as a potential refuge area for hares. Access of hares to pens A or B from pen C was through 30 × 30-cm openings in each adjoining wall (see Figure 1).

Hares moved freely among all three pens, and new animals were always allowed at least five to seven days to acclimate to this experimental configuration. To exert adequate feeding pressure on the experimental material and keep social interactions at a minimum, two hares were used in each trial. All hares used were from our northern study areas at Prince George, B.C. Hares were replaced at regular intervals to overcome potential variability and habituation among animals. Rabbit pellets and water were available ad libitum throughout these trials.

Field Bioassays. Field tests of mustelid scent gland odors were conducted at Tabor Mountain, 25 km southeast of Prince George, B.C., during April–May 1983. This study area was located in the subboreal spruce biogeoclimatic zone (Krajina, 1969). It was overgrown with deciduous brush species, mainly willow (*Salix* spp.) and Sitka alder (*Alnus sinuata*), with some aspen (*Populus tremuloides*), black cottonwood (*P. trichocarpa*), and paper birch (*Betula papyrifera*), and it had experienced coniferous plantation failure because of feeding damage by snowshoe hares. The hare population cycle peaked in this area in 1980–1981, but the peak was much more of a plateau (Sullivan and Sullivan, in preparation) than that described elsewhere (Keith and Windberg, 1978). Thus, the population of hares on Tabor Mountain continued to inflict severe damage to planted coniferous seedlings during the spring of 1983, thereby providing a suitable study area for testing our mustelid compounds. Both spring and fall represent periods when coniferous seedlings are particularly vulnerable to snowshoe hare feeding attacks. Snow cover is not adequate to protect seedlings and alternative summer herbaceous foods are not available.

Scent-Gland Compounds. Anal-gland compounds from the genus *Mustela:* 2,2-dimethylthietane, 2-propylthietane, 3-propyl-1,2-dithiolane, and 3,3-dimethyl-1,2-dithiolane, were prepared according to Crump (1978, 1980a,b, 1982) and were tested in pen and field bioassays. Thietane, 2-methylthietane, and di-*n*-propyl disulfide (acyclic analog of 3-propyl-1,2-dithiolane) were available commercially and tested in pen bioassays to determine the effect of closely related compounds on hare feeding behavior. All compounds were dispensed in 140-μl capillary tubes (75 mm in length), one tube attached by a plastic twist-tie to each seedling. The capillary tubes protected the compounds from adverse weather conditions (in field bioassays) and maintained the respective odor around the seedlings for optimum evaluation purposes. Approximately 30 mg of each compound was placed in a given capillary tube using a 1-ml microsyringe with a 20-gauge needle. A capillary tube filled with 30 mg water was attached to each control seedling to complete the design of a given experiment.

Field Trial. Each of three blocks (each 400 m^2 in area and separated from adjacent blocks by 100 m) was planted with 50 (5×10) 3-year-old (nursery stock) lodgepole pine seedlings on April 6, 1983, at Tabor

Mountain, Prince George. Blocks were assigned treatments as follows (in accordance with available amounts of compounds): control, 3-propyl-1,2-dithiolane mixed with petroleum ether (1:1 ratio) to maintain stability and retard volatility of the compound, and the third block was split between 2,2-dimethylthietane (20 seedlings) and pure 3-propyl-1,2-dithiolane (25 seedlings). Two additional blocks of 25 lodgepole pine seedlings each were planted on April 18, 1983, to test 2-propylthietane with a control. Snow cover (up to 30 cm) was still present in patches on the study area at the start of the trial.

Hare browsing of seedlings was checked on days 5, 12, 17, 23, and finally on day 38 (May 14) when the experiment was terminated. Hares had switched to summer herbaceous foods, and hence were no longer feeding on coniferous material. For the test with 2-propylthietane, seedlings were checked on days 5, 11, and finally on day 26. Feeding (clipping) on the terminal was considered as mortality to a given seedling. Coniferous seedlings rarely recover from terminal feeding damage, and even if they do, growth and form of the young tree are usually severely impaired.

RESULTS

Pen Bioassays. The compounds 2,2-dimethylthietane and 3-propyl-1,2-dithiolane were very effective in suppressing snowshoe hare feeding (Figure 2). Control seedlings were almost (5% remaining) completely eaten after the first day. Upon removal of compounds after the fourth day, hares readily consumed the unprotected coniferous seedlings. There was not a significant difference (chi-square) between 2,2-dimethylthietane and 3-propyl-1,2-dithiolane in suppressing hare feeding during the five-day bioassays. However, both compounds reduced hare browsing more effectively than 2-propylthietane after three days ($P < 0.01$; chi-squares = 7.73; 18.65). This latter compound completely suppressed feeding for the first two days of the trial but seedling survival declined to 30% at day three.

The efficacy of 3,3-dimethyl-1,2-dithiolane was high for two days before declining to 16% at day 3 (Figure 3). The 1:1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane was more effective in suppressing hare feeding than 2-propylthietane by itself. However, seedling survival with this mixture declined to 36% at day 4 which was significantly ($P < 0.01$; chi-square = 9.93) lower than the 80% survival value of 3-propyl-1,2-dithiolane (see Figure 2).

Those compounds closely related to mustelid odors, thietane and 2-methylthietane, were ineffective in altering hare feeding (Figure 3). Similarly, the acyclic analog of 3-propyl-1,2-dithiolane, di-*n*-propyldisulfide, was effective initially, but seedling survival then declined dramatically to 16.7% after three days.

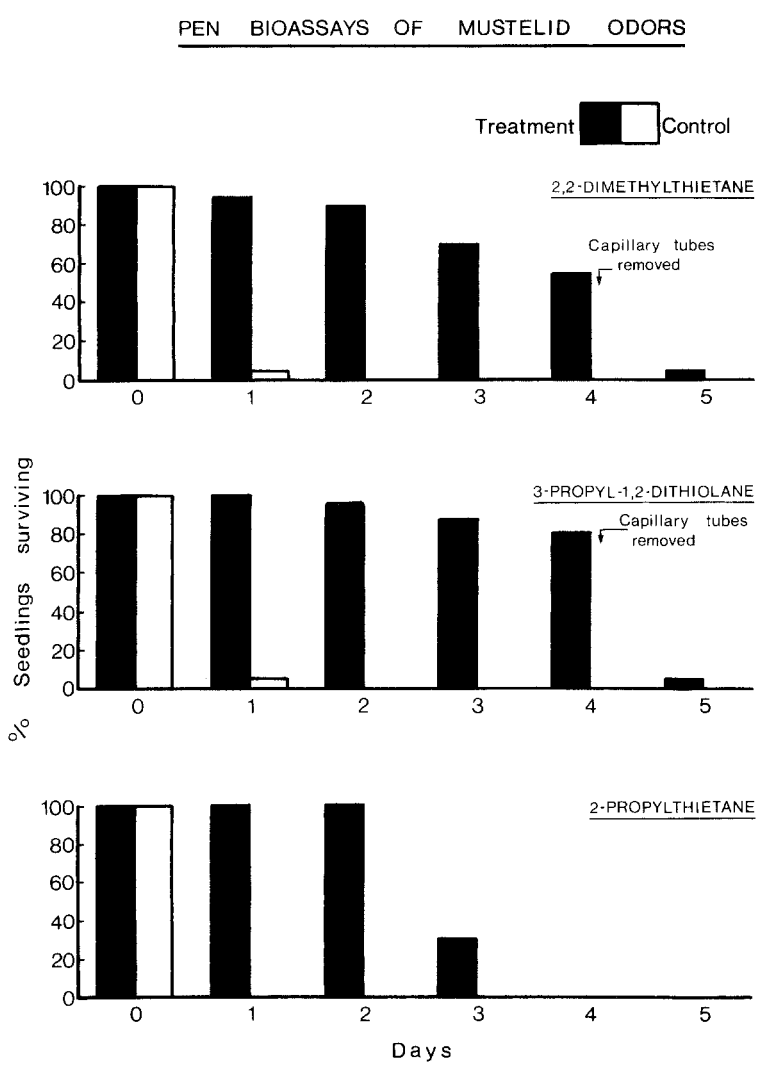

FIG. 2. Effectiveness of mustelid compounds, 2,2-dimethylthietane, 3-propyl-1,2-dithiolane, and 2-propylthietane, in suppressing snowshoe hare feeding on lodgepole pine seedlings in pen bioassays.

Field Bioassays. In the field trials, 3-propyl-1,2-dithiolane in pure form completely suppressed hare feeding on lodgepole pine seedlings during the five-week (38 days) experiment (Figure 4). This compound mixed with petroleum ether was not as effective as the pure form. Control seedlings were essentially all consumed by hares at day 12. 2,2-Dimethylthietane was reasonably effective in suppressing hare feeding (Figure 4), stabilizing at

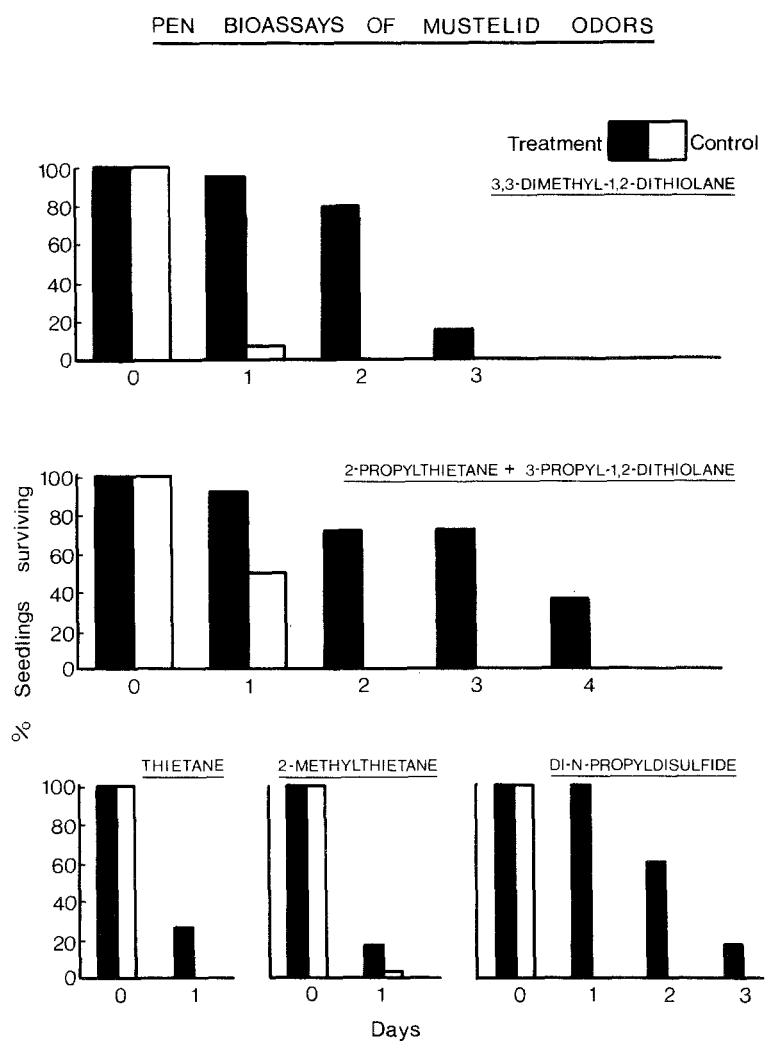

FIG. 3. Effectiveness of mustelid compounds, 3,3-dimethyl-1,2-dithiolane and a mixture of 2-propylthietane and 3-propyl-1,2-dithiolane, and the similar compounds, thietane, 2-methylthietane, and di-*n*-propyldisulfide, in suppressing snowshoe hare feeding on lodgepole pine seedlings in pen bioassays.

78% seedling survival. The difference between 3-propyl-1,2-dithiolane (pure) and 2,2-dimethylthietane was not statistically significant (chi-square), although it is of biological and practical significance when seedling survival and a complete lack of hare feeding pressure are considered. After the twelfth day of the trial, 3-propyl-1,2-dithiolane in a pure form was

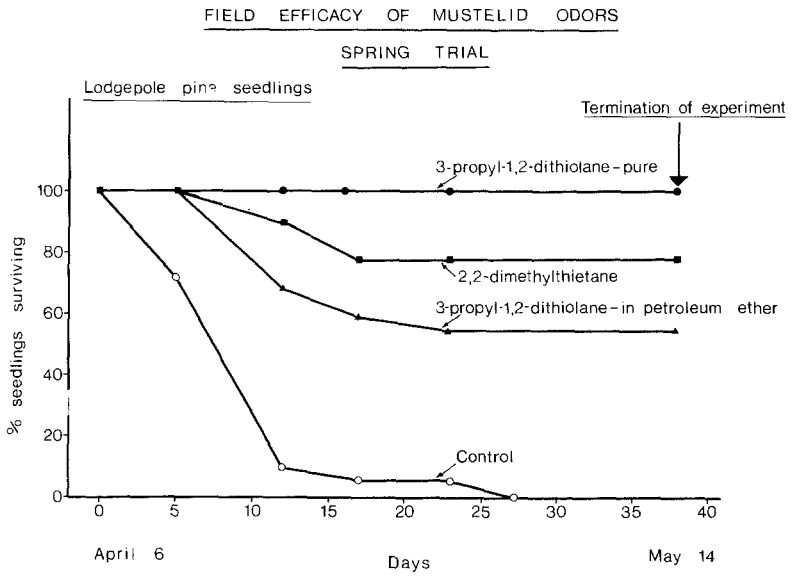

FIG. 4. Field efficacy of mustelid compounds, 3-propyl-1,2-dithiolane (pure and mixed with petroleum ether) and 2,2-dimethylthietane, in suppressing snowshoe hare feeding on lodgepole pine seedlings.

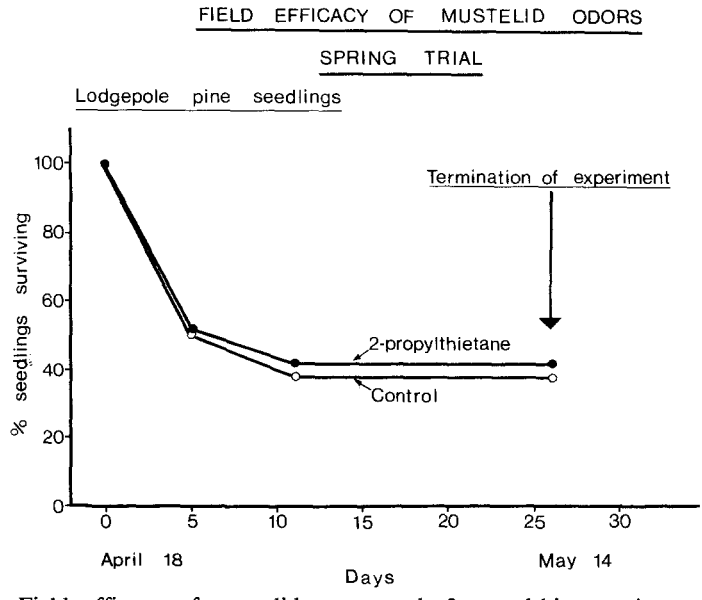

FIG. 5. Field efficacy of mustelid compound, 2-propylthietane, in suppressing snowshoe hare feeding on lodgepole pine seedlings.

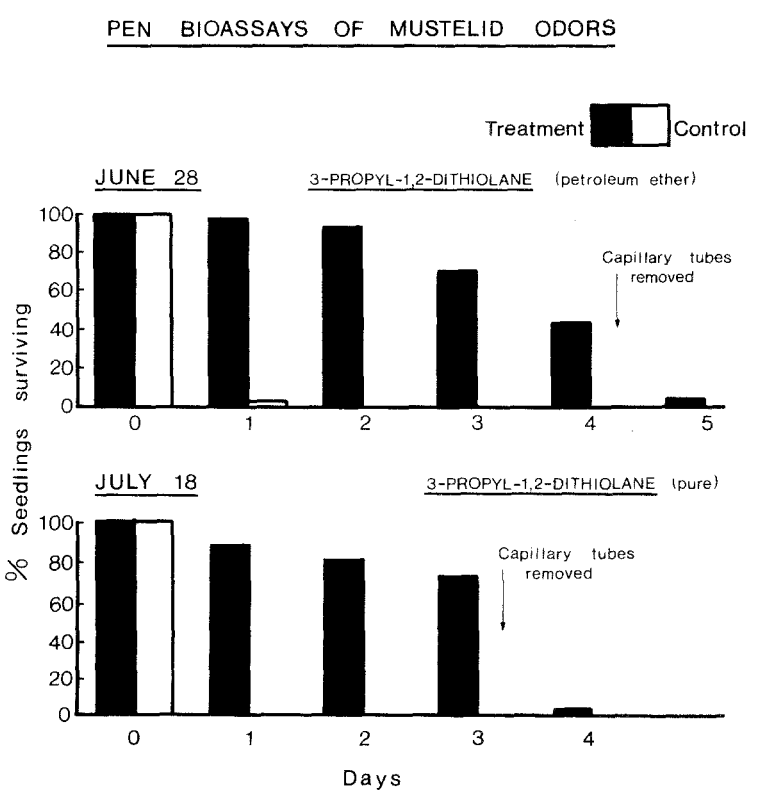

FIG. 6. Effectiveness of 3-propyl-1,2-dithiolane (pure and mixed with petroleum ether) in suppressing snowshoe hare feeding on lodgepole pine seedlings in pen bioassays. Capillary tubes containing this compound from the field bioassays were then tested in pen bioassays.

significantly ($P < 0.01$; chi-square = 10.17) more effective than that mixed with petroleum ether. The third mustelid chemical, 2-propylthietane, did not improve seedling survival over that of control seedlings (Figure 5). These spring field results corroborate those obtained in the pen bioassays.

Replication. To further substantiate the negative effect of 3-propyl-1,2-dithiolane on snowshoe hare feeding, the same capillary tubes with this chemical from the field trial were tested in pen bioassays on June 28 (petroleum ether) and July 18 (pure). These results are presented in Figure 6. Again, 3-propyl-1,2-dithiolane effectively suppressed feeding in a similar manner to earlier pen and field bioassays. The pure 3-propyl-1,2-dithiolane was originally encapsulated on April 6 and was still effective 3.5 months later on July 18. Another pen bioassay was conducted on August 29, at which time the same capillary tubes with the pure 3-propyl-1,2-dithiolane

were tested again. The strength of odor had clearly declined and it was not surprising that hare feeding was unaltered. Subsequent GC-MS analysis indicated that only a trace of the chemical remained in the capillary tubes.

DISCUSSION

This study has clearly demonstrated that certain mustelid scent gland compounds will alter the feeding behavior of snowshoe hares. 3-Propyl-1,2-dithiolane completely suppressed feeding for a 38-day period, which provides ample evidence that hares, in the field, do not habituate to an active repellent compound. This biological activity is presumably a fear response by hares to a component of mustelid (predator) scent-gland odor. If this is the case, then the chemical scent communications within and possibly between species of the genus *Mustela* may also act as an interspecific signal or allelochemic (Whittaker and Feeny, 1971) between predator and prey. A compound such as 3-propyl-1,2-dithiolane may then be regarded as a kairomone (Brown et al., 1970) since it may help hares, for example, detect an area marked by a weasel or stoat. This communication is of adaptive advantage to the hare if it avoids the area and consequently, is less likely to be preyed upon.

As discussed by Sullivan et al. (1984a), stoats or ermine (weasels) are wholly sympatric with and prey on snowshoe hares, particularly at the nest site. Therefore, hares may have an innate fear of these mustelids, as postulated by Lockley (1964), and avoid areas marked with scent and thus frequented by one or more weasels. This relationship is supported by our bioassay results which indicated that 2-propylthietane, the major but highly volatile compound of the stoat anal gland, was not effective compared with 3-propyl-1,2-dithiolane. This latter compound may provide some degree of persistence for naturally deposited anal scent marks. In our field trial, this compound polymerized to a certain degree, which may have contributed to its longevity as an effective repellent.

The major compound of the mink, 2,2-dimethylthietane, also affected hare feeding behavior. Mink do not normally prey, to a significant degree, on snowshoe hares and so this was a somewhat surprising result. However, traces of this compound have also been identified in the stoat secretion (Brinck et al., 1983), as well as the ferret (Crump, 1980b) although this latter species does not naturally occur in North America. Therefore, hares may still categorize the odor as belonging to a potentially important predator.

The specificity of chemical structure of the compounds tested appears to be of critical importance to the snowshoe hare. 3-Propyl-1,2-dithiolane and 2,2-dimethylthietane may have specific receptor sites within the olfactory system of the snowshoe hare and perhaps related lagomorphs. The

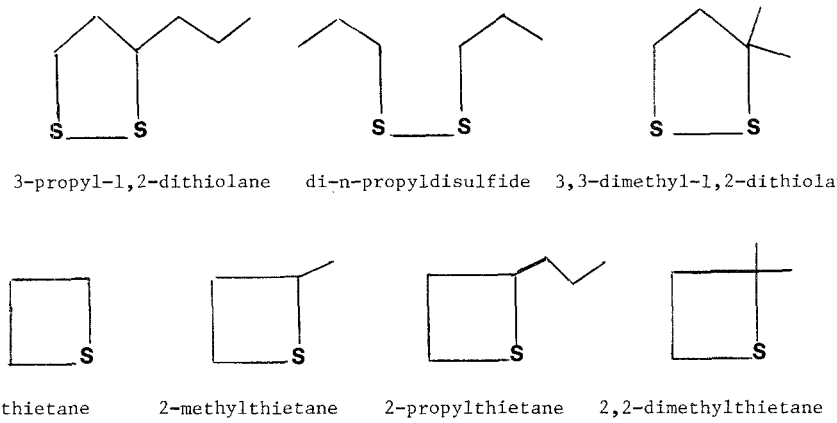

FIG. 7. Structural formulas of compounds.

ineffectiveness of the analogous compounds shown in Figure 7 clearly demonstrates that the suppression of hare feeding on lodgepole pine was not due to a novel odor effect (see Stoddart, 1980a; Sullivan et al., 1984a). In a similar study, a single compound, trimethylthiazoline, was isolated from fox feces and caused alarm reactions in rats (Vernet-Maury et al., 1980). This result, along with those in our study, strongly suggest that one or more specific semiochemicals in the urine, feces, or scent gland deposits of predators may elicit a fear response in prey species.

The use of fear- or avoidance-inducing compounds as area repellents to reduce forest and agricultural crop and livestock depredations are clearly of considerable value. Our study reports one of the first practical utilizations of mammalian semiochemicals for crop protection and wildlife management. The potential use of chemical signals in various aspects of crop protection, wildlife management, and animal husbandry has been discussed by several authors (Muller-Schwarze, 1974; Shumake, 1977; Stoddart, 1980b). In general, odors that occur naturally in the ecosystem have much more durable effects than those selected from nonbiological origins. Muller-Schwarze (1974) has indicated that predator odors are examined by herbivores at close range (a few centimeters). Such repellents would keep herbivores from feeding on treated plants but may not provide a "chemical fence" to keep them out of a given area. However, it is likely that most herbivores would choose to leave a "predator" area rather than risk being preyed upon. This avoidance response would presumably be dependent on the configuration and concentration of the specific chemical signals.

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PLANT-DETERMINED VARIATION IN CARDENOLIDE CONTENT AND THIN-LAYER CHROMATOGRAPHY PROFILES OF MONARCH BUTTERFLIES, *Danaus plexippus*[1] REARED ON MILKWEED PLANTS IN CALIFORNIA

3. *Asclepias californica*[2,3]

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Abstract—Variation in gross cardenolide concentration of the mature leaves of 85 *Asclepias californica* plants collected in four different areas of California is a positively skewed distribution ranging from 9 to 199 μg of cardenolide per 0.1 g dry weight with a mean of 66 μg/0.1 g. Butterflies reared individually on these plants in their native habitats contained a normal distribution of cardenolide ranging from 59 to 410 μg of cardenolide per 0.1 g dry weight with a mean of 234 μg. Cardenolide uptake by the butterflies was a logarithmic function of plant concentration. Total cardenolide per butterfly ranged from 143 to 823 μg with a mean of 441 μg and also was normally distributed. Populational variation of plant cardenolide concentrations occurs within subspecies, but the northern subspecies *A. c. greenei* does not differ significantly from the southern *A. c. californica.* Generally higher concentrations occur in

[1]Lepidoptera: Danaidae.
[2]Apocynales: Asclepiadaceae.
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butterflies from northern populations and in females. No evidence was adduced that cardenolides in the plants adversely affected the butterflies. Low cardenolide concentrations in the leaves and the absence of cardenolides in the latex characterize both *A. californica* and *A. speciosa,* but not *A. eriocarpa.* Thin-layer chromatography in two solvent systems isolated 24 cardenolide spots in the plants, of which 18 are stored by the butterflies. There was a minor difference in the cardenolide spot patterns due to geographic origin of the plants, but as in our previous studies, none in the sexes of the butterflies. Unlike *A. eriocarpa* and *A. speciosa, A. californica* plants lack cardenolides with *Rf* values greater than digitoxigenin. Overall, the cardenolides of both *A. californica* and *A. speciosa* are more polar than those in *A. eriocarpa. A. californica* plants contain cardenolides of the calotropagenin series including calotropin, calactin, and uscharidin, and the latter is metabolically transformed by monarch larvae to calactin and calotropin. Cardenolides of this series also occur in *A. vestita,* and *A. cordifolia* from California, the neotropical *A. curassavica,* and the African *Calotropis procera, Gomphocarpus* spp., and *Pergularia extensa;* they therefore cross established taxonomic lines. *A. californica* is the predominant early season milkweed in California and may be important in providing chemical protection to the spring generation of monarchs in the western United States. *A. speciosa, A. eriocarpa,* and *A. californica* each imparts distinctive cardenolide fingerprints to the butterflies, so that ecological predictions are amenable to testing.

Key Words—*Danaus plexippus,* Lepidoptera, Danaidae, monarch butterflies, *Asclepias californica,* Asclepiadaceae, milkweeds, ecological chemistry, plant–insect interactions, chemical ecology, chemical defense, chemotaxonomy, coevolution, thin-layer chromatography, cardenolide fingerprints, cardenolides, calotropagenin glycosides, calactin, calotropin, uscharidin.

INTRODUCTION

This paper is the third in our series presenting a multifaceted analysis of cardenolides in monarch butterflies sequestered from seven species of larval foodplants of the genus *Asclepias* (Asclepiadaceae) in California. Our purposes, explained in more detail in our studies on *Asclepias eriocarpa* Benth. (Brower et al., 1982) and on *A. speciosa* Torr. (Brower et al., 1984), are to investigate quantitative variation in the cardenolide contents of the butterflies as they relate to the milkweed plants growing in their natural habitats, to adduce evidence about the chemistry and emetic properties of the cardenolides and, by means of thin-layer chromatography, to establish plant-determined cardenolide fingerprints of the butterflies. We now report on cardenolides in monarchs collected as larvae on four populations of two subspecies of *Asclepias californica* Greene.

METHODS AND MATERIALS

Geographical and Ecological Distribution of Asclepias californica

Asclepias californica is an endemic California milkweed common in the foothills and lower mountainous areas of the southern half of California but is absent from the lowlands of the Central Valley. Two subspecies are distinguished by floral morphology (Woodson, 1954), and their distributions are shown in Figure 1 based on Woodson (1954) and Lynch (1977), as well as new data summarized from several herbarium collections (Lynch, as cited in Brower et al., 1982). *A. californica* subsp. *greenei* Woodson occurs to the north in central California and is geographically isolated by several mountain ranges from the southern *A. californica* subsp. *californica* Greene (Munz and Keck, 1959). The nearest the two approach each other is approximately 20–30 km southeast of area 3 (Figure 1) in Tulare and Kern counties where they are separated by the divides of the Sierra Nevada and Greenhorn Mountains.

In the Coast Ranges, the northern limit of *A. c. greenei* is in the vicinity of Mt. Diablo in Contra Costa County. [Lynch, 1977, determined that a record reported from Lake County by Woodson, 1954, was based on a misidentified herbarium specimen of *Asclepias cordifolia* (Benth.) Jepson.] Populations in the western Coast Ranges extend southwards to the Santa Lucia Ranges of northern San Luis Obispo County where they usually occur on south-facing slopes in chaparral clearings or in foothill woodlands at elevations ranging from 250 to 1400 m. The Coast Ranges and Sierra Nevada populations of *A. c. greenei* are isolated from each other by the grasslands and marshes of the Central Valley. In the Sierra Nevada, extensive populations occur along the foothills of the eastern border of the San Joaquin Valley from Mariposa County, southwards to Kern County and the Greenhorn Mountains. Also occurring primarily on south-facing slopes, these populations extend from valley grassland communities at 150 m up to foothill woodland, chapparal, and *Pinus ponderosa* pine forest communities at 1000 m. The northernmost populations of *A. c. californica* are located in western Inyo County along the eastern slopes of the Sierra Nevada and in Kern County along the Kern River. This subspecies extends southward to San Diego County along the slopes of the Paiute and Tehachapi ranges into the Transverse and Peninsular ranges. Although Woodson (1954) extends *A. c. californica* southwards into northern Baja California, we have observed no specimens or citations to verify its distribution south of San Diego County.

Populations in these ecologically diverse areas occur in a variety of plant communities. These include chaparral and foothill woodlands in

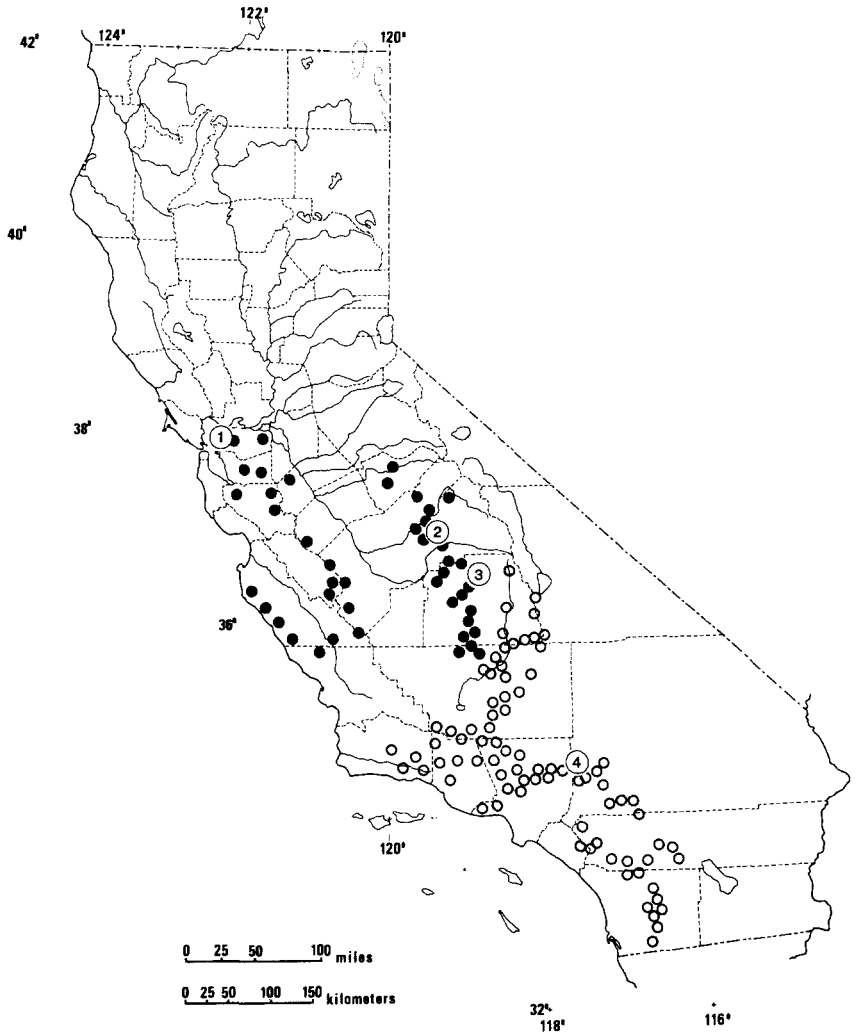


FIG. 1. The known geographic distribution of the milkweed *Asclepias californica* in California and the collection sites of the material analyzed in the study. The numbered open circles are the four areas where the 85 pairwise samples of monarch butterflies and their corresponding individual *A. californica* foodplants were collected. The solid black circles indicate populations of *A. californica* subsp. *greenei* and the open circles indicate those of *A. californica* subsp. *californica*. Area 1 (Mt. Diablo) is located in the central Coast Ranges, area 2 (Pine Flat Reservoir) is in the foothills of the southern Sierra Nevada, area 3 (Sequoia National Park) is in the lower montane zone of the southern Sierra Nevada, and area 4 (Wrightwood) is on the eastern margin of the San Gabriel Mountains which are part of the Transverse Range northeast of Los Angeles.

Santa Barbara, Ventura, Los Angeles, Orange, and San Diego counties and pinon–juniper woodlands, sagebrush scrub–chaparral transitions, and foothill to woodland–Jeffrey pine forest transitions in parts of Kern, Inyo, Los Angeles, Riverside, and San Bernardino counties. The southern subspecies extends beyond this habitat and also occurs on flats and gentle slopes with easterly and westerly exposures. Elevations for the known populations of *A. c. californica* range from 300 to 2200 m (Lynch, 1977).

Locations, Methods, and Dates of Sample Collections

We collected immature monarchs (*Danaus plexippus plexippus* L., western U.S.A. population) from milkweeds over an extensive area of California during the summer of 1975 and the spring and summer of 1976. Whereas previously (Brower et al., 1982, 1984), most individually matched plant–butterfly samples were obtained by collecting fifth-instar larvae and chrysalids directly from the wild plants, most of the butterflies utilized in this study were obtained by bagging wild eggs or young larvae on individual plants in nylon net bags and allowing them to mature therein until subsequently collected as fifth instars or chrysalids. Lack of sufficient numbers of breeding monarchs in some areas required that we transfer to selected plants eggs or young larvae obtained either in the wild or by confining one or more females in netting over a milkweed plant. Most transferred eggs did not survive, whereas most first- or second-instar larvae did.

Figure 1 indicates the four areas from which we obtained a total of 85 paired butterfly and plant samples. Those from area 3 were obtained during the summer of 1975, while those from the rest were collected during the spring and summer of 1976. We selected the areas to represent a diversity of biological communities across the range of both subspecies of *A. californica.* Geographical and altitudinal data were confirmed using various California regional maps (Anon. 1972a,b, 1974, 1975) and U.S. Geological Survey topographical maps (Anon., 1965, 1967, and 1968a,b).

Area 1 (Mt. Diablo) is in the central Coast Ranges in Contra Costa County on the southwesterly facing hillsides of Mt. Diablo at an elevation of 415 m. The community is open foothill woodland vegetation with a heavily grazed herbaceous layer composed largely of introduced annual grasses and forbs. Because we found no immatures here, we transferred eggs and early instar larvae collected on *A. californica* plants in area 2 on April 23 and bagged them on the Mt. Diablo plants. We then collected them on May 7, 1976. The twenty paired leaf–butterfly samples are Nos. 724, 727, 733–735, 737–739, 741, 742, and 745–754.

Area 2 (Pine Flat Reservoir) is in the foothills of the southern Sierra Nevada in Fresno County. This area is in the Sierra National Forest located

approximately 18 km north of Piedra on Trimmer Springs Road along the northern shore of Pine Flat Reservoir. Numerous plants were found on the south-facing slopes at an elevation of 400 m. These slopes are vegetated by heavily grazed European grasses interspersed with foothill woodlands dominated by digger pine (*Pinus sabiniana* Dougl.), blue oak (*Quercus douglasii*) H. and A., and interior live oak (*Q. wizlizenii*) A. DC. (Barbour and Major, 1977). We found many first- and second-instar larvae here on April 20, 1976, and bagged and subsequently collected them (as well as completely wild individuals) on May 15 797A, 798, 800, 802, 804, 807–809, 812, 813, 816, 821–825, 827, 828, and 830).

Area 3 (Sequoia National Park) is in the montane zone of the southern Sierra Nevada in Tulare County, northeast of Three Rivers at Potwisha Campground in Sequoia National Park, at an elevation of 750–850 m. The vegetation was transitional between the Sierran foothill woodland and an open ponderosa pine forest. We found numerous plants in partial clearings on the steep southwesterly facing slopes above the campground and obtained 26 paired samples including wild Nos. 43, 44B, and 45 on June 11, 1975; Nos. 86–89 on June 27; Nos. 175–176 and 184–185 on July 8; and Nos. 259–260 on July 19. Bagged eggs and first- or second-instar larvae produced Nos. 178 and 181–183 collected on July 8, and Nos. 261–266 and 268–270 on July 19.

Area 4 (Wrightwood) is located at an altitude of 1850 m on the southwestern facing slopes of Swartout Valley on the eastern slope of the San Gabriel Mountains of the Transverse Ranges in western San Bernardino County. This area is immediately north of the San Andreas fault and has vegetation which is transitional between a pinon pine (*Pinus monophylla* Torr. and Frem.) -juniper woodland (*Juniperus californica* Carr.) and Jeffrey pine forest (*Pinus jeffreyi* Grev. and Balf. in A. Murr.), with few grasses and forbs. Other dominant species included sagebrush *Artemesia tridentata* Nutt., California buckwheat *Eriogonum fasciculatum* Benth., and flannel bush *Fremontodendfron californicum* (Torr.) Cov. More than 100 *A. c. californica* plants were found scattered over the steep open hillside. Twenty paired samples were obtained by bagging first or second instar larvae on May 16, 1976. (We transferred these larvae from wild *A. eriocarpa* plants growing in the vicinity of area 2.) Collections made on June 6, 1976, are sample Nos. 914, 916, 918, 919, 921, 923, 924, 929, 931, 933, 934, 936–941, 943, 946, and 947.

Analytical Procedures

Gross Cardenolide Content. During the summer of 1977, we determined the gross cardenolide concentrations of the 85 butterflies (44 males and 41 females) and the leaves from the respective individual plants upon which they had been reared from the four areas. All butterfly samples

were reared on separate plants. Each sample was spectroassayed by the same procedures used in Brower et al. (1982) which give results in micrograms of cardenolide (equivalent to digitoxin) per 0.1 g dry weight.

Thin-Layer Chromatography. The cleanup of *A. californica* plant and butterfly extracts utilized the procedure described in Brower et al. (1984). Prior to cleanup, the plant extracts had a mean cardenolide concentration of 3.49×10^{-5} *M* (95% confidence limits = 0.17, $N = 5$, SD = 0.14), and the cleaned extracts 1.70×10^{-5} M (95% confidence limits = 0.19, $N = 5$, SD = 0.15) giving a cardenolide recovery efficiency of 49%. Respective values for the butterfly samples were 6.95×10^{-5} M (95% confidence limits = 0.06, $N = 5$, SD = 0.05) and 5.86×10^{-5} M (95% confidence limits = 0.12, $N = 5$, SD = 0.10), giving a recovery efficiency of 84%. The conditions for TLC development in two separate solvent systems [chloroform–methanol–formamide (90:6:1) = CMF, and ethyl acetate–methanol (97:3) = EAM], visualization, and photography of developed chromatograms were also as described previously. We carried out the TLC analyses in April–May 1979. Our TLC protocol spotted extracts of three male and three female butterflies and their respective plants on each of eight plates with two plates for each of the four areas, giving a total of eight plates with 48 butterfly–plant pairs. The rationale for selecting the samples and the methods of quantifying the $R_{\text{digitoxin}}$ and spot intensity (SI) values were as described previously. Eight of the plant and one of the butterfly samples contained insufficient cardenolide to replate in the EAM system.

We ran commercial digitoxin and digitoxigenin as reference standards along with calactin, calotropin, and uscharidin on each of the plates. As in the *A. speciosa* study, we found that interfering plant substances resulted in lower cardenolide recoveries and therefore weaker TLC spot profiles than expected, especially in those channels in which less than an estimated 50 μg of total cardenolide were spotted.

The mean $R_{\text{digitoxin}}$ (R_d) value for digitoxigenin in the CMF system was 2.12 (range = 1.89–2.29, SD = 0.11, $N = 16$) which is intermediate to that found in the *A. speciosa* (1.89) and *A. eriocarpa* (2.67) studies. These differences contribute to difficulty in quantitative comparisons of *Rd* values in different TLC runs, but do not affect the relative positions of the cardenolides in the TLC regions below and above digitoxin. In addition to quantifying the relative positions of the various spots, comparisons of TLC patterns are also based on the mean and standard deviations of each spot's intensity value (SI) as well as each spot's probability of occurrence (PO).

Statistical Analyses

Cardenolide Concentrations, Total Cardenolide, and Dry Weights. Statistical analyses were performed via the University of Florida Northeast Regional Data Center utilizing SAS, Release 79.6 (Anon., 1976). We

analyzed dry weights of the butterflies, cardenolide concentrations, and the total cardenolide contents of the butterflies, and cardenolide concentrations of the plants. Proc. Univariate and Freq. Plot Normal tests were used to examine frequency distributions and to test for normality (Kolmogorov D statistic). Data sets which proved to be not normally distributed ($P < 0.05$) were transformed ($\log_{10}$) which resulted in normality. We carried out two-way analyses of variance (two-way ANOVAs) separately on the plant and butterfly data to determine the significance of the influence of sex, area, and their interaction upon cardenolide concentration. Because of unequal numbers in the cells, we used the general linear models procedure (GLM). The minimum number of observations in any cell was nine (Table 1). Type IV sum of squares statistics were used if there was one or more significant interactions in the data, whereas type II sum of squares were used if there was none. We used Duncan's multiple range tests to compare the significances of the differences between mean values for the geographic areas with $P \leq 0.05$.

To relate cardenolide concentrations, total cardenolide contents, and dry weights of individual butterflies to the cardenolide concentrations of their respective plants, we ran regressions of the butterfly data (Y, the dependent variable) against the plant concentrations (X, the independent variable). Plots of residual values further examined the data for possible relationships not explained either by the linear or logarithmic models. Linear regressions of the butterfly data (concentrations and total cardenolide per butterfly) against the $\log_{10}$ values of the plant concentration data best conformed (highest r^2 values) to the relationship $Y = b(\log X) + a$ (see also Brower et al., 1984). We carried out each regression in two steps. The first considered the overall relationship of Y to X and calculated r^2, the slope, and the intercept. The significance of the difference of the slope and Y intercept values from zero is based on the t statistic. The second set of regressions broke down the dependence of the butterfly data on the cardenolide concentrations in the plants by sex, by area, and by their interactions. Where appropriate, sequential retesting dropped insignificant interactions to assess the significance of main effects per se.

TLC $R_{digitoxin}$ *and Spot Intensity Values.* As pointed out previously (Brower et al., 1982, 1984), the reliability of cardenolide fingerprinting depends upon a high degree of correlations between R_d values of the respective cardenolides in the plants and butterflies. While our TLC spot comparison methodology predetermines the correspondence of the respective plant and butterfly spots for each plant butterfly pair, the SAS linear regression program allows for the simultaneous testing of the dependence of the R_d values for all butterfly spots on the R_d values of their respective plant spots as affected by geographic area, sex, and interplate variation in the entire sample. We ran these same regressions on the spot intensity data.

TABLE 1. SUMMARY OF MEANS AND GRAND STANDARD DEVIATIONS OF CARDENOLIDE CONCENTRATIONS OF 85 WILD-COLLECTED INDIVIDUAL *Asclepias californica* PLANTS AND MONARCH BUTTERFLIES REARED THEREON[a]

	Plant material[b]			Butterfly material[b]		
	Males	Females	Means[c]	Males	Females	Means[c]
Area 1: central Coast Ranges, Mt. Diablo ($N = 10, 10$)	111.3	121.1	116.2	259.4	316.3	287.9
Area 2: southern Sierra Nevada, foothills, Pine Flat Reservoir ($N = 9, 10$)	30.2	56.7	43.5	163.3	211.8	187.6
Area 3: southern Sierra Nevada, Montane, Sequoia Nat. Pk. ($N = 15, 11$)	60.4	65.0	62.7	257.9	255.8	256.9
Area 4: San Gabriel mountains, Wrightwood ($N = 10, 10$)	39.5	43.7	41.6	188.2	200.7	194.5
Grand means[d]	61.0	71.5	66.1	223.0	246.4	234.3
Grand SDs[d]	42.0	48.7	45.4	70.5	75.6	73.1
Grand Ns	44	41	85	44	41	85
Ranges	9–199			59–410		

[a]Collections are from the four areas shown in Figure 1. Data are μg (equivalent to digitoxin) per 0.1 g dry weight of butterfly or plant material.
[b]The plant material corresponds to the butterflies according to the sex of the butterfly; the butterfly material represents the butterflies reared on their respective plants.
[c]The means above the grand means are the mean male value added to the mean female value divided by 2.
[d]Based on all 44 males, 41 females, and their respective plants.

Because we did not spot samples from each area on all eight plates, there were too few area replicates to provide sufficient degrees of freedom in the regression model to estimate the simultaneous effects of plate, area, sex, and their interactions on the main butterfly–plant regression. To rectify this, we ran two initial regressions: the first dropped area as an independent variable, and the second dropped plate. These two regressions showed that sex had no significant main or interactive effects with either plant R_d, plate (or area), or with any two- or three-way interactions in the regressions (P values ranged from >0.09 to <0.74). We then dropped sex and reran the regression with plate nested within the area variable (Tables 8A and 9A). In

future studies, a more robust statistical analysis can be achieved by running samples of all areas and both sexes on each plate.

RESULTS

Gross Cardenolide Concentrations

Quantitative Variation in Plants. The pattern of variation is shown in Figure 2. All 85 plants contained cardenolide and ranged from 9 to 199 μg/0.1 g with a grand mean of 66.1 μg and a grand standard deviation of 45.4 μg (Table 1). This variation encompasses approximately one order of magnitude, and in terms of cardenolide as a percent of dry weight represents from 0.01 to 0.20% (mean = 0.07%). The curve over the histogram in Figure 2 displays the expected normal distribution calculated by the z statistic (Steel and Torrie, 1960) and shows that the variation is positively skewed (moment of skewness = 0.91; mean and median = 66.1 and 54.0; $D = 0.130$; $N = 85$; $p < 0.01$), i.e., the majority of plants contain less than the average concentration of cardenolide. However, the $\log_{10}$ transformed data do not depart significantly from normality: mean and median = 1.719 and 1.740; $D = 0.068$, $P > 0.15$. Further statistical analyses of the plant concentration data are therefore based on the $\log_{10}$ transformed data.

Inspection of the means in Table 1 suggests differences both in the cardenolide concentrations of the plants from the four geographic areas and in the plants fed on by the males vs. the females. The two-way ANOVA (Table 2A) indicates that the area difference is highly significant ($P < 0.0001$) but that the difference in the plants according to the sex of butterfly that fed upon them is not and that the interaction is also not significant. The order of the cardenolide concentrations from highest to lowest in the four areas is area 1, 3, and 2 $\simeq$ 4. Duncan's test indicates that plants in area 1 (116 μg/0.1 g) are significantly higher than all the other areas. Concentrations in area 3 (62 μg/0.1 g) differ from area 2 (44 μg/0.1 g), but not from area 4 (42 μg/0.1 g), and area 2 and 4 also do not differ significantly from each other. The grand variance of the cardenolide concentration of the plants upon which the females were reared is not significantly different than those on which the males were reared (for the s^2 female/s^2 male, $F = 0.964$; $df = 40, 43$; $0.25 < P < 0.50$).

These data indicate significant populational variation in the gross cardenolide concentrations of *Asclepias californica* plants. The central Coast Range population has substantially higher concentrations than the three other populations, two of which in turn differ among themselves but without any obvious geographic pattern. Most importantly, there are no significant differences based on the two subspecies.

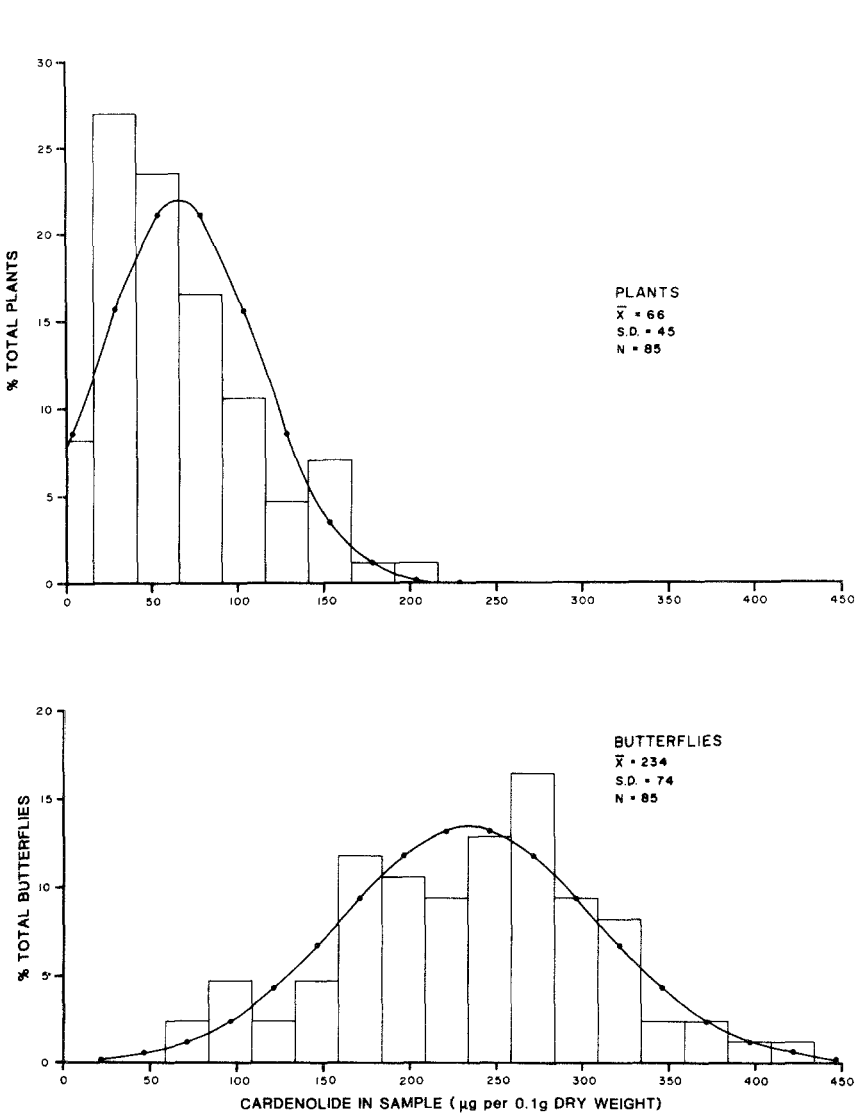


FIG. 2. Frequency distributions of the gross cardenolide content (as μg per 0.1 g dry weight, equivalent to digitoxin) of 85 *Asclepias californica* plant samples and 85 adult monarch butterflies reared thereon. All individual butterflies were collected as fifth-instar larvae or chrysalids on the respective individual milkweed plants growing in their natural environments from the four geographic areas shown in Figure 1. The width of each bar is 25 μg. The curves above the histograms are the calculated normal distributions.

TABLE 2. TWO-WAY ANOVA OF CARDENOLIDE CONCENTRATION DATA IN TABLE 1.

Source of variation	*df*	SS	MS	*F*	*P*
A. Plant material ($r^2 = 0.40$)[a]					
Model	7	3.51795	0.50256	7.45	<0.0001
Error	77	5.19549	0.06747		
Corrected total	84	8.71344			
Area	3	3.28745		16.24	<0.0001
Sex	1	0.11847		1.76	>0.18
Sex × area	3	0.13054		0.64	>0.59
B. Butterfly material ($r^2 = 0.37$)[b]					
Model	7	169895	24271	6.57	<0.0001
Error	77	284427	3694		
Corrected total	84	454322			
Area	3	145271		13.11	<0.0001
Sex	1	15065		4.08	=0.047
Sex × area	3	13058		1.18	>0.32

[a]Type II sum of squares, data are $\log_{10}$ of μg/0.1 g.
[b]Type II sum of squares, data are μg/0.1 g.

Quantitative Variation in Butterflies. All 85 butterflies contained cardenolide. The concentration range for the four geographic areas was 59–410 μg/0.1 g with a grand mean of 234.3 μg/0.1 g (Table 1). This range spans approximately one order of magnitude and in terms of cardenolide per gram dry weight represents from 0.06 to 0.41% (mean = 0.23%), i.e., from about 2 to 7 (mean = 3.5) times that in the plants.

As seen in Figure 2, the pattern of variation in cardenolide concentration in the butterflies is normally distributed (mean and median = 234.3 and 238.0, respectively; $N = 85$, $D = 0.059$; $P > 0.15$). The grand variance of the 66 males does not differ significantly from that of the 45 females ($F = 1.15$, $df = 40, 43$, $0.75 > P > 0.55$). The grand butterfly standard deviation is 75.6 μg, i.e., 1.55 times that of the plants they were reared upon (Table 1, Figure 2). In other words, the range of variation in the butterflies is substantially increased over that of the plants, as well as normalized.

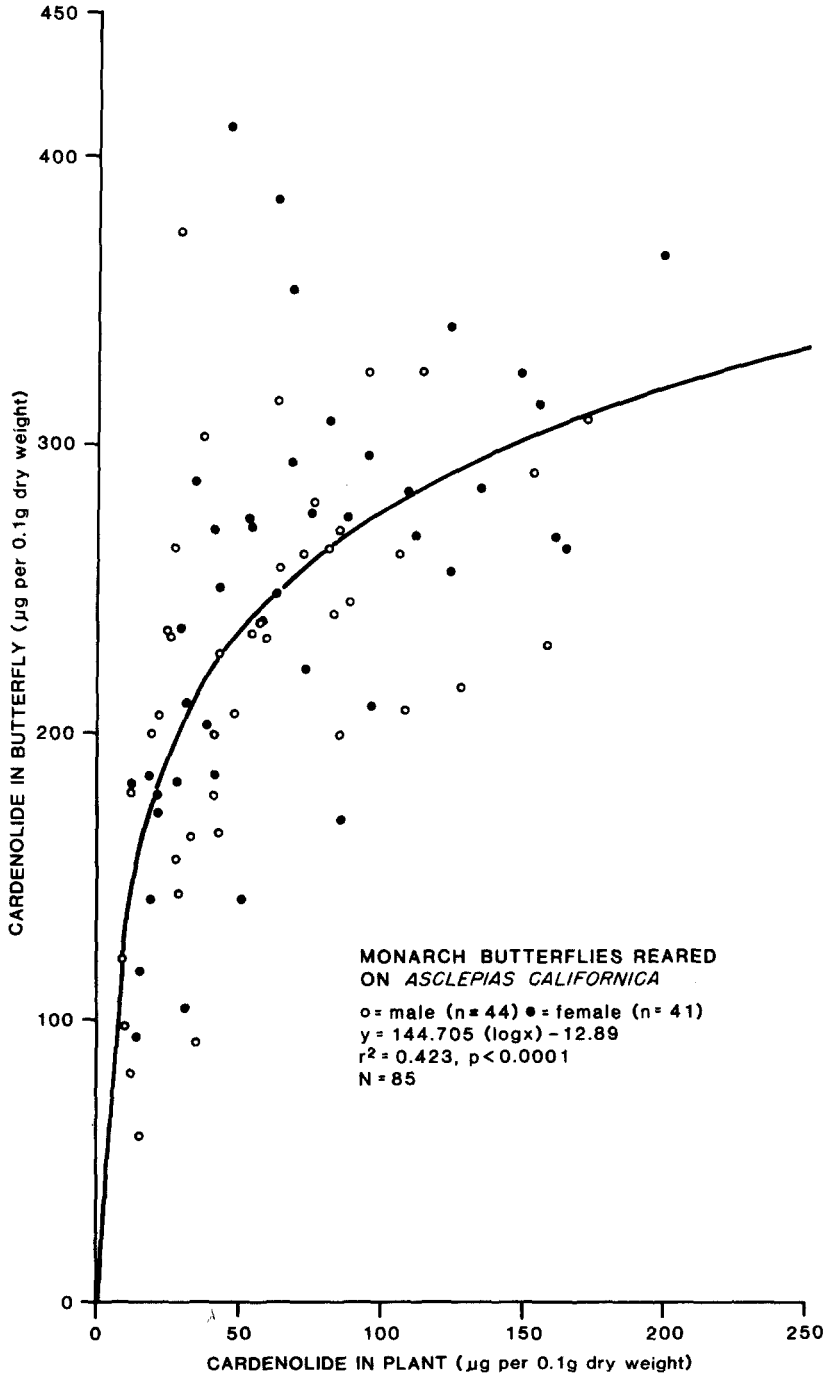
The two-way ANOVA of the two sexes and four areas indicates that the butterfly concentrations differ significantly by both sex and by area, but without a significant interaction (Table 2B). Overall, females have higher cardenolide concentrations in three of the four areas. Duncan's comparison of the four geographic areas indicates that butterflies from area 1 (288 μg/0.1 g) and area 3 (257 μg/0.1 g) have significantly higher concentrations than those from area 4 (194 μg/0.1 g) and area 2 (189 μg/0.1 g). Areas 1 and 3 do not differ significantly, nor do areas 4 and 2. The order of the mean cardenolide concentrations in the butterflies from highest to lowest (area 1,

3, 4, and 2) is similar to that of the plant concentrations (area 1, 3, and $2 \simeq 4$).

Cardenolide Concentrations of Butterflies as Function of Cardenolide Concentrations of Plants. The mean cardenolide concentration in the butterflies is 3.54 times that of their respective plants (grand mean ratio) and the overall range in the butterflies (59–410 μg/0.1 g) is shifted substantially upwards from that of the plants (9–199 μg/0.1 g). Figure 3 plots butterfly concentrations against the respective plant concentrations for both males and females and the main regression model (Table 3A) confirms the logarithmic relationship $Y = 144.705 \log_{10}X - 12.89$ ($r^2 = 0.423$; $P < 0.0001$). The slope is significantly different from 0 at the $P < 0.0001$ level, whereas the intercept is not ($P > 0.69$). Plotting of the residual values in both the linear and log regressions substantiated this logarithmic relationship. The regression equation predicts that butterflies which ate plants

TABLE 3. LINEAR REGRESSION ANALYSES OF CARDENOLIDE CONCENTRATIONS (μg/0.1 g DRY WT) IN BUTTERFLIES (DEPENDENT VARIABLE) VS. CARDENOLIDE CONCENTRATIONS ($\log_{10}$ μg/0.1 g DRY WT) IN THEIR RESPECTIVE PLANTS, ACCORDING TO FUNCTION $y = b\ (\log_{10}x) + a$.

Source of variation	*df*	SS	MS	*F*	*P*
A. Overall regression pooling sex and geographic areas ($r^2 = 0.423$; type IV sum of squares)					
Plant concentration	1	192390	192390	60.96	<0.0001
Error	83	261932	3156		
Corrected total	84	454322			
Estimated value of parameters		SE	*T* for H=O		*P*
$a = Y$ intercept $= -12.889$		32.24	−0.40		>0.69
b = slope $= 144.705$		18.53	7.81		<0.0001
Equation for the line: $Y = 144.705\ (\log_{10}x) - 12.89$					
B. Butterfly concentrations on plant concentrations by area, by sex, and for all interactions ($r^2 = 0.533$; type II sum of squares)					
Model	15	242052	16137	5.25	<0.0001
Error	69	212270	3076		
Corrected total	84	454322			
Log plant conc	1	67181		21.84	<0.0001
Area	3	6236		0.68	>0.57
Sex	1	262		0.09	>0.77
Log plant × area	3	4113		0.45	>0.72
Log plant × sex	1	88		0.03	>0.86
Sex × area	3	995		0.11	>0.95
Log plant × sex × area	3	832		0.09	>0.96


450
400
300
200
100
0
0
50
100
150
200
250
CARDENOLIDE IN BUTTERFLY (µg per 0.1g dry weight)
CARDENOLIDE IN PLANT (µg per 0.1g dry weight)
MONARCH BUTTERFLIES REARED
ON ASCLEPIAS CALIFORNICA
o = male (n = 44) ● = female (n = 41)
y = 144.705 (logx) - 12.89
r² = 0.423, p < 0.0001
N = 85

containing as little as 2 μg/0.1 g would contain approximately 31 μg/0.1 g of cardenolide, those which fed upon 50-μg plants would contain 233 μg/0.1 g, those on 100-μg plants, 276 μg/0.1 g, and those on 300-μg plants, 346 μg/0.1 g, etc. The second regression analysis of these data (Table 3B) indicated that the high dependence of the butterfly concentration on the plants is unaffected by the sexes, the four geographic areas, or their interactions (all P values > 0.57).

Dry Weights of Butterflies

The dry weights of the butterflies (Table 4) are normally distributed (grand mean = 0.190 g, median = 0.193 g), $D = 0.055$; $P > 0.15$) and the variances of the two sexes do not differ significantly ($F = 1.54$; df = 65, 44; $0.25 > P > 0.10$). Two-way ANOVA indicates a significant difference of the means both by sex and by geographic area, but no significant interaction (Table 5A). The average dry weight of the males is 0.196 g and the females 0.183 g (Table 4), i.e., the males on average weigh 1.07 times the females. Duncan's test indicates a significant difference among the geographic areas grouped as follows: area 4 (0.197 g) and area 3 (0.196 g), area 1 (0.190 g), and area 2 (0.173 g). Areas 3 and 1 do not differ from each other, but both areas 4 and 3 differ from area 2.

Dry Weights of Butterflies as Function of Cardenolide Concentrations in Plants and Butterflies. The same linear regression model as in Table 3A was run to relate the dry weights of the butterflies (Y) to the $\log_{10}$ cardenolide concentrations of the plants (X). The overall model indicated no correlation ($r < 0.0003$; $F = 0.0211$ $P > 0.89$). The butterflies' overall dry weights likewise showed no correlation with the cardenolide concentration of the butterflies ($r^2 < 0.03$, $F = 2.48$, $P > 0.11$).

Total Cardenolide in Butterflies.

The data for all four areas are normally distributed (mean = 441.2 μg; median = 459.6 μg; $D = 0.080$; $P > 0.15$). Total cardenolide per butterfly ranged from 143 to 823 μg with a standard deviation of 144 μg (Table 4). The variances of the males and females do not differ significantly ($F = 1.43$; $0.25 > P > 0.10$). The males contain an average of 431 μg and the females 452 μg. The two-way ANOVA indicates a highly significant difference

FIG. 3. Gross cardenolide concentrations of adult monarch butterflies (Y axis) as a function of the gross cardenolide concentrations of their larval foodplants (X axis). Each of the 85 data points represents one corresponding individual butterfly-plant rearing experiment. Open circles are males and solid circles are females. The line is derived from the regression equation $Y = b(\log_{10} X) + a$, transformed back to standard coordinates.

TABLE 4. SUMMARY OF DRY WEIGHTS AND TOTAL CARDENOLIDE CONTENT (EQUIVALENT TO DIGITOXIN) OF 85 ADULT MONARCH BUTTERFLIES REARED ON *A. californica* PLANTS POOLED FROM FOUR GEOGRAPHIC AREAS IN TABLE 1.

		Dry weights (g)			Total cardenolide (μg)		
Sample size		Means	SD	Range	Means	SD	Range
Male	44	0.196	0.030	0.128–0.287	431	131	143–684
Females	41	0.183	0.026	0.127–0.235	452	157	151–823
Both	85	0.190	0.029	0.127–0.287	441	144	143–823

among the areas but neither the sex nor the interaction is significant (Table 5B). Duncan's test of the geographic differences indicates that butterflies from area 1 (543 μg) and area 3 (493 μg) do not differ significantly; those from area 4 (384 μg) and area 2 (323 μg) also do not differ significantly, but those from the first two differ from the latter two. The order of the mean total cardenolide per butterfly from highest to lowest in the four areas (area 1, 3, 4, and 2) is identical to the butterfly concentrations and is similar to the mean plant concentrations (area 1, 3, and $2 \simeq 4$).

Total Cardenolide in Butterflies as Function of Cardenolide Concentrations in Plants. Regression analyses (Table 6A) indicated that total

TABLE 5. TWO-WAY ANOVAS OF DRY WEIGHTS AND TOTAL CARDENOLIDE CONTENT OF BUTTERFLIES BY FOUR AREAS AND TWO SEXES[a].

Source of variation	*df*	SS	MS	*F*	*P*
A. Dry weight of butterflies ($r^2 = 0.17$)					
Model	7	0.01134	0.00162	2.19	<0.044
Error	77	0.05689	0.00074		
Corrected total	84	0.06823			
Area	3	0.00677		3.05	<0.034
Sex	1	0.00331		4.48	<0.038
Sex × area	3	0.00075		0.34	>0.80
B. Total cardenolide per butterfly ($r^2 = 0.38$)					
Model	7	667421	95346	6.93	<0.0001
Error	77	1075158	13963		
Corrected total	84	1742579			
Area	3	613558		14.65	<0.0001
Sex	1	17711		1.27	>0.26
Sex × area	3	44398		1.06	>0.37

[a]Type II sum of squares (data are normally distributed).

TABLE 6. LINEAR REGRESSION ANALYSES OF TOTAL CARDENOLIDE (μg) PER BUTTERFLY (Y AXIS, DEPENDENT VARIABLE) VS. CARDENOLIDE CONCENTRATIONS ($\log_{10}$ μg/0.1 g DRY WT.) IN THEIR RESPECTIVE PLANTS, ACCORDING TO FUNCTION $Y = b(\log_{10} X) + a$

Source of variation	*df*	SS	MS	*F*	*P*
A. Overall regression pooling sex and geographic areas ($r^2 = 0.403$; type IV sum of squares)					
Plant concentration	1	702932	702932	56.12	<0.0001
Error	83	1039646	12526		
Corrected total	84	1742579			
Estimated value of parameters	SE		*T* for H=O		*P*
a = Y intercept = − 31.33	64.23		− 0.49		>0.62
b = slope = 276.60	36.92		7.49		<0.0001
Equation for the line: $Y = 276.60 (\log_{10} X) - 31.33$					
B. Total cardenolide per butterfly on $\log_{10}$ plant concentrations by area, by sex, and for all interactions ($r^2 = 0.543$; type II sum of squares)					
Model	15	945870	63058	5.46	<0.0001
Error	69	796709	11547		
Corrected total	84	1742579			
Log plant concentration	1	243380		21.08	<0.0001
Area	3	16069		0.46	>0.71
Sex	1	1051		0.09	>0.76
Log plant concentration × area	3	15027		0.43	>0.73
Log plant concentration × sex	1	1119		0.10	>0.75
Sex × area	3	20538		0.59	>0.62
Log plant concentration × sex × area	3	18500		0.53	>0.66

micrograms of cardenolide per butterfly is highly dependent on the $\log_{10}$ plant concentrations ($Y = 276.60 \log_{10} X - 31.33$; $r^2 = 0.403$; $P < 0.0001$), and the slope is significantly different from 0 at the $P < 0.0001$ level, whereas the intercept does not differ significantly from zero. This regression equation predicts that butterflies which fed upon plants containing as little as 2 μg/0.1 g would contain approximately 52 μg of cardenolide, those on 50-μg plants would contain 439 μg, those on 100-μg plants would contain 522 μg, and those on 300 μg plants would contain 654 μg, etc.

The second regression (Table 6B) indicated that the only significant predictor of butterfly concentrations derives from the plant concentrations ($P < 0.0001$): neither the sex of the butterflies, their geographic location nor the interaction has a significant effect (all P values > 0.62). The order of the mean total cardenolide per butterfly in the four areas (area 1, 3, 4, and 2) is similar to that of the mean plant concentrations (Area 1, 3, and $2 \simeq 4$).

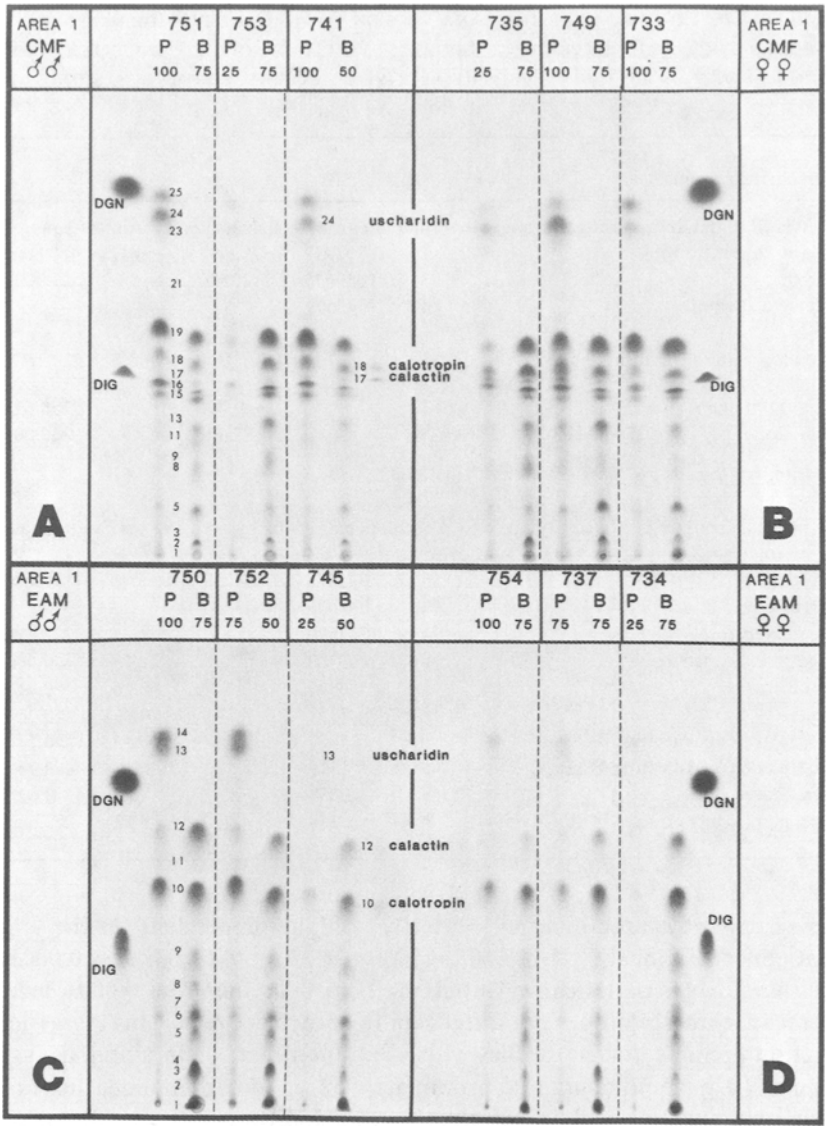


FIG. 4. Photographic reproduction of the thin-layer chromatographic profiles of the cardenolides stored by adult monarch butterflies from their *Asclepias californica* larval foodplants in California. Sections A–D of the plate each show the TLC profiles of three pairs of plants and corresponding individual male or female monarch butterflies reared on these plants form the Mt. Diablo (area 1) population. Five standards were run on each plate including digitoxin (DIG), digitoxigenin (DGN), uscharidin, calactin, and calotropin. Sections A and B were run in the chloroform–

TLC Cardenolide Profiles of Plants and Butterflies: Chloroform–Methanol–Formamide (CMF) System

Figure 4A and B is a photograph of one of the eight TLC plates run in the CMF system and shows six individual plants and the three male and three female butterflies reared thereon from area 1. Table 7 summarizes the means and standard deviations for both $R_{\text{digitoxin}}$ (R_d) and spot intensity (SI) values for all the spots in the 48 plant and butterfly samples from the four areas. Table 7 also gives each spot's probability of occurrence (PO), based on the proportion of plants or butterflies in which each spot occurred, regardless of intensity. Although several spots were sometimes weak or absent in the individual plant or butterfly channels, the standard deviations of the R_d values of all spots are low, i.e., resolution of the cardenolides is good.

Figure 5 is based on the mean R_d and SI values from Table 7 and depicts the average cardenolide profile for the CMF system for the plants and butterflies representing most of the geographic range of *Asclepias californica.* The shapes and sizes of the spots are drawn from a representative TLC plate and are generalized in the figure. Twenty-five discernible cardenolides of varying intensities were resolved in this TLC system. Of these, 24 occurred in the plants and 18 occurred in the butterflies. Spot 10 was not present in any of the plants nor was spot 12 in any of the butterflies.

Figure 5 also shows the R_d values for digitoxin, digitoxigenin, calactin, calotropin, and uscharidin. The mean migration distance for 16 digitoxin spots (two on each of the eight plates) was 43.75 mm, with a range of 38.0–47.5 mm, and a SD of 2.77 mm. Corresponding values for 16 digitoxigenin spots were: 92.40 mm, 86.5–97.0 mm, and a SD of 2.78 mm. The mean $R_{\text{digitoxin}}$ value for digitoxigenin was 2.12, with a range of 1.89–2.29, and a SD of 0.11. Corresponding $R_{\text{digitoxin}}$ values for eight uscharidin spots were 1.86, 1.74–1.98, SD = 0.08; for eight calotropin spots were 1.06, 1.02–1.11, SD = 0.03; and for eight calactin spots were 0.98, 0.97–0.99, SD = 0.01. (One each of these three standards was spotted on the center of each plate.)

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methanol–formamide system which separates a total of 25 spots. Twenty-four of these (all except spot 10) occur in the plants, and 18 (Nos. 1–19, excepting spot 12) occur in the butterflies. Because of the low gross concentrations of cardenolides in these examples, several spots are not visualized (but see the generalized drawing in Figure 5). Sections C and D show a second set of six sample pairs run in the ethyl acetate–methanol system which separates up to 14 cardenolides in the plants and up to 12 in the butterflies. Spots 17 and 18 in the CMF system and spots 12 and 10 in the EAM system correspond, respectively, to calactin and calotropin, and spots 24 and 13 in the two respective systems correspond to uscharidin.

TABLE 7. SUMMARY OF MEANS AND STANDARD DEVIATIONS FOR $R_{digitoxin}$ VALUES AND SPOT INTENSITIES FOR 25 CARDENOLIDES AND PROBABILITY OF THEIR OCCURRENCE IN 48 PAIRED PLANTS AND BUTTERFLIES.[a]

	Means				Standard deviations							
	$R_{digitoxin}$ values		Spot intensity values		$R_{digitoxin}$ values		Spot intensity values		Probability of spot		Subsample sizes	
Spot No.	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly	Plant	Bfly
25	2.05		1.94		0.12		1.10		0.71		34	
24	1.91		2.10		0.10		1.22		0.83		40	
23	1.78		1.40		0.10		0.52		0.21		10	
22	1.64		1.00		0.04		0.00		0.15		7	
21	1.51		1.17		0.06		0.41		0.13		6	
20	1.46		1.00		0.00		0.00		0.02		1	
19	1.19	1.19	2.62	4.21	0.06	0.05	1.34	0.94	0.77	1.00	37	48
18	1.06	1.04	2.16	3.47	0.05	0.04	1.07	1.06	0.77	0.94	37	45
17	0.97	0.96	2.24	2.64	0.04	0.02	1.09	1.14	0.60	0.81	29	39
16	0.93	0.92	2.53	2.91	0.03	0.03	1.34	1.31	0.67	0.73	32	35
15	0.89	0.89	2.05	3.18	0.03	0.03	1.08	1.17	0.40	0.81	19	39
14	0.83	0.83	1.26	3.14	0.03	0.04	0.65	1.32	0.40	0.79	19	37
13	0.72	0.73	1.13	3.04	0.05	0.04	0.46	0.87	0.48	1.00	23	48
12	0.67		1.25		0.06		0.50		0.08		4	
11	0.64	0.64	1.11	2.58	0.03	0.04	0.32	1.07	0.38	0.90	18	43
10		0.62		2.42		0.05		1.02		0.50		24
9	0.52	0.56	1.10	2.00	0.02	0.05	0.32	1.05	0.21	0.67	10	32
8	0.48	0.50	1.18	1.92	0.05	0.05	0.39	0.96	0.38	0.81	17	39
7	0.42	0.44	1.00	1.42	0.03	0.04	0.00	0.61	0.15	0.40	7	19
6	0.35	0.35	1.00	1.11	0.03	0.02	0.00	0.31	0.15	0.77	7	37
5	0.25	0.27	1.19	3.75	0.02	0.02	0.48	0.98	0.56	1.00	27	48
4	0.16	0.20	1.00	1.50	0.01	0.01	0.00	0.67	0.06	0.25	3	12
3	0.12	0.14	1.18	2.90	0.02	0.01	0.40	1.16	0.23	0.88	11	42
2	0.07	0.08	1.03	3.73	0.02	0.01	0.18	1.25	0.63	1.00	30	48
1	0.00	0.00	1.11	2.31	0.00	0.00	0.33	1.00	0.19	0.94	9	45

[a] Based on chromatograms of 48 plants and corresponding butterflies reared thereon (chloroform–methanol–formamide TLC system).

Plants. As pointed out in the methods, the overall SIs in the cleaned plant extracts often were less than anticipated from the spectroassay results obtained with the crude plant extracts. This may have been due, at least in part, to recording a higher concentration of cardenolides (by a factor of about two) in the crude extracts than in the cleaned extracts. There are two possible explanations for this: (1) interfering noncardenolide coextractives

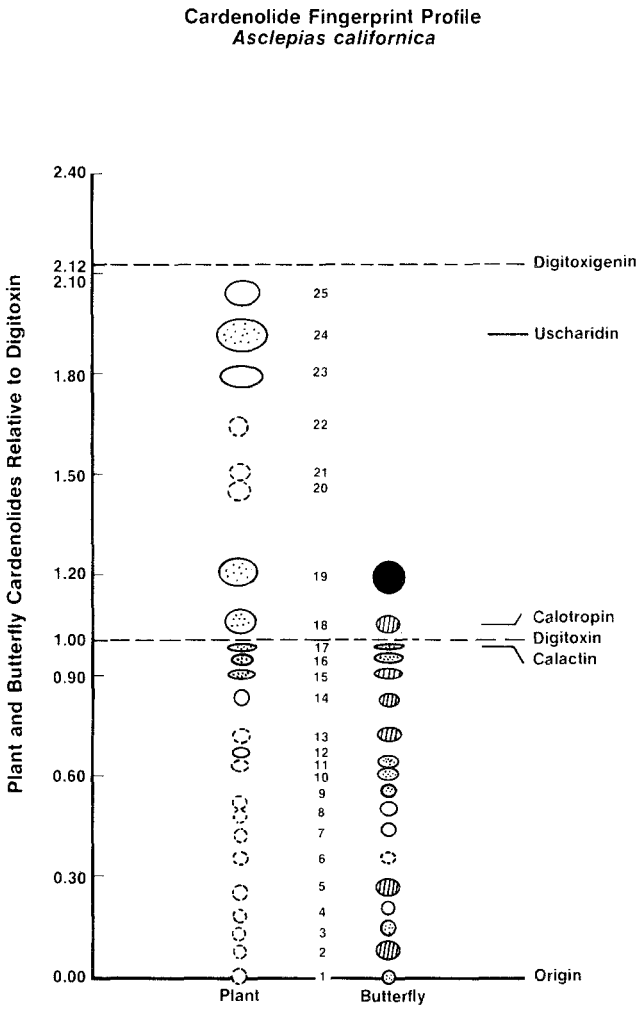

FIG. 5. The cardenolide fingerprint profile for freshly emerged monarch butterflies reared as larvae on *Asclepias californica* based on the CMF (chloroform–methanol–formamide) TLC system. The diagram shows mean $R_{digitoxin}$ and spot intensity values for 24 plant and 18 butterfly cardenolide spots and is based on the data in Table 8 for 48 plants and the 48 respective butterflies (24 males, 24 females) reared thereon. Spot intensities are represented as follows: 1.00 to <1.25, dotted circle; 1.25 to <2.00, closed circle; 2.00 to <3.00, stippled circle; 3.00 to <4.00, hatched circle; and 4.00–5.00, black circle. Fifteen of the 18 spots in the butterflies (all but Nos. 4, 6, and 7) have spot intensity values $\geq$1.50, probabilities of occurrence $\geq$0.50, and are highly diagnostic of the cardenolide fingerprint profile of freshly emerged monarch butterflies which have fed on *Asclepias californica*. Mean R_d values for calactin, calotropin, uscharidin, and digitoxigenin were, respectively 0.98, 1.06, 1.86, and 2.12.

produced a false-positive response in the spectroassay and were removed by the lead acetate cleanup, or (2) some cardenolide was lost during the cleanup. The first explanation is more plausible because the contribution of this responding background, which may occur to some extent in many *Asclepias* species, appears to be exaggerated at low plant concentrations. Seiber et al. (1982) reported 84% cardenolide recovery for another sample of *A. californica* plants which had a mean gross cardenolide concentration of 115 μg/0.1 g—roughly twice that found in this study. Also, our cleanup procedure was identical for both plant and butterfly samples so that, had we lost significant amounts of cardenolide during cleanup, the percentage losses should have been similar for both sample types. In fact, the average recovery for *A. californica* butterflies was 84%—similar to that for plant and butterfly samples from *A. eriocarpa* (Brower et al., 1982) and *A. speciosa* (Brower et al., 1984).

As a result of low concentrations, 13 of the 24 spots in the plants were frequently not detected on the TLC plates and had PO values of less than 0.40 (spots 1, 3, 4, 6–9, 11, 12, and 20–23; Table 7). This leaves 11 spots as particularly diagnostic: 2, 5, 13–19, 24, and 25. The 24 plant spots occur in two distinct R_f regions, those below digitoxin ($N = 16$, or 67%) and those above digitoxin ($N = 8$, or 33%). Unlike both *A. speciosa* and *A. eriocarpa* plants, no spots in *A. californica* occur above digitoxigenin in this TLC system.

Butterflies. The mean SI values for the butterflies (Table 7) are in all instances greater than their corresponding plant SIs. This undoubtedly accounts for their uniformly higher PO values. Thus 16 of the 18 spots (all but 4 and 7) have PO values of greater than 0.40. Moreover, only one of the 18 spots (No. 6) has an SI value of less than 1.25. This leaves 15 of the 18 spots as particularly diagnostic of the butterfly cardenolide pattern. Of these, spots 18 and 19 occur above and the remaining 13 occur below the digitoxin standard.

As was true of *A. eriocarpa* and *A. speciosa,* all the butterfly cardenolides occur in two TLC regions: 16 (89%) of them have R_f values below digitoxin, two (11%) above digitoxin, and none occurs above digitoxigenin. Overall, the butterflies are capable of storing cardenolides within approximately 58% of the R_d range of those found in the plants ($= R_d$ plant spot 19/R_d plant spot 25).

Plant–Butterfly TLC Relationships: Effects of Sex, Geography, and Plate

Because of the low PO values of some of the spots, particularly in the plants, this analysis could use only 317 of the 816 possible spot pairs (48 plant–butterfly pairs × the 17 spots in common). The numbers of matching pairs is shown in the footnote to Table 8. The analyses of SI values (Table 9)

TABLE 8. LINEAR REGRESSION ANALYSES OF $R_{digitoxin}$ VALUES OF 17 BUTTERFLY CARDENOLIDES (Y) AS FUNCTION OF $R_{digitoxin}$ VALUES OF RESPECTIVE PLANT CARDENOLIDES (X).[a]

Source of variation	*df*	SS	MS	*F*	*P*
A. Dependence of butterfly R_ds on plant R_ds as affected by four geographic areas (8 TLC plates nested therein), $r^2 = 0.966$)[b]					
Model	15	44.6374	2.9758	5808	<0.0001
Error	301	0.1542	0.0005		
Corrected total	316	44.7916			
Plant R_d	1	24.4700		47764	<0.0001
Area	3	0.0026		1.67	>0.172
Plate (area)	4	0.0046		2.24	>0.065
Plant R_d × area	3	0.0018		1.18	>0.315
Plant R_d × plate (area)	4	0.0080		3.91	<0.005
B. Dependence of butterfly R_ds on plant R_ds alone ($r^2 = 0.996$)[c]					
Model	1	44.612	44.61	78246	<0.0001
Error	315	0.179	0.0006		
Corrected total	316	44.791			
Estimated value of parameters		SE	*T* for H=O		*P*
a = *Y* intercept = 0.016		0.003	5.79		<0.0001
b = slope = 0.981		0.004	279.72		<0.0001
Equation for the line: $y = 0.981x + 0.016$					

[a] Visualized in the chloroform–methanol–formamide TLC system in 48 samples of plants and respective butterflies reared thereon. The regressions are based on 317 plant–butterfly spot pairs as follows: spot 1 = 9 pairs; 2 = 30; 3 = 11; 4 = 2; 5 = 27; 6 = 5; 7 = 2; 8 = 17; 9 = 9; 11 = 18; 13 = 23; 14 = 17; 15 = 18; 16 = 26; 17 = 29; 18 = 37; 19 = 37.
[b] Type IV sum of squares.
[c] Type II sum of squares.

used this same data subset. Initial regression analyses (see Methods and Materials) indicated that neither the slope nor the intercept of the regression of butterfly values on plant values for either R_d or SI are affected by the sex of the butterflies.

$R_{digitoxin}$ *Values.* Regression analysis with the plate variable nested within the area variable (Table 8A) indicates a highly significant dependence of butterfly R_d on plant R_d ($r^2 = 0.996$, $P < 0.0001$). There is also a significant interaction ($F = 3.91$, $P < 0.005$) involving butterfly and plant R_d values in the different plates as nested within the areas. The overall regression model therefore indicates that the slope of the regression line is slightly different for different areas because of different migration rates on the plates in the four separate TLC runs. This experimental variable is difficult to control, but fortunately its magnitude, even though statistically significant, is not great

TABLE 9. LINEAR REGRESSION ANALYSES OF SPOT INTENSITY VALUES OF 17 BUTTERFLY CARDENOLIDES (Y) AS FUNCTION OF SPOT INTENSITY VALUES OF RESPECTIVE PLANT CARDENOLIDES (X)[a]

Source of variation	*df*	SS	MS	*F*	*P*
A. Dependence of butterfly SIs on plant SIs as affected by four geographic areas (8 TLC plates nested therein), $r^2 = 0.184$)[b]					
Model	15	96.2285	6.4152	4.52	<0.0001
Error	301	427.0522	1.4188		
Corrected total	316	523.2808			
Plant SI	1	45.8543		32.32	<0.0001
Area	3	7.8345		1.84	>0.13
Plate (area)	4	9.6552		1.70	>0.14
Plant SI × area	3	17.0013		3.99	<0.009
Plant SI × plate (area)	4	6.0490		1.07	>0.37
B. Dependence of butterfly SIs on plant SIs alone ($r^2 = 0.085$)[b]					
Model	1	44.57	44.57	29.33	<0.0001
Error	315	478.71	1.52		
Corrected total	316	523.28			
Estimated value of parameters		SE	*T* for H=O		*P*
$a = y$ intercept = 2.763		0.131	21.14		<0.0001
b = slope = 0.355		0.066	5.42		<0.0001
Equation for the line: $y = 0.355x + 2.763$					

[a] Visualized in the chloroform–methanol–formamide TLC system in 58 samples of plants and respective butterflies reared thereon. The regression is based on the same plant–butterfly spot pairs as in Table 8.
[b] Type II sum of squares.

(see variation in R_d values for digitoxigenin, above). We next reran the regression model for the main effect alone (Table 8B) which gives the same high r^2 value (0.996). The estimated main regression equation is $Y = 0.981X + 0.016$, i.e., there is a virtual one-to-one correspondence of the plant and butterfly cardenolide R_d values.

SI Values. The overall relationship of butterfly SIs (Y) to plant SIs (X) is $Y = 0.355X + 2.763$ (Table 9B). The butterfly SIs showed considerably less correspondence with the plants than did the R_d values. The low r^2 value (0.184) for the first test (Table 9A) indicates only a weak (but statistically significant, $P < 0.0001$) correspondence of butterfly SIs on their respective plant SIs, and there was also a significant interaction of plant intensity × area ($P < 0.009$). Neither area per se, plates nested within the areas, nor the plant SI × plate (area) interaction was significant. The dependence of butterfly SIs solely on plant SIs (Table 9B) is also weak ($r^2 = 0.085$).

The A. californica *CMF Fingerprint Profile.* As summarized in Table 7 and Figure 5, the butterflies contain 18 of the 24 cardenolides present in the *A. californica* plants as resolved in the CMF solvent system. Fifteen of these (all but spots 4, 6, and 7) have SI values ≥ 1.50 and PO's ≥ 0.50. These 15 spots therefore are particularly diagnostic of the cardenolide fingerprint profile of freshly emerged monarch butterflies which have fed on *A. californica.*

Bioconcentration of Cardenolides in CMF System. Examination of SI values in Table 7 and Figure 5 confirms the overall quantitative bioconcentration of cardenolides by the butterflies and also suggests that it is greater for some spots, particularly those below an R_d value of approximately 0.85 (i.e., below spot 15) than for the others. Thus butterfly–plant SI value ratios greater than 2.0, from the highest to the lowest ratios, are those for spots 2, 5, 13, 14, 11, 1, and 3. These data do not indicate a consistently increasing trend of bioconcentration from higher to lower R_d values.

TLC Cardenolide Profiles of Plants and Butterflies: Ethyl Acetate–Methanol (EAM) System

As in the *A. eriocarpa* and *A. speciosa* studies, resolution of fewer cardenolides occurred in this TLC system than in the CMF system. We therefore did not quantitatively analyze the data as in Tables 7–9. Examples of three male and three female butterflies from area 1 (Mt. Diablo) and the six plants they were reared upon are in Figure 4C and D. A maximum of 14 spots in the plants and 13 in the butterflies were resolved in this system. Some of the butterflies from area 3 had an additional spot (9a) above spot 9 in this system.

Spot 10 corresponds to calotropin, spot 12 to calactin, and spot 13 to uscharidin. Calotropin occurred at consistently high intensities in the plants, whereas calactin and uscharidin were resolved at generally lower intensities. Spot 11 was present in only a few plant samples. Spots 1–9 occurred below digitoxin and matched the *Rd* values of the corresponding spots in the butterflies, as indicated in Figures 4C and D. However, due to the low gross concentrations in the plant material, these spots frequently were not resolved. In the butterflies nine spots (1, 3–7, 9, 10, and 12) were consistently resolved and usually of high intensity. Spot 10 (calotropin) was the darkest and most consistently resolved, followed by spot 12 (calactin). Spot 11 occurred weakly in a few butterflies. Neither spot 13 (uscharidin) nor 14 occurred in the butterflies. As in the CMF system, most of the spots of lower *Rf* than digitoxin were more concentrated in the butterflies than in the plants. Except for one extra spot at an *Rd* of approximately 1.00 in some of the butterflies from area 3, no obvious differences occurred in the TLC profiles of the 39 plant and 47 butterfly samples studied in the EAM system.

Conclusions from Both TLC Systems

The TLC data indicate that the majority of cardenolides present in the leaves of *A. californica* plants are stored by the butterflies. Spots 20–25 of the CMF system and spots 13 and 14 of the EAM system occur in the plants but not in the butterflies and the evidence suggests that CMF spot 24 and EAM spot 13 are uscharidin which is metabolized in the butterflies to calactin and calotropin (see Figures 4 and 5, and Discussion). As in the *A. eriocarpa* and *A. speciosa* studies, the cardenolide profiles in both TLC systems are highly consistent in the plants, both sexes of butterflies, and throughout California.

DISCUSSION

Quantitative Variation of Cardenolides in Plants and Butterflies. As was found both in the *A. eriocarpa* and *A. speciosa* studies, large variation occurred in the mean cardenolide concentrations of the 85 *A. californica* plants (9–199 μg/0.1 g), in the butterflies reared on these plants (59–410 μg/0.1 g), and in the total cardenolide per butterfly (143–823 μg). The leaves of *A. californica* have a mean concentration of 66 μg/0.1 g which is 73% of *A. speciosa* leaves (90 μg/0.1 g) and only 16% of *A. eriocarpa* leaves (421 μg/0.1 g). However, the butterflies reared on *A. californica* have higher mean concentrations (234 μg/0.1 g) than do butterflies reared on *A. speciosa* (179 μg/0.1 g), but both have lower concentrations than do those reared on *A. eriocarpa* (317.6 μg/0.1 g). *A. californica*-reared butterflies are also similar to those reared on *A. speciosa* in that in the process of sequestration and storage there is an increase of both the mean and the variance of their cardenolide concentrations compared to their plants, whereas they decrease both parameters when reared on *A. eriocarpa*. In other words, the shapes of the plant and butterfly histograms are reversed from those in the *A. eriocarpa* study (compare Figure 2 in this and the *A. speciosa* study with Figure 3 of the *A. eriocarpa* study). The higher concentrations of the *A. californica* butterflies compared to their plants may actually be even greater than 3.5-fold because of overestimation of the plant concentrations (see Methods and Materials).

Our evidence indicates significant populational variation in the gross cardenolide concentrations of *Asclepias californica* plants. The central Coast Range population has substantially higher concentrations than the three other populations, two of which in turn differ among themselves. However, there is no significant difference based on the two subspecies. These results differ from those previously established for both *Asclepias eriocarpa* (Brower et al., 1982) and *A. speciosa* (Brower et al., 1984) in which variation

in the cardenolide contents of the plants over an even wider geographic range was comparatively minor.

The fact that the populations of the distinct subspecies of *A. californica* show no consistent concentration differences suggests an evolutionary conservatism in the gross cardenolide contents of the plants and also implies that ecological factors outweigh genetically based divergence within geographically isolated populations. One such ecological determinant may be the time of the year that the plant material is collected. Thus in another sample of *A. californica* leaves from area 2 on July 25, 1979, Seiber et al. (1982) determined a mean concentration of 115 μg/0.1 g compared to our determination of 43.5 μg/0.1 g in the May 15, 1976, sample. Nelson et al. (1981) also found substantial temporal variation within leaf concentrations of *A. eriocarpa* over the course of one growing season.

The higher mean concentrations in female compared to male monarchs reared on *Asclepias eriocarpa* (Brower et al. 1982), on several other *Asclepias* species (Brower and Glazier, 1975; Brower et al., 1975; c.f. Cohen, 1983), and in collections from wild United States populations (Brower et al., 1972; Brower and Moffitt, 1974) was again found in butterflies reared on *A. californica*. However, the trend of higher concentrations in the butterflies to the north and lower concentrations to the south suggested in the *A. speciosa* study is not sustained by our data on *A. californica*.

Relationship of Cardenolide Concentrations and Total Cardenolide in Butterflies to Cardenolide Concentrations in Plants. Both concentrations and total cardenolide in the butterflies are proportional to the logarithm of the cardenolide concentrations in the plants, i.e., the butterflies significantly increase their cardenolide contents when feeding on low-concentration plants and reach an upper capacity when reared on plants containing high concentrations. This was also true for monarchs reared on *A. speciosa* but not *A. eriocarpa*. The difference occurs because *A. eriocarpa* plants all contain sufficiently high cardenolide concentrations that the lower portion of the uptake curve does not pertain (see also Brower et al., 1984).

The regression analyses also determined that concentration and total cardenolide in the butterflies are solely dependent on the concentrations of the cardenolides in the plants: no significant effects derive from storage differences based either on plants from different geographic areas or from male and female butterflies.

The data also suggest that monarchs are more adept at sequestration of cardenolides from *A. californica* and therefore able to store substantially more cardenolide than when they are reared on correspondingly low-concentration *A. speciosa* plants (compare Figure 3 of both studies).

Inferred Emetic Potencies of Monarchs Reared on A. californica. While we did not determine the emetic potencies of the butterflies reared on

A. californica in this study, we have elsewhere shown that cardenolides of the calotropagenin series (Roeske et al., 1976; Brower et al., in preparation) have a much higher emetic potency than those in *A. speciosa* (Brower et al., 1984). Thus *A. californica* may well be a very important milkweed for providing protection to the founding generation of monarchs during spring in the western United States.

Dry Weights of Butterflies and Relationships to Plant and Butterfly Cardenolide Concentrations. The average dry weight of the males in this study was slightly but significantly higher (1.07) than the females. Lighter females also characterized both the *A. eriocarpa* and *A. speciosa* studies. The butterflies' overall dry weights showed no correlation either with the cardenolide concentration in the plants ($r^2 < 0.0003$, $F = 0.02$, $P > 0.89$) or with the cardenolide concentration in the butterflies ($r^2 < 0.03$, $F = 2.48$, $P > 0.11$). The dry weight data therefore provide no evidence for a metabolic cost of either cardenolide ingestion or storage.

Relationship of Cardenolide Chemistry and Storage in A. californica. Coincidence of TLC R_f values in two solvent systems strongly indicates that spots 23, 18, and 17 in the CMF system and spots 13, 10, and 12 in the EAM system are uscharidin, calotropin, and calactin, respectively. Spot 25 in CMF may be voruscharin, although we were unable to obtain confirmation in the EAM system (Fig. 6). These four cardenolides are members of a series derived from the genin calotropagenin. Much is known of the chemistry of the calotropagenin cardenolides (Bruschweiler et al., 1969). They occur in the neo-tropical *A. curassavica* L. (Reichstein et al., 1968; Singh and Rastogi, 1969), in the African *Calotropis procera* L. (Hesse and Reicheneder, 1936; Hesse et al., 1939), *Gomphocarpus* spp. (Roeske et al., 1976; Cheung et al. 1983), and in *Pergularia extensa* (Roeske et al., 1976). Aspects of their uptake and storage have been studied in detail for monarch butterflies (Reichstein et al., 1968; Roeske et al., 1976; Seiber et al., 1980), the grasshopper, *Poekilocerus bufonius* Klug (von Euw et al., 1967), and in *Aphis nerii* (Malcolm, 1981). The evidence is that calactin and calotropin are the principal cardenolides stored when monarchs feed on plants containing calotropagenin glycosides. The 3′-keto glycoside uscharidin and its 3′-spiro derivatives uscharin and voruscharin are not stored per se by monarchs, but give way instead to calactin and calotropin (isomeric 3′-OH derivatives) by metabolism in the feeding larvae (Marty and Krieger, 1984). A similar metabolic and storage scheme has been proposed for an analogous yet distinct series of epoxy cardenolides in *A. eriocarpa* (Brower et al., 1982) wherein the 3′-keto glycoside labriformidin and its 3′-spiro derivative labriformin are metabolized to desglucosyrioside (3′-OH derivative) which is stored by monarchs. Because the larval metabolites are more polar than their parent cardenolides in the plants, the overall cardenolide TLC profile of monarchs is distinct from the plants, particularly in favoring cardenolides of lower R_f. The tendency of monarchs

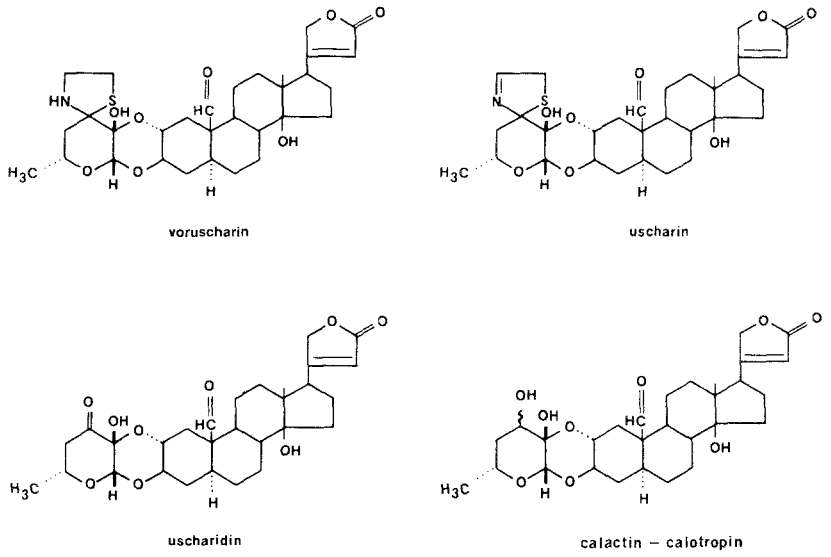

FIG. 6. Structures of five cardenolides determined by TLC analyses to occur in *Asclepias californica* plants. Uschariden, calactin, and calotropin matched perfectly with standards in both TLC systems. Voruscharin and uscharin assignments were not confirmed by cochromatography with standards. These five cardenolides occur together in *Asclepias curassavica* and several other species of Asclepiadaceae.

to store the more polar plant cardenolides, including calactin, calotropin, and desglucosyrioside, as well as compounds of even lower R_f, without metabolic alteration reinforces the trend towards an enrichment of lower R_f cardenolides in the butterflies relative to their larval foodplants.

A. californica plants and monarchs reared upon them conform to this general pattern. CMF spots 24 (uschariden), 25 (possibly voruscharin), and 20–23 are almost certainly metabolized to calactin, calotropin, and perhaps other cardenolide products which, along with cardenolides stored without metabolic alteration, constitute the cardenolide profile of freshly emerged adults. Because the calotropagenin series occurs in *A. vestita* and *A. cordifolia* (Seiber et al., 1982) and probably also in *A. cryptocerus* S. Wats. and *A. solanoana* Woodson (Roeske et al., 1976), the general storage pattern reported here for *A. californica* will likely hold for monarchs reared on these other *Asclepias* species. However, this commonality does not exclude the possibility of distinguishing among the cardenolide patterns for butterflies reared on other calotropagenin-containing *Asclepias* species. For example, *A. californica* plants and butterflies both contain a major TLC spot (spot 19, CMF) which does not occur in *A. curassavica*, although both plant species have calactin, calotropin, and other calotropagenin glycosides. This

spot 19 did not correspond to any standards in our possession and thus was not identified.

Conversion of high to lower R_f cardenolides also occurs in monarchs reared on *A. speciosa* (Brower et al., 1984) and the African milkweed, *Gomphocarpus physocarpus* E. Mey (Roeske et al., 1976; Brower et al., 1982). The general picture thus emerges that monarchs directly store plant cardenolides of moderate to high polarity, but chemically convert very nonpolar cardenolides to those of the moderate polarity range which they then store.

Chemotaxonomic Relationships of Calotropagenin Cardenolides. Woodson (1954) placed *Asclepias californica* in his subgenus 6, Solanoa, which also contains *A. solanoana* and *A. cryptocerus.* The first of these is a highly restricted and specialized California endemic, while the second is more widely distributed and occurs on the edges of the Great Basin from California northwards to Oregon, eastwards to western Colorado, and southwards to Arizona. As pointed out above, all three appear to contain cardenolides of the calotropagenin series. However, calatropogenin cardenolides also characterize the unrelated neotropical *A. curassavica,* the African *C. procera, Gomphocarpus* spp., and two other endemic California milkweeds, *A. vestita* Hook and Arn. and *A. cordifolia* (Benth.) Jepson (Seiber et al., 1982, 1983).

We conclude that cardenolides of the calotropagenin series cut widely across taxonomic lines established by Woodson (1954). Consequently, they either are of little use as chemotaxonomic character or, more likely, the infrageneric taxonomy of *Asclepias* needs reevaluation. Proof of the usefulness of cardenolide profiles in *Asclepias* taxonomy must await the analyses of more of the 108 North American species.

Comparison of Fingerprint Profiles of A. californica, A. speciosa, *and* A. eriocarpa. Twenty cardenolides were resolved in the *A. eriocarpa* plants and/or butterflies in the CMF system, 24 were resolved in *A. speciosa,* and 25 in *A. californica.* Monarchs store 16 cardenolides from *A. eriocarpa,* 21 from *A. speciosa,* and 18 from *A. californica. A. californica* plants have no cardenolides above digitoxigenin in this TLC system and thereby differ from both *A. eriocarpa* and *A. speciosa* plants which have at least two cardenolides above digitoxigenin. In both TLC systems for all three plants, the butterflies stored no spots above digitoxigenin, and in the CMF system the ratio of the R_d range of the spots in the butterflies divided by that of the plants is similar, i.e., 0.62 in *A. eriocarpa,* 0.76 in *A. speciosa,* and in 0.58 in *A. californica.*

Butterfly cardenolide profiles more closely resemble those in the corresponding *A. californica* and *A. speciosa* foodplants than in *A. eriocarpa.* For *A. speciosa,* we ascribed this to the virtual lack of cardenolides in the latex, and this situation also pertains in *A. californica* (Seiber et al., 1982).

In contrast *A. eriocarpa* plants have latex with very high concentrations of the less polar cardenolides, particularly those with an *N*, *S*-spiro ring at C-3′, which monarch larvae are readily able to metabolize and store as indicated above. Thus, in both *A. californica* and *A. speciosa*, only the plant tissue cardenolides are available for uptake and storage by the monarchs, whereas in *A. eriocarpa* cardenolides are additionally available from the latex.

Comparison of the TLC fingerprint profiles (CMF system) of butterflies reared on the three plants can be made with Figure 5 of this paper, Figure 6 in Brower et al. (1982), and Figure 5 in Brower et al. (1984). Major differences shared by both *A. californica* and *A. speciosa* in contrast to *A. eriocarpa* are the greater number of polar cardenolides below digitoxin (16 in *A. speciosa* and 17 in *A. californica* vs. 11 in *A. eriocarpa*). *A. speciosa* butterflies alone have the very prominent spot 10 at $R_d = 0.60$. Although the TLC profile of butterflies reared on *A. californica* is generally similar to that when they are reared on *A. speciosa*, the two profiles differ in several diagnostic ways. Overall, the TLC profile of *A. californica*-reared monarchs is much more uniform in appearance. Spot 18 ($R_d = 1.04$) is major in *A. californica* but has no counterpart in either *A. speciosa* or *A. eriocarpa*. Spots 13–17 in *A. californica* generally coincide with spots in the same general R_d region as those in *A. speciosa*. However, all five are of uniformly high SIs with high POs, whereas in *A. speciosa* they have generally lower SI and PO values. Importantly, although these five spots are roughly coincident in the CMF system, they are chemically distinct in the two milkweeds. Thus in *A. californica* they are calotropin, calactin, and other calotropagenin glycosides, whereas in *A. speciosa* they are epoxy cardenolides related to desglucosyrioside.

Distinction of wild-captured monarch butterflies which fed as larvae on *A. californica*, *A. eriocarpa* (Brower et al., 1982), and *A. speciosa* (Brower et al., 1984) by means of their cardenolide profiles should be clear-cut in the CMF TLC system if no major changes in cardenolide composition occur during the aging of adult monarchs. A recent investigation by Cohen (1983) in fact supports this: no qualitative differences were found in a population of adults of mixed ages which fed on *A. curassavica* in south Florida. These findings bode auspiciously for the utilization of cardenolide fingerprints of monarch butterflies for ecological studies (see also Brower, 1984a).

Ecological Implications. As far as is known, the repopulation of the entire western North American range of the monarch butterfly is dependent upon the reproduction of monarchs which survive the winter in dense aggregations along the California coastline from north of San Francisco to north of Los Angeles (Downes, in Williams et al., 1942; Williams, 1958; Urquhart, 1960; Urquhart et al., 1965, 1970). These colonies begin breaking up from approximately the middle of February through the middle of March with

both the timing and rate of breakup dependent upon local geographic and climatic conditions (Hill et al., 1976; Tuskes and Brower, 1978; Chaplin and Wells, 1982). Extensive research on the spring remigration is lacking, but the evidence suggests that increasingly fecund females fly in a predominantly easterly direction and disperse across the Coast Ranges, the Central Valley, and on into the Sierras and perhaps also into the Great Basin before they die by early summer (Urquhart, 1960; Urquhart and Urquhart, 1977; Brower, 1977; Brower and Huberth, 1977).

Dr. Arthur M. Shapiro's records for 12 years of the first-sighted spring monarchs in the Central Valley (Sacramento, Yolo, and Solano counties) are as follows: for 1972 through 1975: 6, 23, 13, and 3 March; for 1976, 9 February; and for 1977 through 1983, 21, 12, 18, 15, 16, 13, and 8 March. These observations, together with ours on the phenology of California milkweeds (see also Lynch, 1977), indicate that the spring remigration is well underway before any milkweed species have sprouted in the central Coast Ranges, i.e., in those areas closest to the overwintering colonies. Although females can probably oviposit on any *Asclepias* species they encounter (Brower, 1984b), it seems most likely that most remigrants will reach the eastern slopes of the Sierras at about the time the *A. californica* populations are sprouting (see Figure 1). Owing to its early appearance, large size, and abundance, *A. californica* may well be the most important milkweed for monarchs in establishing their spring generation in the western U.S.A. The large numbers of young caterpillars we saw in area 2 in mid-April 1976 is consistent with this interpretation. *A. cordifolia* also is a spring and early summer montane species utilized by monarchs. Although it is more widespread than *A. californica*, its populations tend to be less abundant and more patchy, and it also sprouts slightly later than *A. californica* (Brower et al., in preparation).

If the remigrating monarchs do in fact oviposit predominantly on *A. californica*, then the cardenolides stored from this plant may be of critical importance in providing chemical protection to each new spring generation of monarch adults in western North America. On the other hand, by midsummer both *A. californica* and *A. cordifolia* largely dry out and therefore can be of only marginal importance for monarchs of the last summer generation and therefore of the overwintering populations.

Now that we have established that *A. speciosa*, *A. eriocarpa*, and *A. californica* have distinctive cardenolide fingerprints, these and other ecological predictions are amenable to testing by comparing fingerprints of individuals from the spring and summer breeding populations with those of the fall overwintering populations.

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Book Review

Introduction to Ecological Biochemistry. J.B. Harborne, London: Academic Press, 1982, $35.00, $15.00 paper.

This book, which is now in its second edition, has been prepared in the recognition that ecological biochemistry is a defined, relatively new subject. Its development has been due to the successful identification of organic compounds present in microquantities, and the elucidation of their roles in the complex interactions between plants and animals, especially insects. The need to control pests in an environmentally desirable manner has also contributed to the emergence of this subject.

The book is based on a course taught by the author over a number of years to students in departments of botany, biochemistry, and biological sciences. The chapters have been organized for this purpose, so each is a defined subject. Major books and review articles are listed separately from other references, with the intention that these might form reading lists for students.

The book contains nine chapters, of which the titles and contents are described briefly: I, "The Plant and its Biochemical Adaptation to the Environment" treats the biochemical bases of adaptation to climate, biochemical adaptation to the soil, and detoxification mechanisms. II, "Biochemistry of Plant Pollination" includes the roles of flower color, flower secent, and nectar and pollen. III, "Plant Toxins and Their Effects on Animals" describes different classes of toxins, including the interrelations of cyanogenic glycosides with trefoils and snails, the cardiac glycosides with butterflies and blue jays, and the pyrrolizidine alkaloids with ragworts, moths, and butterflies. IV, "Hormonal Interactions Between Plants and Animals" treats the plant estrogens, insect moulting, and juvenile hormones from plants, and the fruit fly–cactus interaction. V, "Insect Feeding Preferences" is a discussion of the biochemical basis of plant selection by insects, of secondary compounds as either feeding attractants or feeding deterrents, and the evolution of the latter in higher plants. IV, "Feeding Preferences of Vetebrates, Including Man" describes the chemical factors and properties associated with the feeding of animals and man on plant foods. VII, "Animal Pheromones and Defense Substances" lists and discusses a number of insect and mammalian pheromones and defense substances, most of which are associated with insects. VIII, "Biochemical Interactions between Higher

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Plants" is an introduction to the biochemical aspects of the emerging subject of allelopathy. Finally, IX, "Higher Plant–Lower Plant Interactions: Phytoalexins and Phytotoxins" discusses the biochemical basis for disease resistance and the phytotoxins associated with plant disease.

If the book were to be published in a third edition, several subjects perhaps could be added or receive greater emphasis. Toxicants from tropical and desert plants, antibiotics from bacteria, microbial toxins, and natural products affecting fungal development and differentiation might comprise one or two subjects. A broader discussion of phytoalexins, plant growth regulators, photosensitizers, elicitors, and primitive immune systems might also be appropriate. The pheromone field is also rapidly expanding, as is the capability of analyzing the behavior of insects and other animals. Finally, the focus of this book has been somewhat limited in its coverage of the ecological biochemistry associated with pests of crop plants. Although relationships elucidated between "so-called" noneconomic plants and their pests can often be extrapolated to those of crop plants and their pests, there is an urgent, worldwide need to more closely focus research and attention directly on the latter.

In summary, the book competently accomplishes its stated goal of being a textbook for biologically and biochemically oriented students. It will further serve as a valuable reference for scientists conducting research in the general area of chemical ecology, or, as the author describes it, ecological biochemistry.

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