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RESPONSE OF THE EUROPEAN ELM BARK BEETLE, *Scolytus multistriatus* (Coleoptera: Scolytidae), TO ISOMERS AND COMPONENTS OF ITS PHEROMONE

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Abstract—Laboratory bioassays (two methods) and field tests demonstrated synergistic action of the three components [(−)-4-methyl-3-heptanol (I); (−)-2,4-dimethyl-5-ethyl-6,8-dioxabicylo[3.2.1]octane (α-multistriatin) (II); and (−)-α-cubebene (III)] of the pheromone bouquet of *Scolytus multistriatus*. Individually and in pairs the components were slightly attractive; I+II was clearly the most active doublet. Indirect evidence indicates that only one of the four enantiomers of I is active. Of the α, β, γ and δ isomers of II, only the α is active. With the addition of compound I, slightly attractive extract from mated females became nearly as active as extract from virgin females.

Key Words—*Scolytus multistriatus*, bioassay, aggregating pheromone, isomers, enantiomers, 4-methyl-3-heptanol, α-multistriatin, α-cubebene.

INTRODUCTION

The aggregating pheromone of the European elm bark beetle, *Scolytus multistriatus* (Marsham), has been identified as a combination of 3 compounds (−)-4-methyl-3-heptanol (I), (−)-α-multistriatin (II), and (−)-α-cubebene (III) (Pearce et al., 1975). Laboratory bioassays of the three compounds, singly and in all combinations, showed that the compounds act synergistically. Field tests demonstrated that a mixture of synthetic I and II, plus III obtained

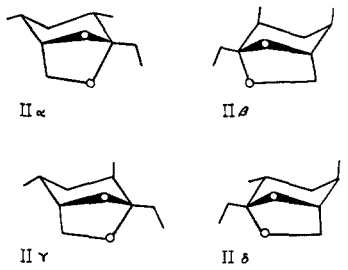

FIG. 1. Isomers of multistriatin: α—natural active; β—natural inactive; γ and δ not known in nature.

from cubeb oil, was as attractive to both sexes of *S. multiastiatus* as virgin females boring in elm logs (Pearce et al., 1975).

Synthetic 4-methyl-3-heptanol (I) consists of a pair of diastereomers, each with two enantiomers; only the (−) enantiomer is produced by virgin female beetles. Multistriatin (II) occurs as four diastereomers (α, β, γ, and δ, Figure 1), each consisting of two enantiomers. Pearce et al. (1975) found α- and β-multistriatin in extracts from aeration of beetle-infested logs, but only the α isomer was clearly attractive in the laboratory.

We present the results of further laboratory and field tests of the relative attractiveness of I, II, and III, their isomers, and various combinations of these compounds.

METHODS AND MATERIALS

Chemicals

Natural (−)-4-methyl-3-heptanol (In), (−)-α-multistriatin (IIn), and (−)-α-cubebene (IIIn) were isolated from the air surrounding virgin female *S. multistriatus* boring in elm logs. The logs were aerated, volatile compounds were collected with Porapak Q®, and crude extracts were obtained by solvent extraction of the Porapak (Byrne et al., 1975; Peacock et al., 1975). The pheromone components were isolated as pure compounds from the Porapak extracts by preparative GLC (Pearce et al., 1975). Porapak extracts of mixed sexes boring in elm logs were prepared in the same manner as the virgin female extracts.

Synthetic 4-methyl-3-heptanol (Is) was obtained from Aldrich Chem. Co., purified by preparative GLC (Carbowax 20M, 5% on AW DMCS Chromosorb G, 6 mm × 6 m silylated glass), and tested as a mixture of diastereomers (approximately 50:50).

Synthetic multistriatin isomers (IIα-δ) were prepared by two routes, and the individual isomers were purified by preparative GLC (Gore et al., 1975; Pearce et al., 1976). The relative stereochemistry of the multistriatin isomers with respect to the C-2 and C-4 methyl groups has been assigned: IIα is 2 *endo*, 4 *endo*; IIβ is 2 *exo*, 4 *exo*; IIγ is 2 *endo*, 4 *exo*; and IIδ is 2 *exo*, 4 *endo* (Gore et al., 1975, Gore and Armitage, 1976).

(−)-α-cubebene (IIIc) was obtained in sufficient quantities for field testing by distillation from cubeb oil (Pearce et al., 1975) and subsequent preparative GLC (SE-30 5% on AW DMCS Chromosorb).

Lab Bioassays

Two laboratory bioassays were used in this study. Bioassay A, conducted by JWP at Delaware, Ohio, employed an olfactometer in which beetles were induced by a weak light source to walk down a walkway covered with filter

TABLE 1. INDICES OF ATTRACTION[a] OF *Scolytus multistriatus* TO COMPONENTS OF ITS PHEROMONE

	Bioassay A[c,d]		Bioassay B		Field[e]	
Material tested[b]	No. of trials	Index	No. of trials	Index	No. of trials	Index
I	3	3	4	13	2	6
II	3	3	4	7	2	4
III	3	6	4	20	2	5
I+II	3	49	4	41	13	72
I+III	3	38	3	33	13	14
II+III	3	6	3	30	13	15
I+II+III	6	100	6	100	15	100
Extract	6	99	6	118		

[a] Index of attraction = No. responding to test/No. responding to standard tripartite mixture. Standard is index 100.

[b] I (4-methyl-3-heptanol) and II (α-multistriatin) were synthetic; III (α-cubebene) was distilled from cubeb oil.

[c] Dosage = the amount of the components produced by 50 female beetles in one hour (beetle-hour equivalents or BH) for lab bioassay A, 10 BH for bioassay B and 1500 BH for field bioassay. A 50-BH aliquot contained absolute amounts of the components as follows: I = 25 ng, II = 1.9 ng, III = 50 ng.

[d] Lab trials consisted of 25 beetles each.

[e] Field tests comparing compounds singly and I+II+III caught an aggregate of 704 beetles; test with doublets and I+II+III caught 4751 beetles. Quantities used were 14 μg I, 7 μg II, and 25 μg III per 4-day test.

paper. A petri dish recessed beneath the paper near the end of the walkway contained materials being tested. Perforations in the paper over the area of the petri dish allowed passage of the odorants. Beetles that made at least two 180° turns or four 90° changes in direction at the perforated area were considered to have responded to the test material (Peacock et al., 1973).

Bioassay B, conducted at Syracuse by GNL, employed the forced-air olfactometer described by Moeck (1970), except that the arena on which the beetles were placed was open rather than glass-covered. An air stream of 60 μl/min through a 3-mm ID glass tube carried odorant being tested. Beetles released at one side of the arena encountered the odorant when they walked toward the source of a light beam intersecting the air stream 10 cm from its outlet. Beetles that interrupted their path toward the light source and walked at least 2 cm up the air stream or that made at least one 360° turn within the air stream were considered to have responded to the odorant being tested. Beetles that did not respond the first time they passed through the air stream were retested. The number of positively responding beetles was the sum of those responding in both trials.

Each replicate in both bioassays used 25 male beetles that had been conditioned under a fluorescent lamp at 23°C for 18–24 hr. A 10-cm petri dish with a dry filter paper floor was used to hold 50–100 beetles during conditioning. Beetles used for bioassay A were from a laboratory colony; those used for bioassay B emerged from naturally infested elm wood. Dosages of the materials tested are given in Table 1.

Field Bioassays

For field tests, four blower olfactometers, described by Vité et al. (1963), were positioned 10 m apart in a row in a residential area in Syracuse, New York. Materials to be tested were dispensed from polyethylene vial caps (Kimble, 60975L) at the bottom of the blower. Beetles responding to the odorants were captured on a vertical plywood sheet, 60 cm^2, covered with polyethylene, and coated with Stikem Special® (Michael & Pelton Co., Emeryville, California 94608). Tests were conducted during the afternoons of warm days in July and August 1974. Positions of the odorants were rotated systematically so that each material would be at each olfactometer during one day of a 4-day replication. Between tests, vial caps containing odorants were held ca. −30°C.

RESULTS AND DISCUSSION

Attractiveness of I, II, and III Individually and in Combinations

Individual components of the pheromone were only slightly attractive

in the laboratory and in the field (Table 1). Any combination of two components was more attractive than single chemicals. Of the doublets, I+II was the most attractive by a slight margin in lab bioassays and clearly superior in the field. In 4 of 13 field tests, the olfactometer containing I+II caught more beetles than that containing I+II+III.

Although positions of the treatments in this comparison were rotated systematically, each doublet was necessarily adjacent to at least one treatment containing the missing component of the tripartite mixture. It was thought that the promity of III might have inflated the catch on the olfactometer containing I+II. The combinations I+II vs. I+II+III were therefore further compared by positioning the two treatments at opposite ends of the line of four blowers and leaving the intermediate positions blank. The obtained indices of 100, 8, 9, and 69 for the triplet, the two blanks, and the doublet, respectively, verify that the I+II doublet is almost as attractive as the triplet, at the concentrations tested. Subsequently, we have learned that increasing the concentration of III relative to I and II results in a corresponding increase in attractiveness (Cuthbert and Peacock, 1977).

Isomers of 4-Methyl-3-heptanol

Initial field tests of the synthetic heptanol (Is) in combination with IIn and IIIn indicated that it was less attractive than an equivalent amount of natural I (In); a fourfold increase in amount of Is resulted in attractiveness equal to that of the mixture containing In (Table 2). These data suggest that, of the 4 enantiomers in synthetic heptanol, only the naturally occurring (−) enantiomer is active.

TABLE 2. ATTRACTION OF SYNTHETIC AND NATURAL 4-METHYL-3-HEPTANOL (I) IN BLOWER OLFACTOMETERS IN THE FIELD[a]

Material tested[b]	No. of trials	Index	Material tested	No. of trials	Index
FI+IIn+IIIn	8	51	Is+IIn+IIIn	4	66
Is+IIn+FIII	8	24	In+IIn+IIIn	4	148
Is+IIn+IIIn	8	34	(4×) Is+IIn+IIIn	4	103
FI+FII+FIII	8	100	FI+IIn+IIIn	4	100

[a] Syracuse, New York, August 1973.

[b] FI, FII, and FIII indicate fractions of Porapak extract (of virgin females boring in elm logs) containing natural 4-methyl-3-heptanol, α-multistriatin and α-cubebene, respectively; Is and In indicate synthetic and natural 4-methyl-3-heptanol, respectively.

TABLE 3. INDICES OF ATTRACTION OF *Scolytus multistriatus* TO THE ISOMERS OF MULTISTRIATIN (II) IN COMBINATION WITH I AND III

Material tested	Lab bioassay A[a]		Lab bioassay B		Field bioassay[b]	
	No. of trials	Index	No. of trials	Index	No. of trials	Index
Is+IIIc	1	24	3	17		
Is+IIIc+IIβ	1	24	3	24	5	4
Is+IIIc+IIγ	1	16	3	12	4	2
Is+IIIc+IIδ	1	24	3	7	4	6
Is+IIIc+IIα	3	114	3	107	5	100
Is+IIIc+IIα (n)[c]	3	100	3	100		
Is+IIIc+IIα	3	105	3	122		
IIβ						
IIγ						
IIδ						

[a] Dosages same as those for the respective assays in Table 1.
[b] Field tests caught an aggregate of 5371 beetles.
[c] IIα (n) is natural; all other isomers of II are synthetic. Is (4-methy -3-heptanol) is synthetic and IIIc (α-cubebene) was distilled from cubeb oil.

Multistriatin

Both α- and β-multistriatin (II) were isolated from Porapak extract, but only the former was attractive in our initial laboratory bioassays (Pearce et al., 1975). Subsequent laboratory and field bioassays of synthetic pure α, β, γ, and δ isomers of II showed that only the α form increased the attractiveness of a mixture of Is+IIIc (Table 3). The β, γ, and δ isomers did not affect the activity of the mixture of Is+IIα+IIIc.

The individual isomers tested in blower olfactometers without I and III attracted relatively few beetles (838 in three heavy-flight days), but the α isomer was clearly superior (indices 100, 8, 26, and 15 for the α, β, γ, and δ forms, respectively).

The individual enantiomers of α-multistriatin were not available for comparison. However, the apparent equality of the attractiveness of the synthetic mixture of enantiomers with an equal amount of natural (−) enantiomer suggests that the (+) enantiomer might also be attractive (Table 3).

Role of 4-Methyl-3-heptanol in Termination of Attraction

After tunneling female *S. multistriatus* are mated by males, the rate

TABLE 4. IMPORTANCE OF 4-METHYL-3-HEPTANOL (I) IN THE PHEROMONE BOUQUET OF *Scolytus multistriatus*

Material tested[a]	Bioassay A		Bioassay B	
	No. of trials	Index	No. of trials	Index
Is	3	0	3	0
Extract mated females	3	59	3	31
Extract mated females + Is	3	82	3	86
Extract virgin females	6	100	3	100

[a] Dosage = 50 beetle-hour equivalents (BH) for bioassay A and 10 BH for bioassay B.

of beetles arriving at the attractive source declines precipitously (Peacock et al., 1971). Elliott et al. (1975) showed that the drop in the attractiveness of females after mating resulted from a decline in the release of the attractant rather than the production of an antiattractant by the mated females or the male. Gore et al. (1977) found that females cease the production of I shortly after mating, but the release of II by the female and III by the host material continues.

In the laboratory bioassays, we found that Porapak extract of boring virgin females was always more attractive than the extract from an equal number of mated females (Table 4). However, extract of mated females to which we added the heptanol was nearly as attractive as material from virgin females. Cuthbert and Peacock (1977) have recently demonstrated reduction in attractiveness of a mixture as the release rate of I declines relative to that of II.

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URINE-MARKING AND THE RESPONSE TO FRESH VS. AGED URINE IN WILD AND DOMESTIC NORWAY RATS

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Abstract—Wild and domestic Norway rats (*Rattus norvegicus*) were compared in regard to their tendency to investigate the odors of fresh vs. aged rat urine and to urine-mark metal rods and wooden blocks placed in their home cages. Castration, sex, and domestication had no effect on the tendency to investigate sources of fresh vs. aged urine odors, but the odor of aged urine was more attractive than fresh urine for most subjects tested. The frequency of urine marking was lower for females and castrated males but generally did not differ between wild and domestic stocks. The implications of these findings for the ecology of the species are discussed.

Key Words—urine, urine marking, odor, castration, domestication, *Rattus norvegicus*.

INTRODUCTION

Domestic stocks of mice (*Mus musculus*) and rats (*Rattus norvegicus*) have been frequently used in studies on olfactory communication (see review by Schultz and Tapp, 1973). Neither of these species possesses discrete scent organs (Stoddart, 1974) but behaviorally active substances are present in the urine and associated secretions of the preputial glands (Krames et al., 1969; Bronson and Caroom, 1971). Female mice in diestrus prefer the urine of intact males to that of castrated males (Scott and Pfaff, 1970). Male albino rats are more strongly attracted to the urine of sexually receptive than

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nonreceptive females (Pfaff and Pfaffman, 1969). Reiff (1956) found that aged or "fermenting" urine of rats is a stronger stimulus than fresh urine. However, as Schultz and Tapp (1973) point out, Reiff's results must be viewed with reservation because of certain problems implicit in his methodology and the experimental report. In addition, Lydell and Doty (1972) found no difference in the response of male rats to externally voided urine and urine taken directly from the bladder.

The frequency of urine marking is under hormonal control in both the rat (Price, 1975) and mouse (Bronson and Desjardins, 1974). Likewise, many conspecific odor preferences disappear when rats are castrated (Carr et al., 1965; Brown, 1975a).

The urine-marks of Norway rats are basically attractive to both strange and familiar conspecifics of both sexes (Eibl-Eibesfeldt, 1950; Calhoun, 1962; Telle, 1966; Brown, 1975b; Richards and Stevens, 1974), suggesting that the response to these urine deposits may be particularly complex. The wild Norway rat (*Rattus norvegicus*) will deposit urine on conspecifics (Steiniger, 1950), trails or runways (Calhoun, 1962; Eibl-Eibesfeldt, 1950; Reiff, 1956; Telle, 1966), live-traps and poison baits (Steiniger, 1950), and prominent objects in its environment such as sticks and stones (Calhoun, 1962). In the laboratory, domestic rats will mark conspecifics (Grant, 1963) and stones or wooden blocks (Brown, 1975b, Grant and Mackintosh, 1963) placed in their home cages.

The urine of rats may contain many messages, including the communication of sexual or social (dominance) status (Calhoun, 1962; Krames et al., 1969; Lydell and Doty, 1972; Desjardins et al., 1973) or it may be a means of labeling the home range of an individual (or group) for use in orientation or to maintain a sense of familiarity or "ownership" of an area (Eisenberg and Kleiman, 1972; Mykytowycz, 1974).

The deposition of urine in the living space of rats and mice can constitute a deliberate activity. Welch (1953) describes how wild house mice establish "urination posts" along frequently traveled pathways by first urinating on a fecal pellet and then, on subsequent visits, depositing urine on the mound, resulting in an accumulation of dust and debris cemented together by urine. Wild Norway rats, likewise, habitually deposit urine along commonly used trails and prominent objects on or near these pathways (Calhoun, 1962; Telle, 1966). In addition to urine, individuals of both sexes rub their urogenital regions on these sites, leaving clearly recognizable traces. This results in a mixture of secretions, desquamated skin, hairs, and sebum from glands located at the base of the tail, which undergoes oxidation and bacterial decomposition (Mykytowycz, 1974, page 335). Although these scent posts do not actively exclude strangers, they may facilitate the establishment and maintenance of dominance by the residents (Telle, 1966). Mykytowycz (1973)

has demonstrated that the outcome of a confrontation between two male wild rabbits in a neutral area can be "fixed" by placing fecal pellets or chin-gland secretion of one of the rabbits in the arena. In the presence of its own odor an individual moves confidently, attacks first, and usually dominates another. The second individual will subsequently dominate the former if an encounter is staged in the presence of its own, familiar odor.

The present study is designed to determine whether sex and physiological status influence olfactory preferences and the propensity to urine-mark. Both fresh and aged urine were presented and both laboratory-reared wild and domestic rats were used as subjects.

METHODS AND MATERIALS

Experiment 1

Subjects. Thirty-six wild and 36 domestic hooded (Long-Evans) Norway rats were divided equally among the following four categories: (1) intact males, (2) castrated males, (3) sham-operated males, and (4) intact females. Wild rats were the laboratory-reared offspring of 14 mated pairs of field-caught rats trapped in a landfill near Syracuse, New York. Domestic subjects were the offspring of 14 pairs of rats obtained from Huntington Farms, West Conshohocken, Pennsylvania. No fewer than 7 mated pairs were represented in each of the eight samples. Ages and body weights of the subjects at the start of testing are summarized in Table 1.

Test subjects were reared in like-sexed groups of two or three animals

TABLE 1. MEAN AGES AND BODY WEIGHTS (±STANDARD DEVIATIONS) AT THE START OF TESTING AND MEDIAN OLFACTORY INVESTIGATION (SNIFF) LATENCIES

	Castrated males	Sham males	Intact males	Females
Age (days)				
Wild	194±12	204±7	192±14	244±18
Domestic	180±10	183±9	193±17	228±1
Body weight (g)				
Wild	271±40	300±21	300±55	212±34
Domestic	361±25	425±47	434±42	273±25
Latency to sniff (sec)				
Wild	174	212	324	852
Domestic	12	16	39	53

in $36 \times 36 \times 18$-cm cages from weaning (25 ± 1 days) until testing. Castrations and sham operations were performed under ether anesthesia when the animals were 30–40 days of age.

Apparatus. Scent Tray. Four olfactory stimuli were simultaneously presented to the test subjects on scent trays composed of a 15.0×15.0-cm sheetmetal base to which $3.7 \times 3.7 \times 3.7$-cm wooden blocks were attached at each corner. Olfactory stimuli were contained in plastic vials, measuring 1.2 cm diameter and 2.4 cm deep, inserted in similar size holes drilled in the wooden blocks.

Threaded Rods. Norway rats will deposit urine on a 0.64-cm (1/4-in.) threaded metal rod suspended 2–3 cm above the floor of their cage (Price, 1975). Most urine-marking occurs as the rat crosses the rod perpendicularly, although rats will occasionally mark while straddling the rod. The marking posture is very subtle and consists of arching the back slightly, thus placing the perineum lower and slightly forward of its position during normal locomotion. Forward movement may slow momentarily as urine is deposited on the object in small quantities.

Placing the marked rod under ultraviolet light increases the visibility of the urine (Desjardins et al., 1973). A ruler is put alongside the rod and the presence or absence of urine at every 1/2-cm point is recorded. By totaling the number of points at which urine is present, a urine marking score is obtained that is highly correlated ($r = 0.94$, $P < 0.001$) with the actual quantity of urine deposited on the rod as determined by fluorometric analysis with a spectrophotofluorometer.

Procedure. Six days before testing the subjects were placed in individual $36 \times 36 \times 18$-cm cages in a $1.4 \times 2.4 \times 2.0$-m room and exposed to a reversed L:D cycle with darkness commencing at 07.00 hr. Each subject was given two tests with the scent tray and two with the threaded rod. Tests were conducted on Tuesday and Thursday of two successive weeks between 09.00 and 15.00 hr. Administration of scent tray and rod tests were balanced so that an animal which was given a scent tray test on the first test day (first week) was given its second scent tray test on the last test day (second week) and so forth.

At the time of testing, the animal (in its home cage) was placed on a small table and the scent tray or the threaded rod was placed in its cage. A dim (7.5-W) incandescent light permitted the investigator to observe the test subject from outside the sound-deadened experimental room. The 15-min trial was started when the subject first sniffed one of the olfactory stimuli or when it crossed the rod for the first time. If the animal did not respond in 30 min, the cage was returned to its rack.

Stimuli presented on the scent tray were: (1) distilled water, (2) excreted fresh urine—collected from a single Sprague–Dawley male rat within 24 hr

of testing, (3) fermented urine—collected from the same male rat 7–8 days before testing, and (4) an empty vial. Urine samples were collected while the donor male was housed in a cage designed to minimize contamination of the urine with feces and food crumbs. During the 15-min observation period the investigator recorded the frequency of sniffing each stimulus on the scent tray. A sniff was recorded when the animal's nose was momentarily positioned within 1 cm of the mouth of the open vial or inserted into the vial.

In addition to recording the latency to cross the threaded rod for the first time, the investigator recorded the total number of bar crossings during the 15-min test period.

Both scent trays and rods were viewed under ultraviolet light after 15-min tests to determine whether the wood blocks (on the scent trays) had been marked with urine and to quantify urine deposition on the rods. Scent trays and rods were then returned to their respective cages where they remained for 24 hr, after which the same measures were taken again.

Mean scores (averaged across the two test periods) were used in the statistical analysis (ANOVA) of each variable. Female subjects were compared with only the intact males in these tests.

RESULTS

Olfactory Investigation Tests. Olfactory investigation (sniff) latencies of wild rats were longer than those of domestic rats (Tables 1 and 2). Neither treatment (male subjects only), sex (intact subjects only), nor interaction effects were significant.

Domestic rats sniffed more per trial than wild rats (Table 2 and Figure 1). Neither treatment, sex, nor interaction effects were significant.

Frequency of sniffs differed for odor stimuli, treatment, and stocks (Table 3 and Figure 2). None of the interaction effects of these three variables were significant. Student–Newman–Keuls analysis of the four odor stimuli indicated that more sniffs ($P<0.05$) were directed at the aged urine than the empty vial but that the number of sniffs directed at aged urine, fresh urine, and water did not differ. Of the 22 male domestic subjects and the 21 wild rats that showed a preference for either aged or fresh urine, 16 (73%) of the domestic males and 15 (71%) of the wild rats investigated the aged urine more than the fresh urine ($\chi^2 = 8.4$, $df = 1$, $P<0.005$). The main effect for treatment was significant in the odor × treatment × stock analysis (Table 3). Intact males sniffed less ($P<0.05$, Student–Newman–Keuls test) than castrated rats. However, sham-operated subjects did not differ from either intact or castrated animals.

Only the main effect for stocks was significant in the odor × sex × stock

TABLE 2. SUMMARY OF STATISTICAL ANALYSES (ANOVA) OF SNIFFING

	F value	*df*	*P*<
A. Latency to sniff			
1. Male stocks × treatments			
Stocks	27.2	1	0.001
Treatments	0.6	2	N.S.
Interaction	0.1	2	N.S.
2. Intact stocks × sexes			
Stocks	22.0	1	0.001
Sexes	0.3	1	N.S.
Interaction	0.3	1	N.S.
B. Total number of sniffs			
1. Male stocks × treatments			
Stocks	41.2	1	0.001
Treatments	1.4	2	N.S.
Interaction	0.5	2	N.S.
2. Intact stocks × sexes			
Stocks	15.6	1	0.001
Sexes	0.1	1	N.S.
Interaction	0.0	1	N.S.

TABLE 3. SUMMARY OF STATISTICAL ANALYSES (ANOVA) OF NUMBER OF SNIFFS DIRECTED AT EACH ODOR STIMULUS

	F value	*df*	*P*<
Male stocks × treatments × odors			
Stocks (S)	113.5	1	0.001
Treatments (T)	3.8	2	0.025
Odors (O)	3.8	3	0.025
ST	1.5	2	N.S.
SO	0.3	3	N.S.
TO	0.2	6	N.S.
STO	0.4	6	N.S.
Intact stocks × sexes × odors			
Stocks (S)	43.1	1	0.001
Sexes (X)	0.2	1	N.S.
Odors (O)	0.5	3	N.S.
SX	0.0	1	N.S.
SO	0.2	3	N.S.
XO	0.9	3	N.S.
SXO	0.3	3	N.S.

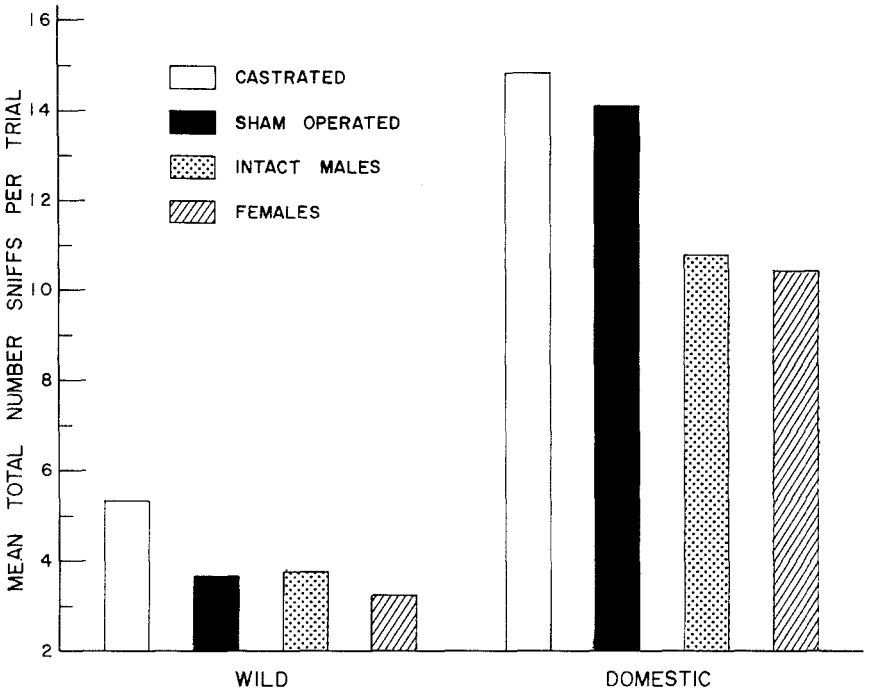

FIG. 1. Mean total number of sniffs per trial for stocks (wild and domestic), treatments (castration, sham controls and intact subjects), and sexes (intact males and females).

analysis (Table 3). As before, domestic subjects sniffed more than their wild counterparts.

Urine-Marking. In the 15-min tests (Table 4) domestic subjects had shorter latencies than wild rats to cross the rod for the first time. Neither treatment, sex (intact subjects only), nor interaction effects were significant (Table 5).

Domestic rats crossed the rod more frequently than wild rats (Tables 4 and 5). Castration and the sham operation had no effect on the frequency of rod crossings, but females crossed significantly more often than intact males (Tables 4 and 5).

Stocks did not differ in urine-marking scores (rods) during either 15-min or 24-hr tests (Table 6 and Figures 3 and 4). But castrated males urine-marked less ($P<0.05$ Student–Newman–Keuls) than sham-operated subjects in both 15-min and 24-hr rod-marking tests (Figures 3 and 4), while intact males were intermediate. Intact males marked more than females in 15-min rod tests and the same trend was noted in 24-hr tests (Table 6 and Figures 3 and 4).

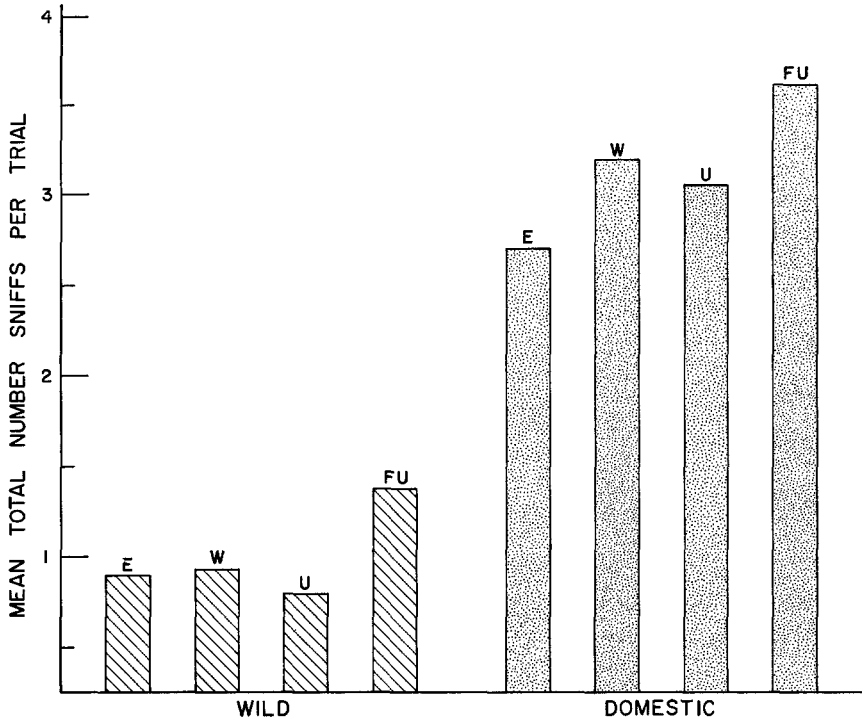

FIG. 2. Mean total number of sniffs per trial for each odor stimulus: E = empty vial, W = water, U = fresh urine, FU = aged or fermented urine.

Correlation coefficients for individual rod-marking scores and the frequency of rod crossings during 15-min tests were nonsignificant when the eight experimental samples were tested separately ($N = 9$) or in combination

TABLE 4. MEDIAN LATENCIES FOR FIRST ROD CROSSING AND MEAN TOTAL NUMBER OF ROD CROSSINGS IN 15-MIN TESTS

	Castrated males	Sham males	Intact males	Females
Latency to cross rod (sec)				
Wild	183	242	264	141
Domestic	27	14	28	47
Total number of rod crossings				
Wild	13.7	11.0	11.0	17.9
Domestic	16.4	18.7	15.8	32.2

TABLE 5. SUMMARY OF STATISTICAL ANALYSES (ANOVA) OF DATA FOR LATENCY TO CROSS AND TOTAL NUMBER OF ROD CROSSINGS PER 15-MIN TRIAL

	F value	*df*	*P*<
A. Latency to cross the rod			
1. Male stocks × treatments			
Stocks	16.7	1	0.001
Treatments	0.2	2	N.S.
Interaction	0.3	2	N.S.
2. Intact stocks × sexes			
Stocks	8.4	1	0.01
Sexes	0.8	1	N.S.
Interaction	1.6	1	N.S.
B. Total number of rod crossings			
1. Male stocks × treatments			
Stocks	6.3	1	0.025
Treatments	0.3	2	N.S.
Interaction	0.5	2	N.S.
2. Intact stocks × sexes			
Stocks	7.1	1	0.025
Sexes	10.5	1	0.005
Interaction	1.8	1	N.S.

($N = 72$). Urine-marking scores of intact and sham-operated males obtained during the first and second 24-hr rod-marking tests were correlated for both wild ($r = 0.71$, $P<0.01$) and domestic rats ($r = 0.84$, $P<0.01$).

The mean number of wooden blocks (on the scent trays) marked with urine during 24-hr test periods was lower for castrated males than intact or sham-operated males, which did not differ (Table 7 and Figures 5). Neither stock nor treatment × stock interaction effects were significant in the analysis on male subjects. However, in the stock × sex analysis, males marked more blocks than females and domestic subjects marked more blocks than wild rats (Table 7 and Figure 5).

Experiment 2

The results of Experiment 1 concern the behavior of only one population of wild Norway rats and the Long-Evans strain of domestic rats. Thus, the question arises as to the generality of these results. Consequently, 24-hr rod-marking tests were conducted on three additional rat populations.

Ten adult male wild Norway rats were trapped at the New York State Fairgrounds in Syracuse, New York. These rats averaged 327 ± 32 (SD) g at the time of capture (ages unknown) and were maintained in the laboratory

TABLE 6. SUMMARY OF STATISTICAL ANALYSES (ANOVA) OF URINE-MARKING SCORES FOR 15-MIN AND 24-HR TESTS

	F value	*df*	*P*<
A. 15-min urine-marking tests			
1. Male stocks × treatments			
Stocks	1.5	1	N.S.
Treatments	4.1	2	0.025
Interaction	0.9	2	N.S.
2. Intact stocks × sexes			
Stocks	2.7	1	N.S.
Sexes	4.6	1	0.05
Interaction	0.5	1	N.S.
B. 24-hr urine-marking tests			
1. Male stocks × treatments			
Stocks	0.1	1	N.S.
Treatments	8.9	2	0.001
Interaction	1.0	2	N.S.
2. Intact stocks × sexes			
Stocks	1.2	1	N.S.
Sexes	2.6	1	N.S.
Interaction	0.2	1	N.S.

7–12 days before testing. The 8 male Sprague–Dawley albino rats were the offspring of 4 breeding pairs originally obtained from Blue Spruce Farms, Altamont, New York. They averaged 534±53 g and 290 (range = 259–327) days of age at the start of testing. In addition, 10 male hybrid rats were obtained from 5 cross-strain pairings of wild female and Long-Evans male domestic stock. The mean body weight of this group was 433±35 g, and they averaged 271 (range = 265–279) days of age at the start of testing. Care and handling was the same as described for the animals used in Experiment 1.

The testing procedure was basically the same as described for Experiment 1 except that the two 24-hr exposures to the threaded rod were separated by only one day. Data, including the scores of the intact male laboratory-reared wild rats and Long-Evans domestic stock from Experiment 1, were statistically treated by analysis of variance.

RESULTS

Experiment 2

Differences in the urine-marking scores of the 5 male groups were of doubtful significance ($F = 2.65$, $df = 4/41$, $P<0.10$). Rod-marking scores

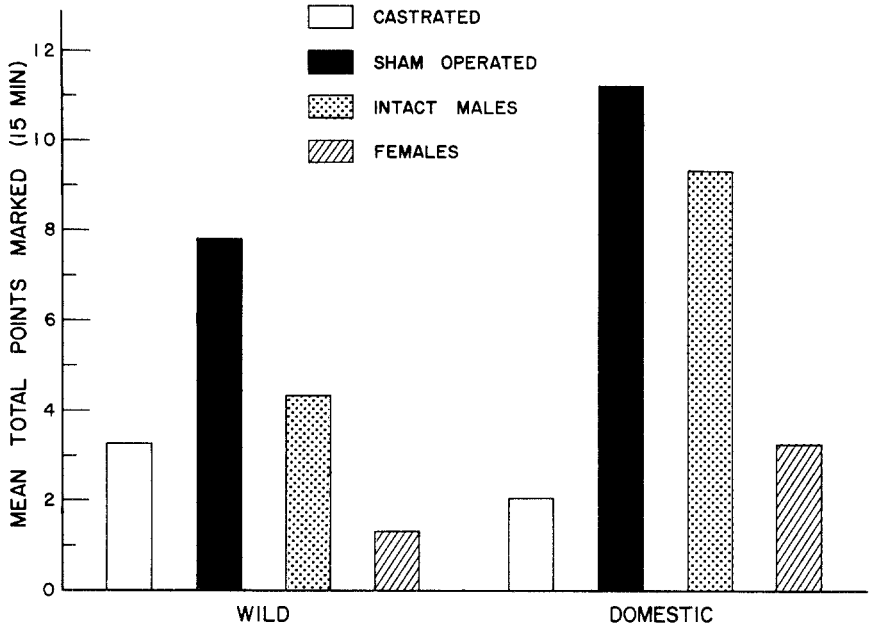

FIG. 3. Mean rod-marking scores (total points marked) for stocks, treatments, and sexes during 15-min trials.

tended to be higher for Sprague–Dawley males than for first generation laboratory-reared wild males (Figure 6). Field-trapped wild males also yielded relatively high urine-marking scores. Four wild-caught females scored similarly ($\bar{x} = 17.3$) to the first-generation laboratory reared females tested in Experiment 1.

DISCUSSION

Castration resulted in a clear decrease in urine marking. The androgen dependence of this behavior was also revealed in the relatively low urine-marking scores of the intact females and by the reinstatement of urine-marking by castrated males after testosterone replacement (Price, 1975). The significance of this finding from a population standpoint may reside in a correlation between the frequency of urine-marking and dominance behavior. The smaller contribution of immature males and females to the scent posts of the rat colony may reflect their lesser role in territorial defense (Barnett, 1975).

In contrast, castration had little impact on the propensity of males to investigate (sniff) the olfactory stimuli provided. If any change was noted, it

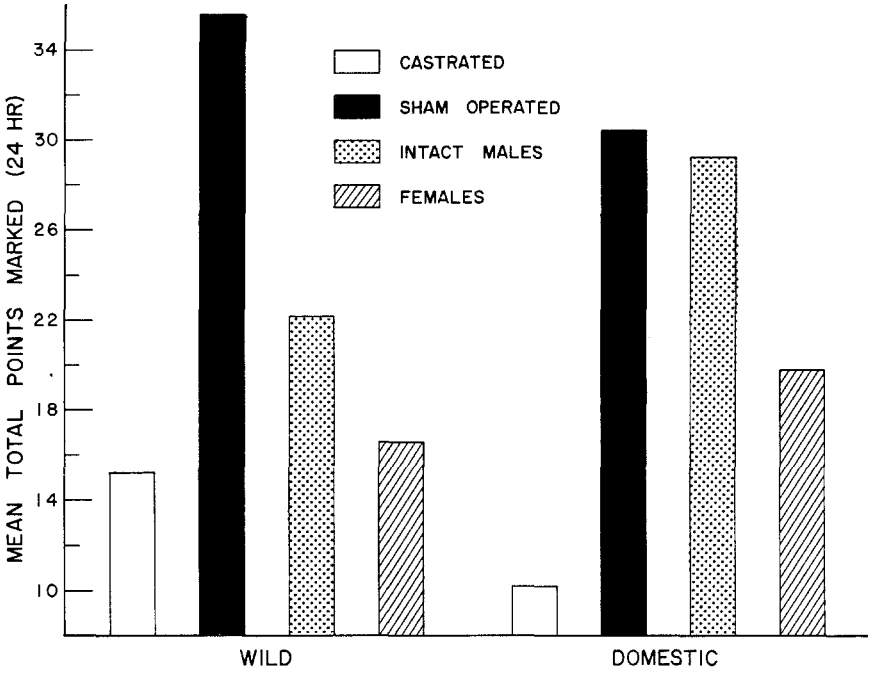

FIG. 4. Mean rod-marking scores (total points marked) for stocks, treatments, and sexes during 24-hr test periods.

was in the direction of increased investigation by the castrated subjects (Figure 1). The loss of androgen-mediated responsivity may result in an animal more concerned with stimulus reception than stimulus dissemination. But the sham-operated males also sniffed rather more, suggesting that factors other than low androgen levels might have contributed to this result. Sham-operated animals also scored higher than intact males in the rod-marking test, a result that was more noticeable for the wild stock (Figures 3 and 4). The mechanism responsible for this phenomenon remains obscure.

Nearly all subjects investigated the aged urine more frequently than the other stimuli; the blank received the fewest sniffs. To the human nose, aged or "fermented" urine presents a stronger stimulus than fresh urine; the greater attractiveness of the former may therefore be based on its strength alone. Since the attractiveness of fresh urine and water was similar it is doubtful that the fresh urine was aversive, as has been proposed for the mouse (Jones and Nowell, 1973). Only two adult males provided urine for the entire experiment. This procedure provided some standardization of the urine samples used, but urine from other donors might have produced slightly different results.

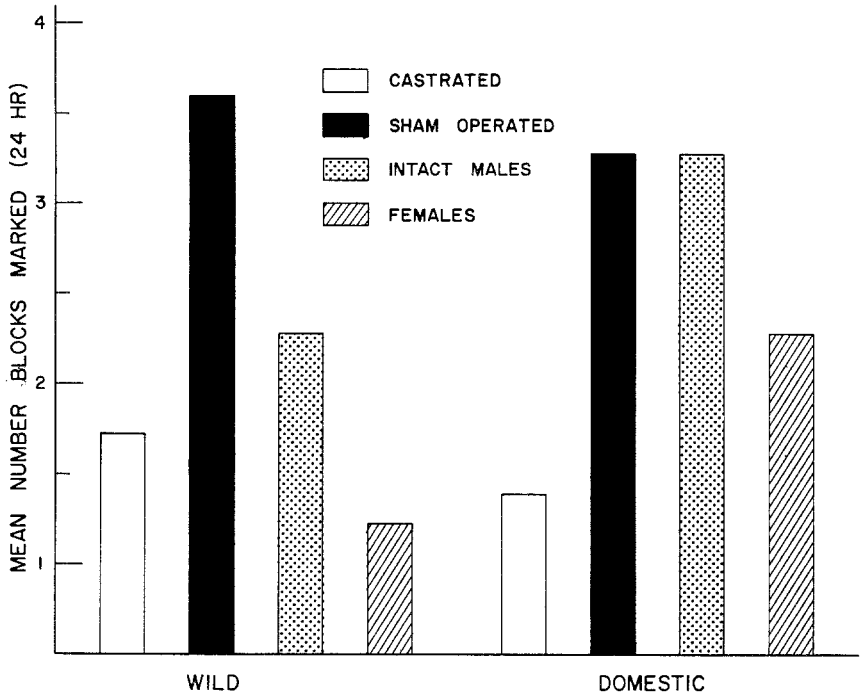

FIG. 5. Mean number of wooden blocks marked with urine during 24-hr periods (stocks, treatments, and sexes separate).

TABLE 7. SUMMARY OF STATISTICAL ANALYSES (ANOVA) OF MEAN NUMBER OF WOODEN BLOCKS MARKED WITH URINE DURING 24-HR SCENT-TRAY TESTS

	F value	*df*	*P*<
Male stocks × treatments			
Stocks	0.1	1	N.S.
Treatments	11.6	2	0.001
Interaction	1.9	2	N.S.
Intact stocks × sexes			
Stocks	5.3	1	0.05
Sexes	5.3	1	0.05
Interaction	0.0	1	N.S.

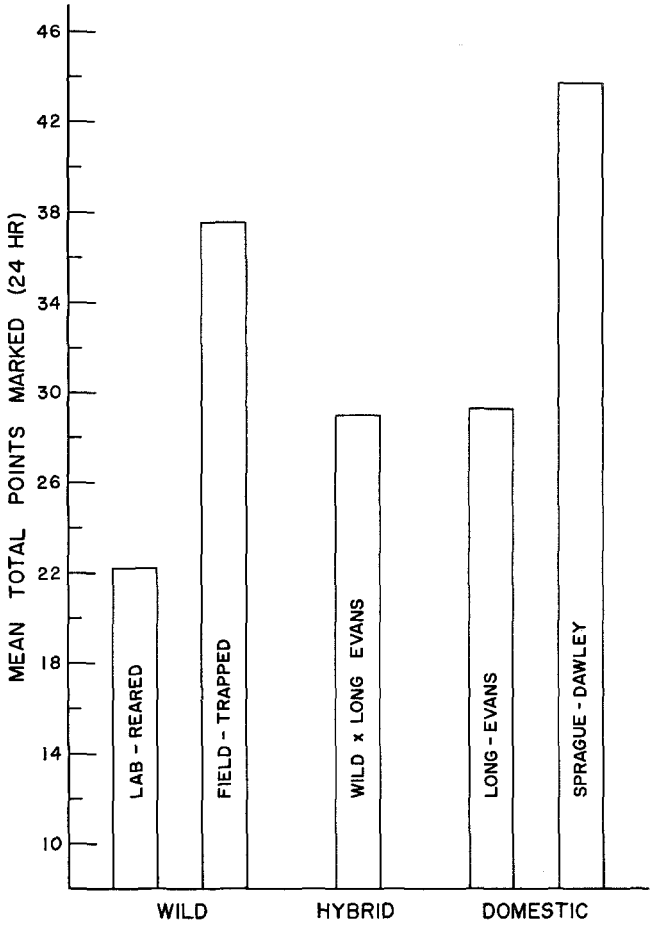

FIG. 6. Mean rod-marking scores (total points marked) of wild, hybrid, and domestic males during 24-hr period.

The finding that aged urine was investigated more frequently than fresh urine supports the suggestion of Reiff (1956) but conflicts with the conclusion of Lydell and Doty (1972). The latter investigators, however, used the urine of female donors, and did not allow the urine to age as long as in the present study.

Despite the shift in selective pressures accompanying the transition from field to laboratory environments and the marked change in group dynamics associated with current husbandry practices (Price and King, 1968), no differences were found in the propensity of wild and domestic Norway

rats to urine-mark rods and wooden blocks. In addition no stock differences were noted in the relative attractiveness of the olfactory stimuli provided in the scent-tray test. However, domestic subjects were more active than wild rats in nearly all other variables measured (latencies to sniff and cross the rod and total number of sniffs and rod crossings) during the 15-min test periods. These differences are best explained by the disturbing effect of placing the novel scent trays and threaded rods in the cages of the wild subjects immediately before testing, and by the reluctance of wild rats to investigates novel objects placed in their familiar living space (Barnett, 1958; Cowan and Barnett, 1975; Mitchell, 1976). In contrast the domestic rat is relatively undisturbed by changes in its environment and will quickly and thoroughly investigate novel objects placed in its home cage.

In general, wild and domestic Norway rats fall within the same range of variation for urine-marking behavior. Differences in urine-marking might, however, occur under different conditions. The tendency for laboratory-reared wild rats to score lower than field-trapped wild rats is also a point worth pursuing in future investigations.

The present study does little to clarify the various functions of urine-marking in free-living populations of the wild Norway rat, but it provides a broader foundation on which such investigations may be conducted. Moreover, domestic stocks may be just as suitable as wild rats for studying the odor preferences and urine-marking behavior of this species.

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DETERMINATION OF ENANTIOMER COMPOSITION OF SEVERAL BICYCLIC KETAL INSECT PHEROMONE COMPONENTS

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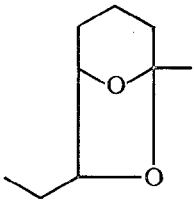
Abstract—Details are given for the determination by a chiral shift reagent of enantiomer compositions of several bark beetle pheromone components, which are bicyclic ketals. The procedure was carried out on three samples in the range of 200 micrograms. For one sample, the determination was achieved at the level of 5 micrograms.

Key Words—pheromone, *exo*-brevicomin, frontalin, multistriatin, *Dendroctonus brevicomis*, *Dendroctonus frontalis*, *Scolytus multistriatus*, enantiomeric composition, optical purity, chiral shift reagent.

INTRODUCTION

Bicyclic ketals have been described as pheromone components in three species of two genera of bark beetles: *exo*-brevicomin (I) (and *endo*-brevicomin) in *Dendroctonus brevicomis* (western pine beetle), frontalin (II) in *D. brevicomis* and *D. frontalis* (southern pine beetle), and α-multistriatin (III) in *Scolytus multistriatus* (European elm bark beetle) (Silverstein et al., 1968; Kinzer et al., 1969; Pearce et al., 1975, 1976).



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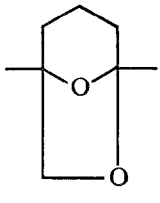

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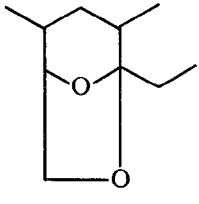

III

These are chiral compounds, and, in view of the recent reports that both sexes of *D. brevicomis* discriminate between the enantiomers of *exo*-brevicomin and of frontalin (Wood et al., 1976) and males of *D. frontalis* discriminate between enantiomers of frontalin (Dickens and Payne, private communication), it is of interest to determine the enantiomeric composition of bicyclic ketals isolated from the above sources. Most pheromones are mixtures of several components which have been shown to be different compounds, structural isomers, or geometric isomers. These mixtures may play a critical role in speciation. It seems likely that enantiomers may play a similar role in some species (Silverstein and Young, 1976).

We have reported details of two methods to determine the enantiomeric composition of several alcohols that are components of insect pheromones (Plummer et al., 1976). We now report the application of a chiral lanthanide shift reagent to several bicyclic ketals; in one instance, results are reported on a 5-μg sample of frontalin.[1]

METHODS AND MATERIALS

Synthetic *exo*-brevicomin and frontalin were obtained from Chemical Samples, Inc. The enantiomers of *exo*-brevicomin and of frontalin were synthesized by K. Mori (1974, 1975). α-Multistriatin was synthesized by the procedure of Pearce et al. (1976) and Gore et al. (1975). All samples were purified by gas–liquid chromatography (GLC).

Natural *exo*-brevicomin was isolated by aerating ponderosa pine bolts infested with female *D. brevicomis* and trapping the volatiles by the total freeze-out procedure of Browne et al. (1974). The condensate was saturated with NaCl and continuously extracted with ether for 24 hr. The dried (Na_2SO_4) condensate was concentrated to 2 ml by removal of the solvent through a glass-bead-packed column at atmospheric pressure. The concentrated sample was sequentially fractionated by GLC as follows: column A, 4% Carbowax 20M on Chromosorb G 60/80 mesh, 5.5 m $\times$ 6.3 mm (OD), 60 cm^3/min He flow rate, 110°C isothermal, collect fraction 17–20 min. Column B, 5% Apiezon L on Chromosorb G 60/80 mesh, 5.5 m $\times$ 6.3 mm (OD), 60 cm^3/min He flow rate, 125°C isothermal, collect fraction 25–27 min.

Natural frontalin was isolated from a hexane extract of 100 hindguts of *D. brevicomis* males and fractionated by GLC: Column A, 10% Carbowax 20M on Chromosorb W, 50/80 mesh 1.5 m $\times$ 9.5 mm (OD) 80 cm^3/min He flow rate, 100°C isothermal, collect fraction 15–25 min. Column B, 5%

[1] A preliminary report of these studies was presented by Dr. E.L. Plummer at the 168th National Meeting of the American Chemical Society, Atlantic City, September 1974.

Apiezon L on Chromosorb G 60/80 mesh, 6.1 m × 6.3 mm (OD), 60 cm^3/min He flow rate, 100°C isothermal, collect fraction 24–26 min. Column C, 4% Carbowax 20M on Chromosorb G 60/80 mesh, 6.1 m × 6.3 mm (OD), 60 cm^3/min He flow rate, 100°C isothermal, collect fraction 14–15 min.

Natural frontalin from *D. frontalis* was isolated from the volatile compounds collected on Porapak Q by aeration of females boring in short-leaf pine bolts (Byrne et al. 1975). The Porapak Q was extracted with pentane in a Soxhlet apparatus for 24 hr, dried over Na_2SO_4, and concentrated to 5.5 ml volume by distillation of solvent through a glass-bead-packed column at atmospheric pressure. The concentrated sample was fractionated by GLC as above for frontalin from *D. brevicomis*.

Natural α-multistriatin was isolated by aerating elm bolts infested with female *S. multistriatus* and trapping the volatiles on Porapak Q, which was then extracted with hexane; the concentrated extract was fractionated by GLC (Pearce et al., 1975; Peacock et al., 1975; Byrne et al., 1975).

GLC fractionations were carried out on a Varian 2740 instrument fitted with flame ionization detectors, a ~100:1 splitter, and a thermal gradient collector with 30.5 cm × 1.6 mm glass capillary tubes (Brownlee and Silverstein, 1968).

The tris[3-(heptafluoropropylhydroxymethylene)-*d*-camphorato]europium III, $Eu(hfbc)_3$ (Aldrich Chemical Co.) was sublimed at 190–200°C, 0.05 Torr, and sealed under vacuum prior to use. Chloroform-*d*, 100% deuterated, was obtained from Stoler Isotopes, Inc. Carbon tetrachloride, spectranalyzed grade and benzene-d6 (100%) was obtained from Fisher Scientific. These solvents were maintained over 4A molecular sieves.

Coaxial tubes (50 μl) in thin-wall (5 mm OD) NMR tubes (Wilmad Glass Co., Inc.) were used for all samples except for the racemic frontalin sample (Figure 2a), which was run in $CDCl_3$ in a 150-μl Wilmad cell, and the studies on frontalin from *D. brevicomis* hindguts: in this case, 1.1 mm OD capillaries (Varian Associates) were used.

Proton NMR spectra were obtained on a Varian XL 100 Fourier Transform spectrometer. The solution of sample, shift reagent, and tetramethylsilane in CCl_4 was filtered through a small, dry, silanized, glass wool plug into the inner tube; $CDCl_3$ in the annular space served as the lock. Sample sizes were in the range of 200 μg. Attempts to use CCl_4 solutions in the 1.1-mm capillary with an external lock were not successful, presumably because of drift. Shoolery (1975) demonstrated that C_6D_6 (containing 0.1% tetramethylsilane) as a solvent in the capillary tube served as an internal lock. However, as previously mentioned (Plummer et al., 1976), shift reagents are less effective in C_6D_6 than in CCl_4. The spectrum in Figure 3d was run after installation of a single side-band crystal filter, which increased sensitivity by about 40%.

Transfer from the 1.6-mm GLC collector capillary to the 1.1-mm NMR capillary was effected by drawing a tip on the collector capillary, inserting the tip, and washing the sample into the NMR capillary with a 7-μl slug of the solution of shift reagent in C_6D_6 containing about 0.1% of TMS. The NMR capillary was centrifuged, sealed, sonicated to mix, and recentrifuged. It was convenient to clamp the collector capillary in place and control the movement of the slug with an Adams Suction Apparatus (Clay Adams Div. of Dickinson & Co., Parsippany, New Jersey) connected with a 2-mm OD Teflon tubing to the collector capillary.

All manipulations involving the shift reagent were performed in the dry box, except for transfer from the collector capillary to the NMR capillary.

RESULTS AND DISCUSSION

Ketals are weaker Lewis bases than alcohols and are therefore rather poor donors towards lanthanide shift reagents. However, the shift reagent, Eu(hfbc)$_3$, was found to be effective with ketals, producing adequate downfield shifts and separations. Peak assignments were made by "spiking" the natural compound with racemic material since peak positions cannot be reliably reproduced with these small amounts of material.

The molar ratio of shift reagent to substrate (L/S) must be determined for each compound for a given concentration in a given solvent. Effects of temperature were demonstrated in the previous paper (Plummer et al., 1976). The optimum ratio for solutions of the bicyclic ketals in CCl_4 was in the range of about 0.25 to 0.4, but a ratio of about 2.8 was required for comparable separation in the solution of frontalin in benzene in the capillary tube. With these small amounts, the L/S ratios are approximate.

The induced chemical shifts have been correlated with the stereochemistry of the multistriatin diastereomers (Gore and Armitage, 1976).

exo-Brevicomin

Figure 1a shows an NMR spectrum of racemic *exo*-brevicomin, and Figure 1b shows a spectrum of racemic *exo*-brevicomin in the presence of Eu(hfbc)$_3$, which causes a downfield shift dependent on the molar ratio of the lanthanide shift reagent to substrate (L/S). The single signal at 1.23 ppm in Figure 1a corresponds to the protons of the methyl group on the bridgehead carbon (C-5). In the presence of the shift reagent, it moves downfield and separates into two peaks (1.98 and 2.01 ppm), representing the methyl groups of the diastereomers produced from the interaction of the two enantiomers and the chiral shift reagent. The spectrum is presumably

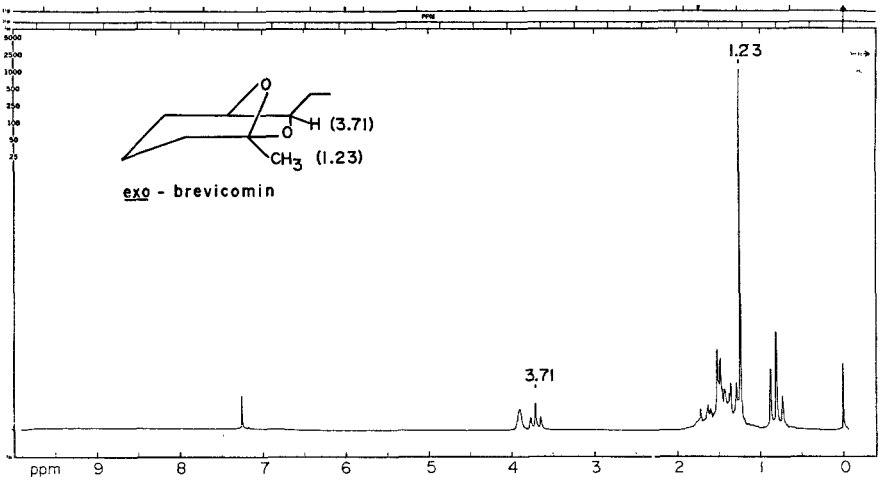

FIG. 1a. *exo*-Brevicomin (racemic), in CCl_4, 50 μl.

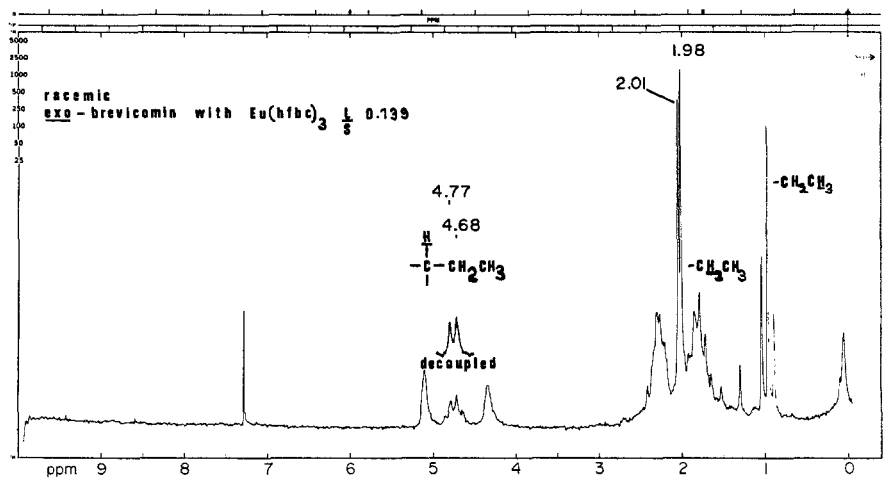

FIG. 1b. *exo*-Brevicomin (racemic), Eu(hf bc)$_3$ L/S = 0.139 in CCl_4, 50 μl.

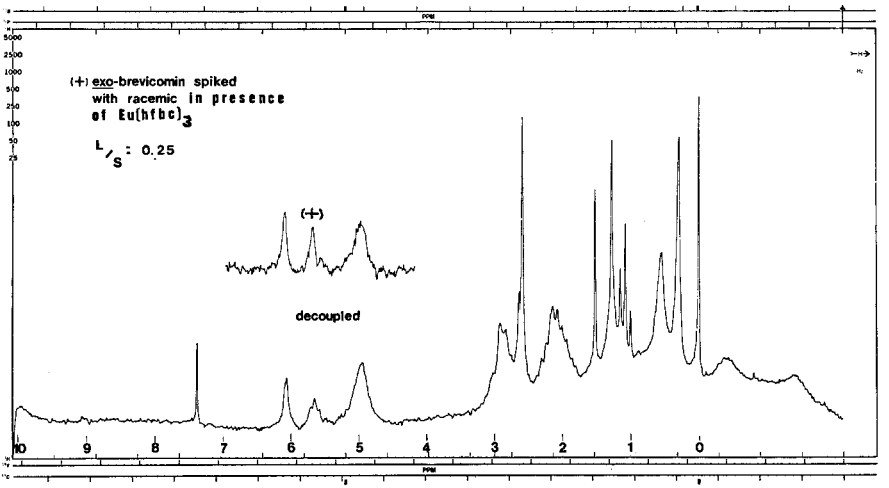

FIG. 1c. (+)-*exo*-Brevicomin spiked with racemic, Eu(hf bc)$_3$ L/S = 0.25, in CCl_4, 50 μl.

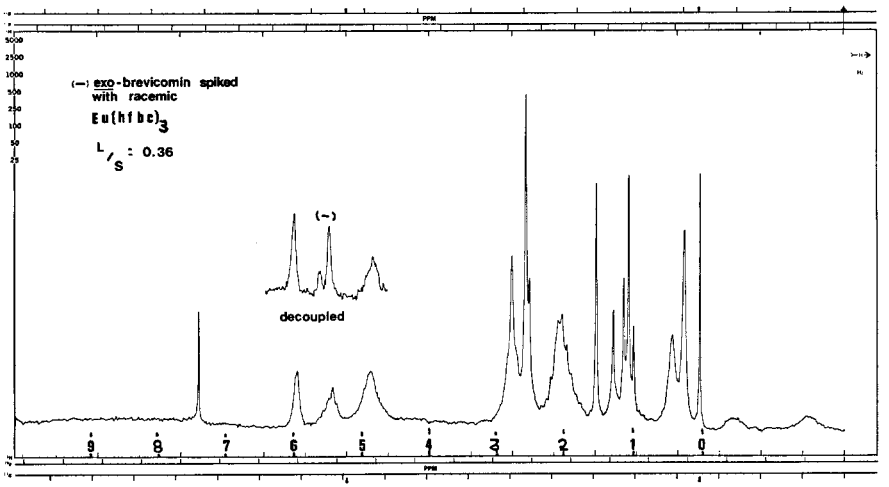

FIG. 1d. (−)-*exo*-Brevicomin spiked with racemic, Eu(hf bc)$_3$ L/S = 0.36, in CCl_4, 50 μl.

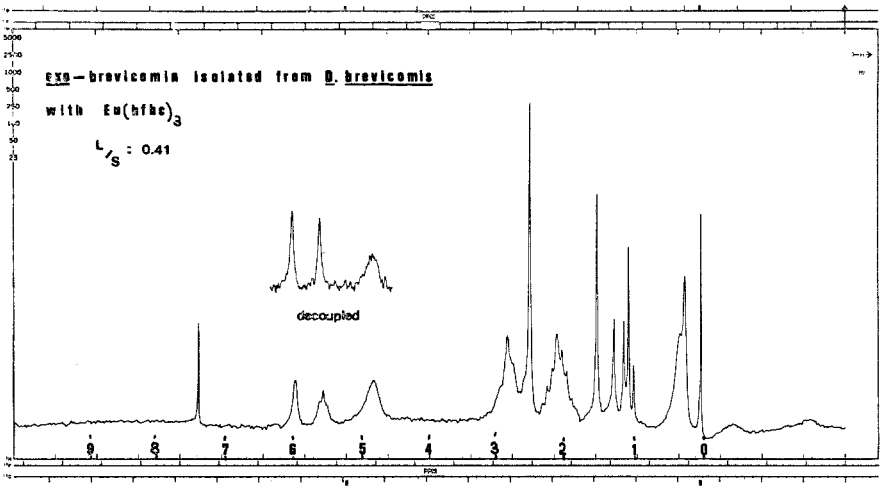

FIG. 1e. *exo* Brevicomin isoated from *D. brevicomis*, Eu(hf bc)$_3$ L/S = 0.41, in CCl_4, 30 μl.

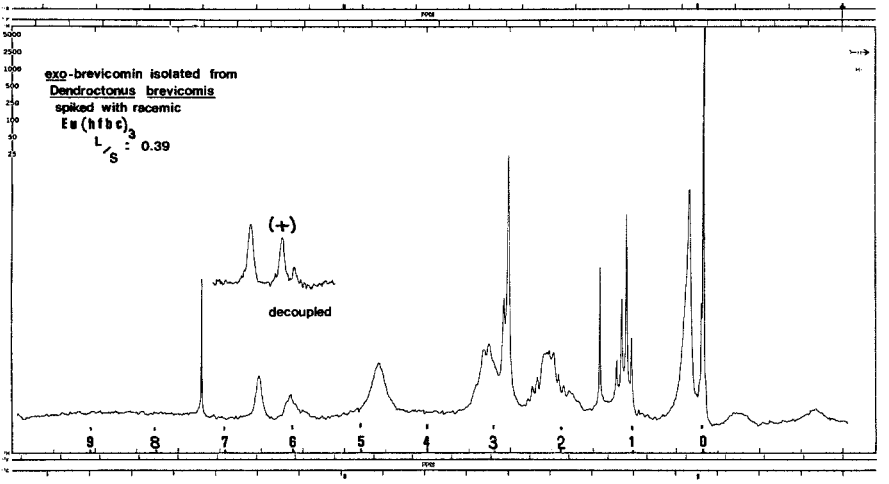

FIG. 1f. *exo*-Brevicomin isolated from *D. brevicomis* spiked with racemic, Eu(hf bc)$_3$ L/S = 0.39, in CCl_4, 50 μl.

a weighted average, resulting from the rapid interchange of the Eu complex between the possible coordinating sites (McCreary et al., 1974; Gore and Armitage, 1976). The ratio of the areas of the peaks gives the ratio of the enantiomers. When the methine proton on C-7 is decoupled by irradiation of the methylene protons of the ethyl group (Figure 1b), one enantiomer peak is seen at 4.68 ppm and the other at 4.77 ppm. This proton is effectively decoupled from the C-1 proton because of the dihedral angle of $\sim 90°$.

Because of the small amounts of material involved, there is some uncertainty in the L/S ratio, and it is difficult to replicate a peak shift. To avoid this difficulty, the sample containing a single enantiomer is "spiked" with a small amount of the racemic mixture. Figures 1c and 1d represent "spiked" samples of the (+) and (−) enantiomers, respectively, of *exo*-brevicomin. In Figure 1c, the upfield signal of the two methyl signals and the downfield signal of the two methine signals represent the (+) enantiomer. In Figure 1d, the opposite assignments can be seen.

Figure 1e is a spectrum of a naturally occurring sample of *exo*-brevicomin with Eu(hfbc)$_3$; the *exo*-brevicomin was isolated from the cold-trap condensate of aerated *D. brevicomis* female beetles. It is clear that this sample consists of one enantiomer. Figure 1f, which is a spectrum of natural *exo*-brevicomin spiked with the racemic compound in the presence of Eu(hfbc)$_3$, shows it to be the (+) enantiomer. It has been found that *D. brevicomis* beetles respond more readily to the (+) enantiomer (Wood et al., 1976).

Frontalin from D. frontalis

Figure 2a shows an NMR spectrum of racemic frontalin with the signals at 1.32 and 1.43 ppm arising from the protons of the methyl groups on C–1 and C–5. In the presence of Eu(hfbc)$_3$, as shown in Figure 2b, the methyl groups at C–1 and C–5 experience a downfield shift and each separates into two peaks (near 2.5 and 3.9 ppm). Figures 2c and 2d show (+) and (−) frontalin, respectively, in the presence of Eu(hfbc)$_3$, spiked with about one-half the amount of racemic frontalin. The upfield signals (2.51 and 3.87 ppm) of each enantiomeric pair correspond to the (+) enantiomer, while the (−) enantiomer is represented by the downfield signals.[2]

Figure 2e shows a sample of frontalin isolated by aerating *D. frontalis* females and collecting the volatiles on Porapak Q. The predominant signal of each methyl pair corresponds to the (−) enantiomer, and integration leads to an enantiomeric ratio of approximately 85(−):15(+). *D. frontalis* males respond more readily to the (−)-enantiomer than to the (+)-enantiomer (Dickens and Payne, private communication).

[2] Dr. K. Mori (1975) has reported that the chiral shift reagent, Eu(facam)$_3$ separates the methyl peaks of racemic frontalin.

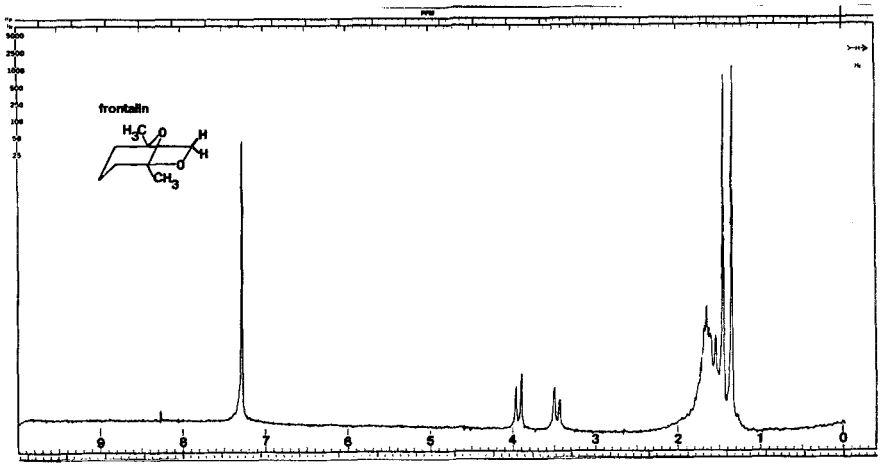

FIG. 2a. Frontalin (racemic), in $CDCl_3$, 150 μl.

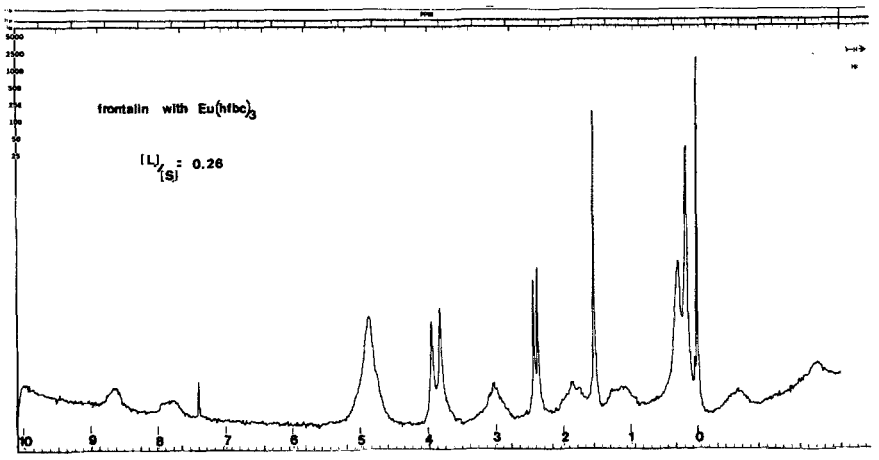

FIG. 2b. Frontalin (racemic), Eu(hfbc), $L/S = 0.26$, in CCl_4, 50 μl.

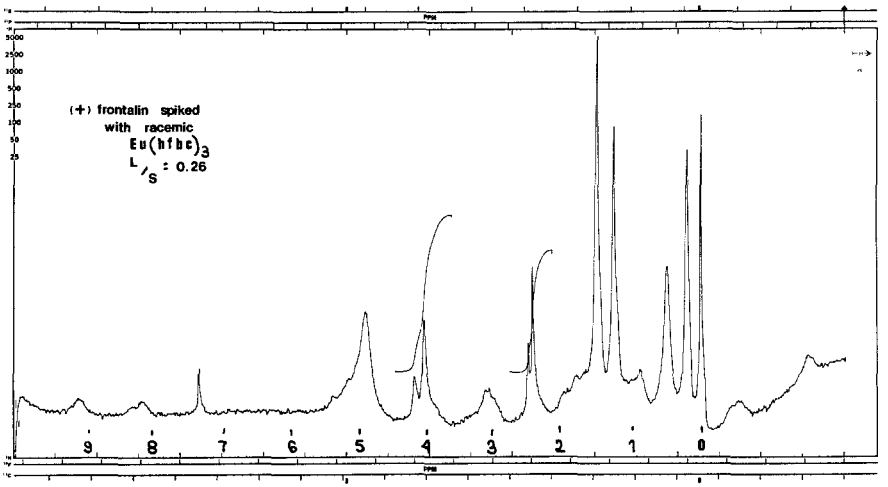

FIG. 2c. (+)-Frontalin spiked with racemic, Eu(hf bc)$_3$ L/S = 0.26, in CCl_4, 50 μl.

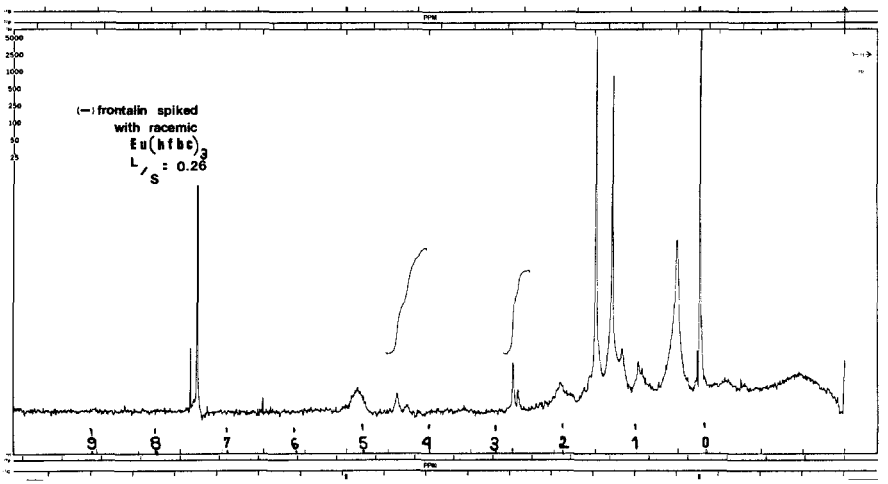

FIG. 2d. (−)-Frontalin spiked with racemic, Eu(hf bc)$_3$ L/S = 0.26, in CCl_4, 50 μl.

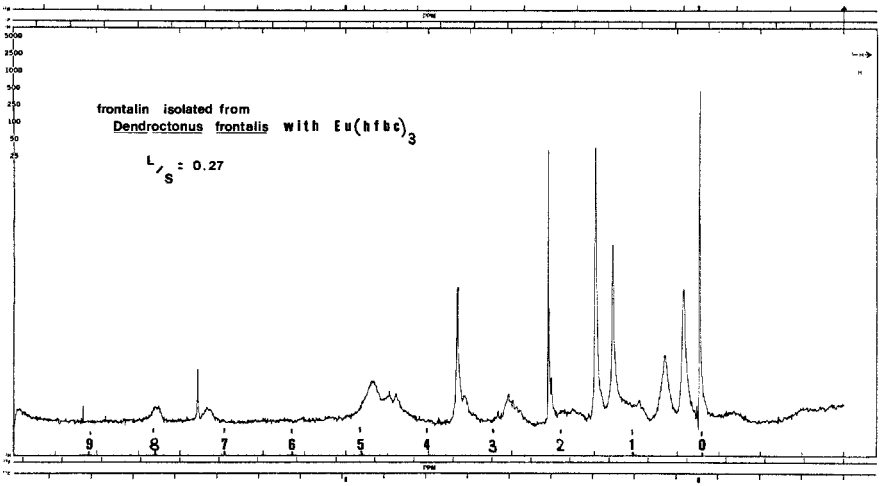

FIG. 2e. Frontalin isolated from *D. frontalis*, Eu(hf bc)$_3$ L/S = 0.27, in CCl_4, 50 μl.

Frontalin from D. brevicomis

Only about 5 μg of frontalin was isolated from the 100 male *D. brevicomis* hindguts available. Since results with the 1.1-mm capillary tube with the external lock were not promising, we adopted Shoolery's (1975) procedure of using C_6D_6 as both the solvent and internal lock, even though C_6D_6 is inferior to CCl_4 as a solvent for shift-reagent studies (Goering et al., 1974; Plummer *et al.*, 1976). Indeed a much higher ratio of shift reagent in benzene was required to bring about optimum separation of each of the methyl peaks; optimum separation of the enantiomeric peaks of racemic frontalin in benzene was achieved at an L/S ratio of about 2.8 in both the 50-μl concentric cell and in the capillary NMR tube.

Figure 3a is a spectrum of 5 μg of racemic frontalin in C_6D_6 in the 1.1-mm capillary, showing the methyl singlets at 1.13 and 1.54 ppm. Figure 3b shows the downfield shifts and enantiomeric peak separations (near 1.5 and 2.3 ppm) achieved on racemic frontalin at an L/S ratio of 2.8.

Figure 3c shows 5 μg of (+)-frontalin with the shift reagent (L/S = 2.8). Figure 3d shows 5 μg of (+)-frontalin with shift reagent spiked with ~3 μg of racemic frontalin; an expanded spectrum (full scale = 200 Hz) is shown as an insert. The upfield peak of each pair (near 1.6 and 2.6 ppm) corresponds to the (+) enantiomer; in this case the L/S ratio is probably less than the nominal L/S ratio of 2.8 because of mechanical difficulties in the transfer. In Figure 3e, 5 μg of the natural frontalin (from *D. brevicomis*) in the presence of the shift reagent (L/S = 2.8) shows only single peaks for the methyl groups.

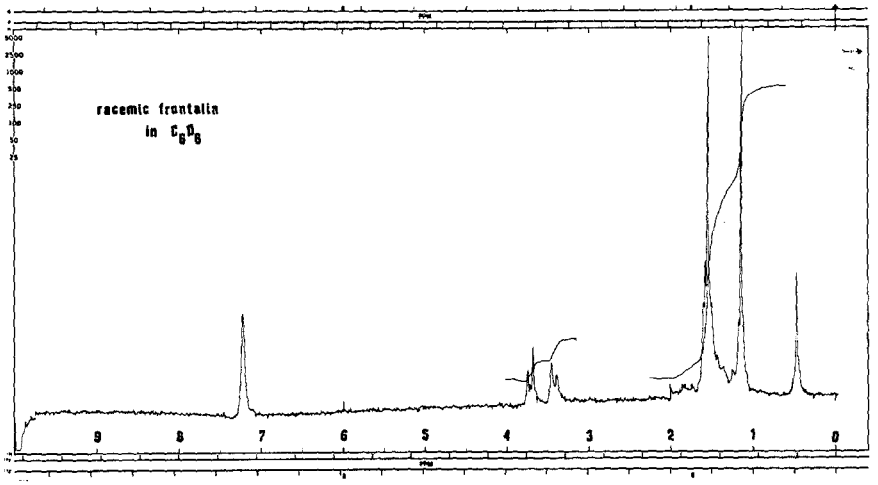

FIG. 3a. Frontalin (racemic) 5 μg in C_6D_6, 1.1 mm capillary.

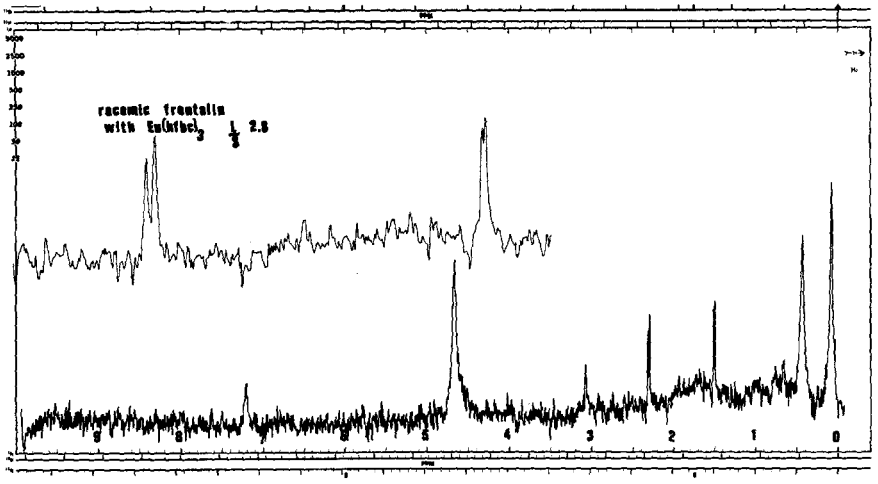

FIG. 3b. Frontalin (racemic) 5 μg, Eu(hf bc)$_3$ L/S = 2.8, in C_6D_6, 1.1 mm capillary.

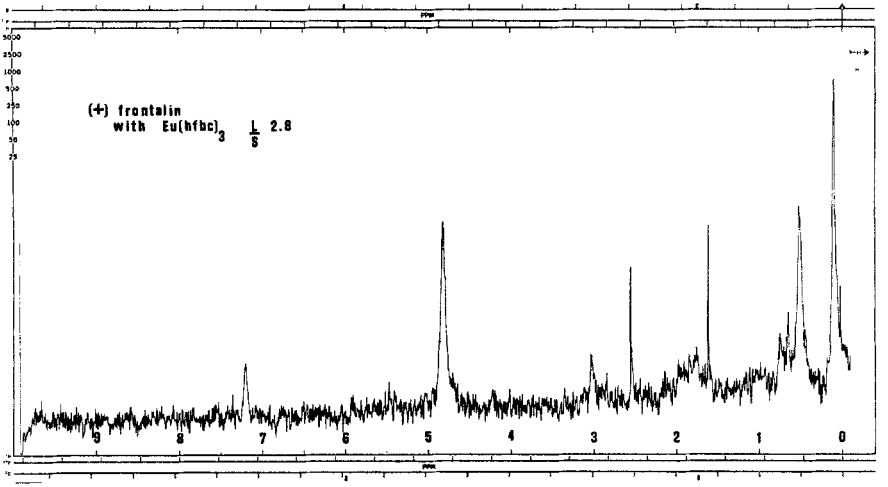

FIG. 3c (+)-Frontalin (5 μg), Eu(hf bc)$_3$ L/S = 2.8, in C_6D_6, 1.1 mm capillary.

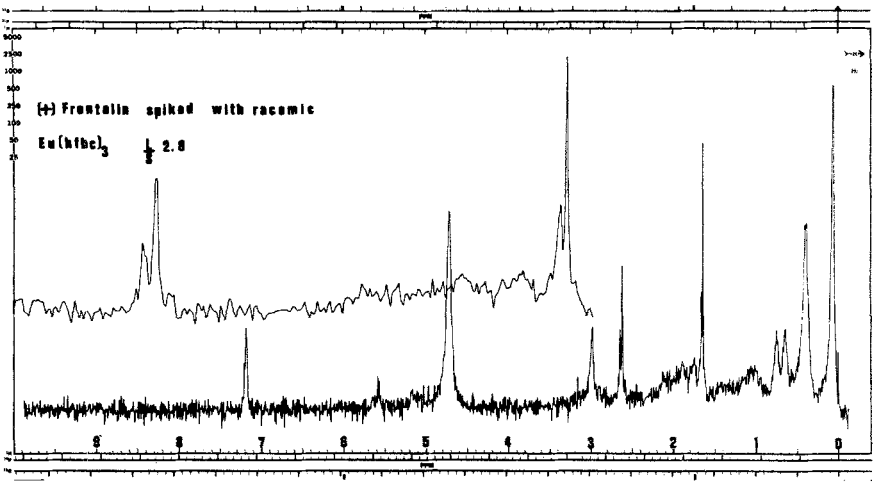

FIG. 3d. (+)-Frontalin (5 μg) spiked with racemic frontalin (2.5 μg), Eu(hf bc)$_3$ L/S <2.8, in C_6D_6, 1.1 mm capillary. Insert 200-Hz full scale.

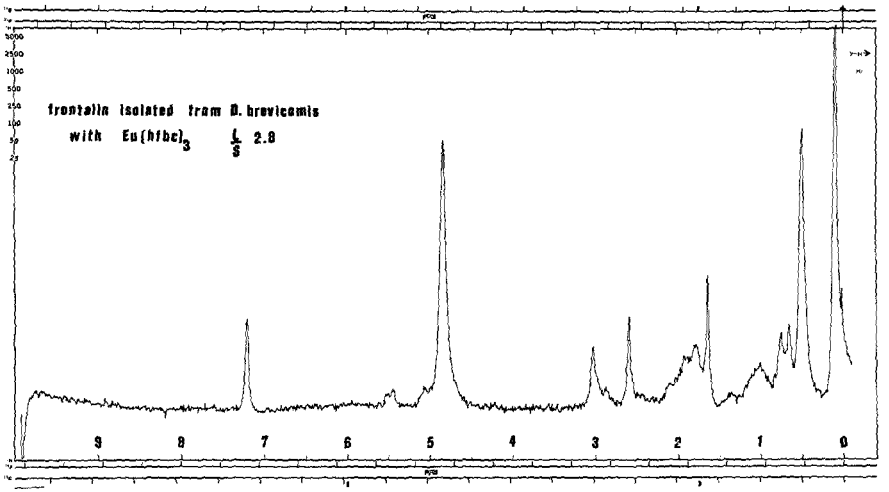

FIG. 3e. Frontalin (5 μg) isolated from *D. brevicomis*, Eu(hfbc)$_3$ L/S = 2.8, in C_6D_6, 1.1 mm capillary.

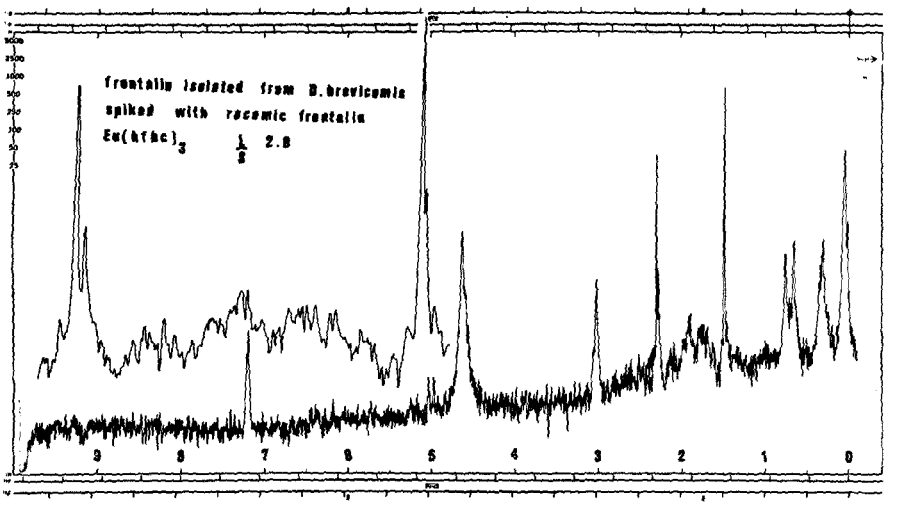

FIG. 3f. Frontalin (5 μg) isolated from *D. brevicomis* spiked with racemic frontalin (2.5 μg), Eu(hfbc)$_3$ L/S = 2.8, in CCl_4, 50 μl. Insert 200-Hz full scale.

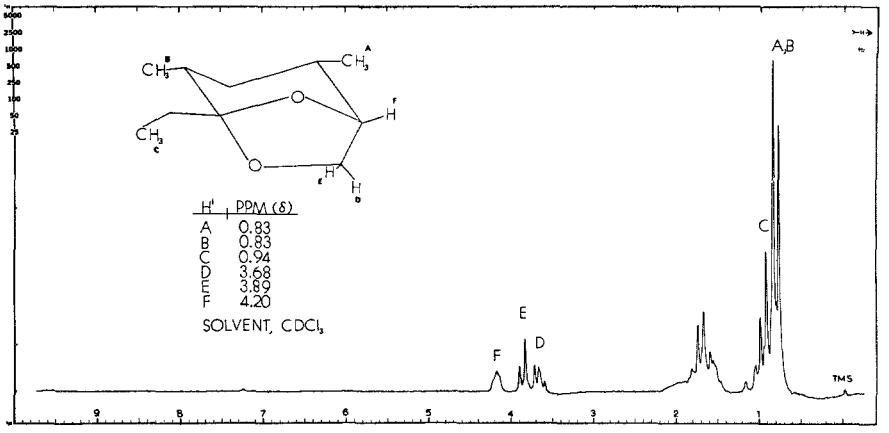

FIG. 4a. α-Multistriatin from *S. multistriatus*, in $CDCL_3$, 50 μl.

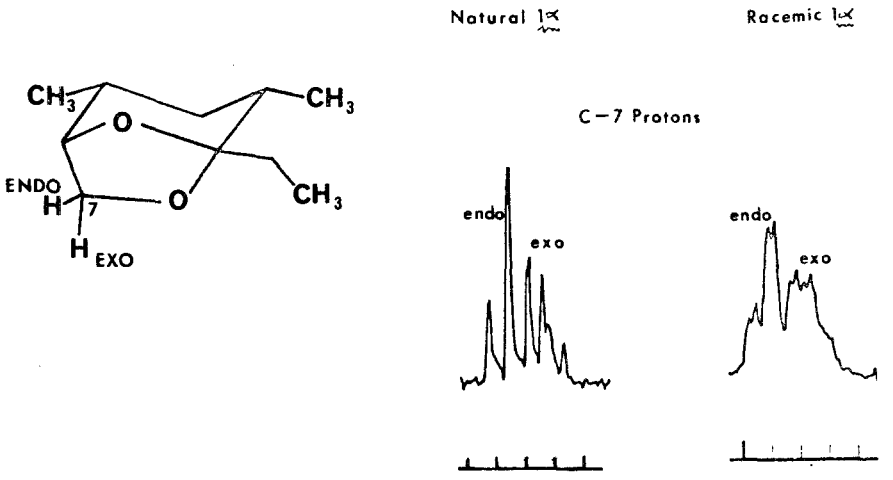

FIG. 4b. α-Multistriatin from *S. multistriatus* and racemic multistriatin with $Eu(hf\ bc)_3$ in CCl_4, 50 μl, $L/S = 0.39$.

"Spiking" this sample with ~3 μg of racemic frontalin shows (Figure 3f) that the natural frontalin is the (−) enantiomer (peaks near δ1.5 and δ2.3); the insert is an expanded spectrum, full scale = 200 Hz. Because of the noise level in these small-scale experiments, we cannot preclude the possibility that up to about 10% of the other enantiomer may be present. *D. brevicomis* beetles respond more readily to the (−) enantiomer (Wood et al., 1976).

α-*Multistriatin*

The natural α-multistriatin was found to be optically active ($[\alpha]_D^{25}$, −47°) (Pearce et al., 1975), but the question remained as to its optical purity. Figure 4a shows the ^{1}H NMR spectrum of natural α-multistriatin, and Figure 4b shows the effect of Eu(hfbc)$_3$ on the C–7 protons. The natural product appears to be a single enantiomer, but the broadening due to the Eu(hfbc)$_3$ prohibits any precise evaluation. Corroborating evidence by ^{13}C shift-reagent NMR spectra for this assignment of enantiomeric composition is presented by Pearce et al. (1976). The pure enantiomers have not yet been synthesized for bioassays, although the absolute configurations have been determined (Pearce et al., 1976).

Acknowledgments—We thank Dr. Kenji Mori for samples of the enantiomers of *exo*-brevicomin and frontalin. This investigation was supported by grants from the USDA Southern Pine Beetle Program (Coop. Agmt. 18–473), Rockefeller Institute, National Science Foundation, and Elm Research Institute. The Varian XL-100 NMR instrument was obtained through a grant from the National Science Foundation. *D. frontalis* material was furnished by Drs. T.L. Payne and L.J. Edson; *D. brevicomis* material by Dr. D.L. Wood and Mr. L.E. Browne; and *S. multistriatus* material by Dr. J.W. Peacock and Mr. R.A. Cuthbert.

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THE MAJOR VOLATILE CONSTITUENTS OF THE SCENT MARK OF A SOUTH AMERICAN PRIMATE *Saguinus fuscicollis, Callithricidae*

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Abstract—The major volatile constituents of the scent mark of a South American primate, the marmoset *Saguinus fuscicollis* have been isolated and identified to be a series of saturated, mono- and diunsaturated fatty alcohols esterified with *n*-butyric acid. Squalene was also found in the scent mark. The identification and possible significance of these components in marmoset chemical communication are discussed.

Key Words—chemical communication, marmoset, primate, skin secretions, scent mark, *n*-butyrate esters, saturated and unsaturated fatty alcohol esters.

INTRODUCTION

It is now well established that chemical signals play a significant role in the reproductive physiology and social behavior of many higher animals. Studies by Bronson (1974), Eisenberg, and Kleiman (1972), Müller-Schwarze et al. (1973) and Epple (1974a, b), indicate that the phenomenon is widespread. In addition, Comfort (1974) has recently speculated that it may, in fact, extend to man.

The South American marmoset monkey, *Saguinus fuscicollis*, for example, utilizes the scent mark to communicate to conspecifics a variety of information including the sex, social status, and individual identity (Epple, 1974a, b).

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Scent-marking behavior in the marmoset consists of rubbing circumgenital and sternal body regions against objects in the environment. This behavioral pattern results in the deposition of material consisting of a complex mixture of secretions from the specialized sebaceous and apocrine skin glands (Perkins, 1966), urine, and in the case of the female, possibly vaginal discharge (Epple, 1975). Although the use of such chemical cues in sexual and social communication has been reported in several primate species, studies on their chemical nature remain limited (Curtis et al., 1971; Goldfoot et al., 1976).

As part of our continuing study (Smith et al., 1976) of the scent-marking behavior of *Saguinus fuscicollis*, we now wish to report in detail the isolation and identification of the major volatile constituents (1–16) present in the scent mark.

METHODS AND MATERIALS

Collection of Scent Marks

Scent marks from each of 16 donors (8 ♂♂ and 8 ♀♀) were obtained by providing preselected animals with frosted glass plates (1 1/2 × 1/8 × 22 in.) attached to an aluminium bar (2 × 1/4 × 22 in.). The animals were allowed to scent mark the plates for approximately 3 min, after which the perches were removed from the cages, separated according to sex, and washed with 100 ml each of CH_2Cl_2 and CH_3OH. The marks collected in this manner consisted mainly of material from the circumgenital gland mixed with urine and, in the case of the female, possibly vaginal discharge. Interestingly, compared to the circumgenital glands, the sternal glands situated on midchest are used less frequently for marking by *Saguinus fuscicollis*. The CH_2Cl_2 and CH_3OH solutions were then combined and concentrated under nitrogen at 40°C to yield an oil which was largely soluble in 0.2 ml hexane. Hexane solutions of this oil not immediately examined were stored under nitrogen at −60°C.

The hexane-soluble material was selected for further analysis because preliminary behavioral studies demonstrated that marmosets respond to the hexane fractions in a manner similar to their response to intact marks (Epple, Smith, and Yarger, unpublished results, 1975). That is, when a subject animal is given the choice between intact scent material from male and female donors, the subjects, in a highly replicable fashion, scent mark significantly more frequently in response to male odor (Epple, 1974a, b).

Analytical Methods

Gas chromatographic (GLC) analyses were performed on a Perkin-

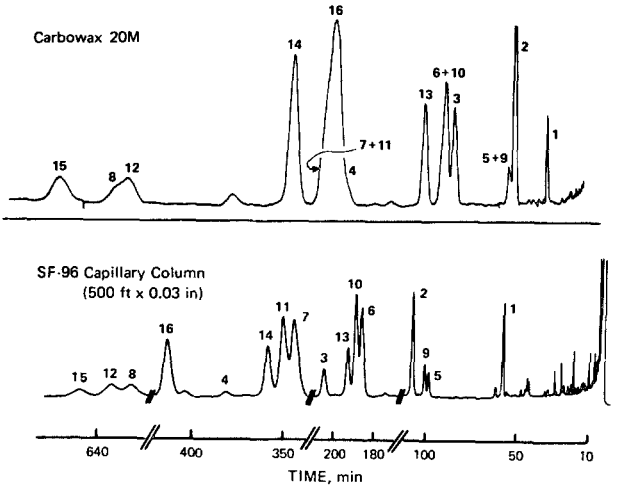
Elmer Model 990 chromatograph equipped with a flame ionization detector and a 10-ft × 1/4-in. (OD) glass column packed with either 5% Carbowax 20 M or 5% APOLAR-10C on Chromosorb G 80/100 mesh (Applied Science Laboratories Inc.), operated at a helium (He) flow rate of 60 ml/min and at 100° for 4 min followed by temperature programming to 230° at 3°/min. High-resolution GLC analyses were performed on a nonpolar SF-96 stainless-steel wall-coated capillary column (485 ft × 0.03 in.) operated at 220°C with a He flow of 11 ml/min. Gas chromatographic fractions were collected in glass capillary tubes (30 cm × 2 mm OD) utilizing a thermal gradient collector (Brownlee and Silverstein, 1968).

Infrared (IR) spectra were recorded on a Perkin-Elmer Model 237 spectrophotometer fitted with focusing beam condensers and 4-μl microcells containing the isolated components dissolved in spectroquality carbon tetrachloride. Low-resolution mass spectra (MS) were obtained on a Hitachi/Perkin-Elmer RMU-6L mass spectrometer interfaced with a Perkin-Elmer 990 gas chromatograph employing either the APOLAR-10C glass column or the SF-96 capillary column. High-resolution mass spectra were obtained by direct insertion of the sample into the ion source of a DuPont Model 492 mass spectrometer. All mass spectra were recorded at an ionization potential of 70 eV. The 220-MHz NMR spectra were recorded on a Varian HR 220 NMR spectrometer employing the Fourier transform mode of operation. Microozonolyses were performed at −65°C on 50 μg samples in spectroquality hexane or carbon disulfide (Beroza and Bierl, 1967).

Identification

After careful micropreparative gas chromatography of the hexane fraction, derived from either male or female donors, the major volatile components of the marmoset scent mark were identified as long-chain normal butyrate esters. These esters (**1–15**) are displayed in Figure 1. The structural assignments, as discussed below, were based on spectroscopic properties, analytical data, and reported chemical transformations. The 220-MHz NMR and IR spectral data for components **1–15** are presented in Tables 1 and 2, respectively, while the mass spectrum for butyrate **3** is reproduced in Figure 2. Figure 3 displays the NMR spectra for butyrates **3, 6, 10,** and **13.**

The distinctive fragmentation patterns found in the low-resolution MS initially suggested that these esters were *n*-butyrates of saturated, mono- and diunsaturated straight-chain alcohols. Characteristic of these spectra were the ions *m/e* 89, M^+, M^+-43, and M^+-88. For most butyrates, *m/e* 89 was the base peak. This intense and diagnostic ion can easily be rationalized by double hydrogen atom rearrangement as shown in Scheme I.

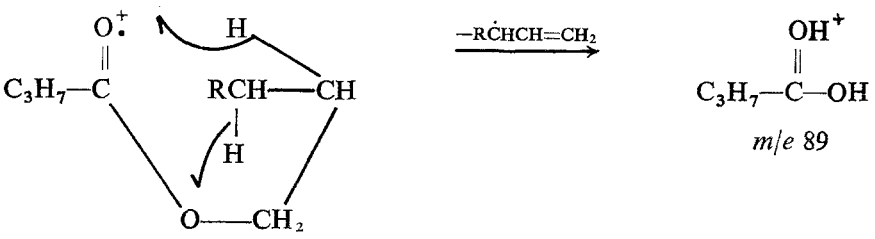


Peak	Molecular Formula	Identification
		SATURATED
1	$C_{20}H_{40}O_2$	
2	$C_{22}H_{44}O_2$	
3	$C_{24}H_{48}O_2$	
4	$C_{26}H_{52}O_2$	
		MONOENES–TYPE A
5	$C_{22}H_{42}O_2$	
6	$C_{24}H_{46}O_2$	
7	$C_{26}H_{50}O_2$	
8	$C_{28}H_{54}O_2$	
		MONOENES–TYPE B
9	$C_{22}H_{42}O_2$	
10	$C_{24}H_{46}O_2$	
11	$C_{26}H_{50}O_2$	
12	$C_{28}H_{52}O_2$	
		DIENES
13	$C_{24}H_{44}O_2$	
14	$C_{26}H_{48}O_2$	
15	$C_{28}H_{52}O_2$	
		SQUALENE
16	$C_{30}H_{50}$	

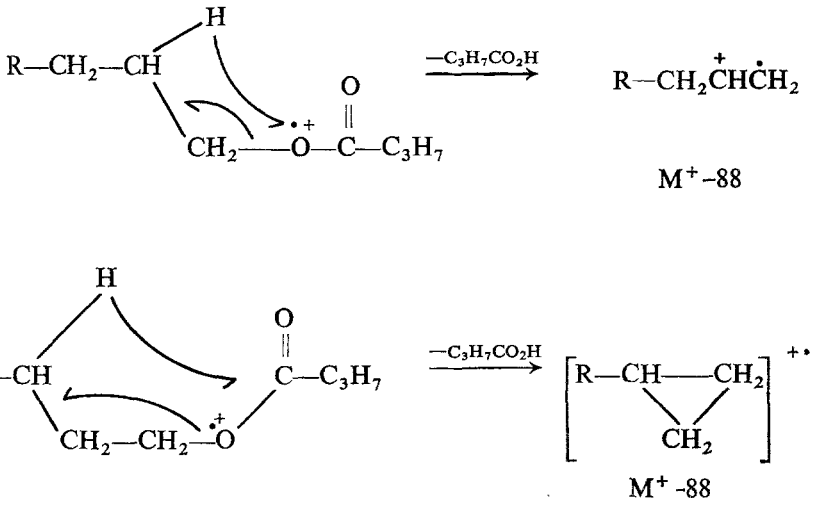
FIG. 1. Major volatile constituents of male marmoset scent mark.

Such rearrangements typically arise in esters having long-chain alkyl alcohol moieties (Ryhage and Stenhagen, 1959).

Likely mechanisms for loss of butyric acid (e.g., $M^+ - 88$) (Scheme II)

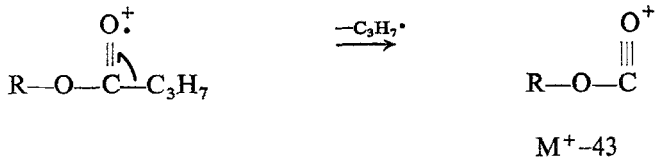


Scheme I



Scheme II

involve the transfer of a hydrogen atom from either the β or γ carbon centers of the alcohol portion of the ester to the ether oxygen, thus yielding neutral butyric acid and the ionized olefinic fragments (Benz and Biemann, 1964). Finally, loss of C_3H_7 ($M^+ - 43$) most probably arises via a simple α-cleavage process as shown in Scheme III (Budzikiewicz et al., 1967). In addition to the low-resolution MS data, high-resolution spectra in each case yielded parent ions consistent with the reported empirical formulae.



Scheme III

TABLE 1. 220-MHz NMR DATA FOR BUTYRATE ESTERS (**1–15**) ($CDCl_3$; δ)

1	4.00 (*t*, 2H), 2.21 (*t*, 2H), 1.60 (*m*, 4H), 1.30 (*m*, 26H) 0.94 (*m*, 6H)
2	4.05 (*t*, 2H), 2.20 (*t*, 2H), 1.60 (*m*, 4H), 1.30 (*m*, 28H), 0.94 (*m*, 6H)
3	4.00 (*t*, 2H), 2.21 (*t*, 2H), 1.60 (*m*, 4H), 1.30 (*m*, 30H), 0.93 (*m*, 6H)
4	4.05 (*t*, 2H), 2.21 (*t*, 2H), 1.60 (*m*, 4H), 1.30 (*m*, 32H), 0.93 (*m*, 6H)
5	5.38 (*m*, 2H), 4.08 (*t*, 2H), 2.30 (*t*, 2H), 2.05 (*m*, 4H), 1.68 (*m*, 2H), 1.30 (*m*, 26H), 0.95 (*m*, 6H)
6	5.38 (*m*, 2H), 4.06 (*t*, 2H), 2.30 (*t*, 2H), 2.05 (*m*, 4H), 1.66 (*m*, 2H), 1.30 (*m*, 28H), 0.94 (*m*, 6H)
7	5.36 (*m*, 2H), 4.06 (*t*, 2H), 2.30 (*t*, 2H), 2.05 (*m*, 4H), 1.68 (*m*, 2H), 1.31 (*m*, 30H), 0.93 (*m*, 6H)
8	5.36 (*m*, 2H), 4.06 (*t*, 2H), 2.30 (*t*, 2H), 2.05 (*m*, 4H), 1.65 (*m*, 2H), 1.30 (*m*, 32H), 0.94 (*m*, 6H)
9	5.38 (*m*, 2H), 4.08 (*t*, 2H), 2.30 (*t*, 2H), 2.02 (*m*, 4H), 1.65 (*m*, 2H), 1.30 (*m*, 26H), 0.91 (*m*, 6H)
10	5.35 (*m*, 2H), 4.06 (*t*, 2H), 2.30 (*t*, 2H), 2.05 (*m*, 4H), 1.65 (*m*, 2H), 1.30 (*m*, 28H) 0.92 (*m*, 6H)
11	5.36 (*m*, 2H), 4.06 (*t*, 2H), 2.30 (*t*, 2H), 2.05 (*m*, 4H), 1.65 (*m*, 2H), 1.30 (*m*, 30H), 0.93 (*m*, 6H)
12	5.38 (*m*, 2H), 4.08 (*t*, 2H), 2.30 (*t*, 2H), 2.05 (*m*, 4H), 1.65 (*m*, 2H), 1.30 (*m*, 32H), 0.95 (*m*, 6H)
13	5.45 (*m*, 4H), 4.05 (*t*, 2H), 2.75 (*m*, 2H), 2.55 (*t*, 2H), 2.08 (*m*, 4H), 1.65 (*m*, 4H), 1.30 (*m*, 24H), 0.94 (*m*, 6H)
14	5.45 (*m*, 4H), 4.05 (*t*, 2H) 2.75 (*m*, 2H), 2.55 (*t*, 2H), 2.08 (*m*, 4H), 1.65 (*m*, 4H), 1.30 (*m*, 26H), 0.94 (*m*, 6H)
15	5.42 (*m*, 4H), 4.02 (*t*, 2H), 2.75 (*m*, 2H), 2.55 (*t*, 2H), 2.10 (*m*, 4H), 1.65 (*m*, 4H), 1.30 (*m*, 28H), 0.94 (*m*, 6H)

Further evidence for the predominant occurrence of the normal butyrate functionality was obtained by heating the oil derived from the scent mark at reflux with 10% aqueous KOH for 24 hr. After acidification and extraction with diethyl ether, *n*-butyric (98%) and isobutyric acid (2%) were the only acids detectable by gas chromatography. However, neither isobutyric nor *n*-butyric acid were found in the hexane-soluble portion of the scent mark. Thus, the presence of ~2% isobutyric acid obtained upon basic hydrolysis could arise either from trace components present in the mark, which at this time have gone undetected, or from the fact that the esters were indeed a mixture of both iso- and normal butyrates. To eliminate the latter possibility, the normal and isobutyrate esters of both 1-hexadecanol and 1-octadecanol were prepared by simple Fisher esterification. In both cases the isobutyrate esters gave distinctly shorter GLC retention data on either packed (APOLAR-

TABLE 2. IR DATA FOR BUTYRATE ESTERS **1–15** (CCl_4)

1	1748 (*s*), 1460 (*m*), 1175 (*s*), 1080 (*m*) cm^{-1}
2	1748 (*s*), 1460 (*m*), 1175 (*s*), 1080 (*m*) cm^{-1}
3	1748 (*s*), 1460 (*m*), 1175 (*s*), 1080 (*m*) cm^{-1}
4	1745 (*s*), 1460 (*m*), 1175 (*s*), 1080 (*m*) cm^{-1}
5	3010 (*w*), 1745 (*s*), 1080 (*m*) cm^{-1}
6	3010 (*w*), 1756 (*s*), 1080 (*m*) cm^{-1}
7	3012 (*w*), 1745 (*s*), 1080 (*m*) cm^{-1}
8	3010 (*w*), 1745 (*s*), 1080 (*m*) cm^{-1}
9	3010 (*w*), 1745 (*s*), 1080 (*s*) cm^{-1}
10	3010 (*w*), 1745 (*s*), 1080 (*s*) cm^{-1}
11	3010 (*w*), 1745 (*s*), 1080 (*s*) cm^{-1}
12	3010 (*w*), 1745 (*s*), 1080 (*s*) cm^{-1}
13	3010 (*w*), 1748 (*s*), 1020 (*s*) cm^{-1}
14	3010 (*w*), 1745 (*s*), 1020 (*s*) cm^{-1}
15	3010 (*w*), 1745 (*s*), 1020 (*s*) cm^{-1}

10C) or capillary (SF-96) GLC columns, thus eliminating the possibility that the isolated components (**1–15**) were a mixture of butyrate isomers.

Finally, the 220-MHz NMR data for esters (**1–15**) were consistent only with the proposed straight-chain structures. In all instances the spectra were characterized in the saturated methyl region by a distinctive multiplet pattern arising from the near superposition of two triplet resonances (cf. Figure 3). Likewise, characteristic triplet patterns were observed at δ 4.10 and 2.20, respectively, for two hydrogens on a carbon bearing an oxygen atom and two hydrogens α to a carbonyl group.

With the carbon skeleton of the esters (**1–15**) established, only the location and configuration of the sites of unsaturation remained to be assigned. To this end microozonolysis in either hexane or carbon disulfide at $-65°C$ (Beroza and Bierl, 1967) was employed. Because component pairs **5/9**, **6/10**, **7/11**, and **8/12** were resolved only on a 485-ft SF-96 capillary column, these component pairs were collected as single fractions and analyzed as such. For example, ozonolysis of the **5/9** pair yielded four products, e.g., *n*-heptanal, *n*-nonanal, 9-oxononyl butyrate, and 11-oxoundecyl butyrate (GLC retention times of 10.8, 19.8, 48.4, and 65.7 min, respectively; Carbowax 20M, 70° and 3 min, then 3°/min to 220°C). Similarly, pairs **6/10**, **7/11**, and **8/12** yielded *n*-heptanal, *n*-nonanal, and their respective aldehyde esters (13-oxotridecyl butyrate, 87.5 min; 15-oxopentadecyl butyrate, 121 min; 17-oxoheptadecyl butyrate, 168 min), while ozonolysis of dienes **13**, **14**, and **15** afforded *n*-hexanal and 11-oxoundecyl butyrate, 13-oxotridecyl

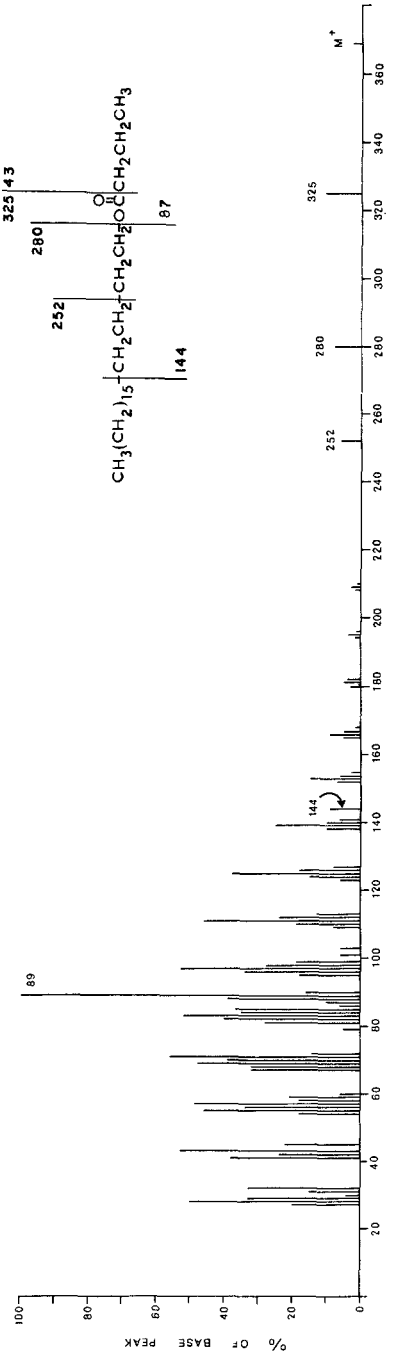


FIG. 2. Mass spectrum of butyrate **3**.

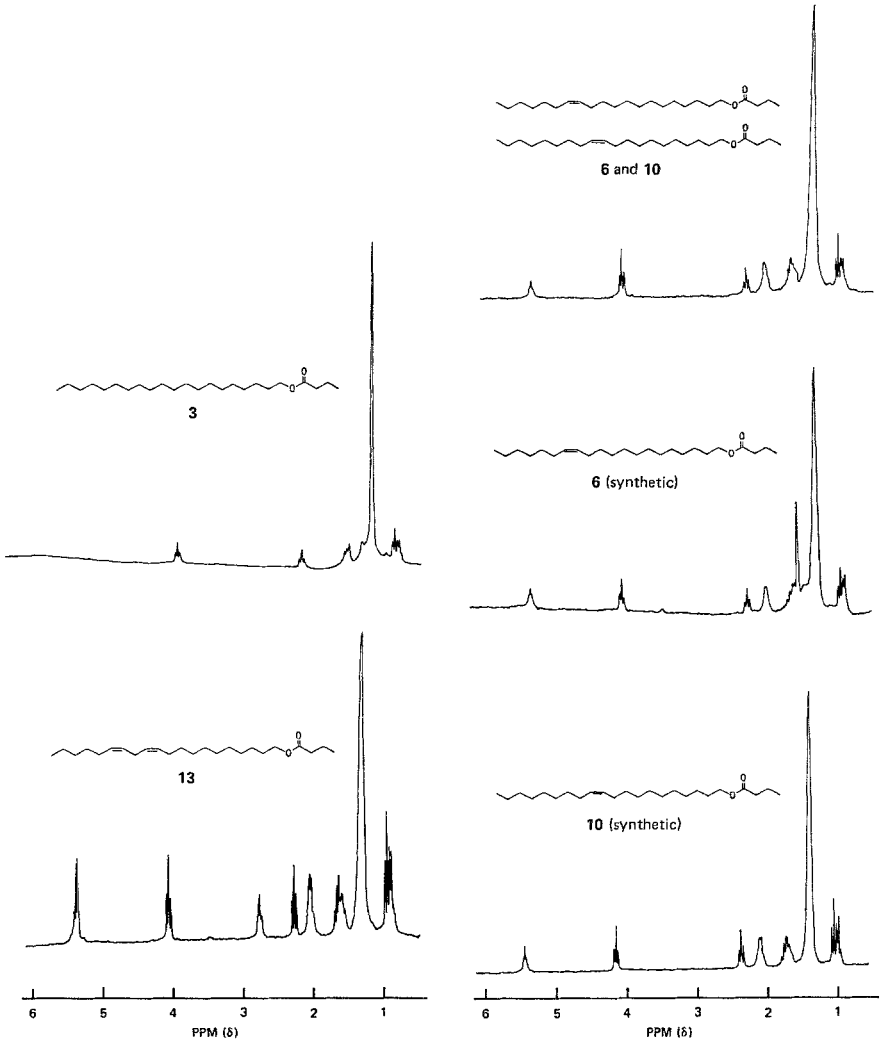


FIG. 3. Nuclear magnetic resonance spectra for butyrates **3**, **6**, **10**, and **13**. Spectra arising from synthetic butyrates **6** and **10** are also reproduced for comparison.

butyrate, and 15-oxopentadecyl butyrate, respectively. The above identifications were based both on MS data and, in the case of the simple aldehydes, comparison of the MS and GLC retention data with that obtained from authentic samples. The three-carbon fragment, 1,3-propanedial, was not observed.

Assignment of the *cis* configuration at each olefinic linkage (*s*) in butyrates (**5–15**) was based on the absence of absorption in the 975 cm^{-1} region of the infrared spectrum. As a control experiment here we examined various mixtures of methyl oleate (C_{18}-*cis*) and methyl elaidate (C_{18}-*trans*). Under our infrared and gas-chromatographic conditions, 5% methyl elaidate was easily detected when added to methyl oleate. Thus the unsaturated butyrates are >95% *cis*.

Squalene (**16**), found in the marmoset scent mark, was identified by comparison of its NMR and MS spectra as well as its GLC retention data with those of authentic material.

Finally, confirmation of structures (**1–15**) was obtained by comparison of their spectroscopic and chromatographic properties with representative authentic samples prepared by alternative synthesis. Documentation of our synthetic approach to butyrates (**1–15**) will be forthcoming.

RESULTS AND DISCUSSION

The major components comprising >96% by weight of the volatile material of the marmoset scent mark are presented in Figure 1. This is the first report of the isolation and identification of these fatty butyrate esters. Examination of these butyrates reveals four major classes, namely the saturated butyrates (**1–4**), the monounsaturated butyrates (**5–11**) of two structural types (A and B) dependent on the location of the olefinic linkage and fourth, the diunsaturated butyrates (**12–15**). In addition, as occurs with other mammalian fatty acid systems, each member of a class differs from the next by a two-carbon unit. Presumably these components originate from the large specialized scent glands located in the circumgenital region of the marmoset rather than from urinogenital secretions. Not surprisingly, these components resemble the fatty acids normally found on the epidermal surface of humans (Nicolaides, 1974) and other mammalian species (Nicolaides, et al., 1968).

Representative gas chromatograms (Carbowax 20M and SF-96, respectively) of the hexane extract of male scent marks are illustrated in Figure 1. Interestingly, when material from several donor animals of both sexes was analyzed separately and on a daily basis for a period of one week, consistent "ester profiles" were obtained. That is, the relative amounts of the various butyrate esters remained constant although patterns from individual donors differed one from another.

Although the exact function of the butyrate esters is currently unknown, it is quite possible that they serve as a matrix or fixative for more volatile components present at much lower concentration (cf. Figure 1). For example, the high-molecular-weight esters and alcohols isolated from the subauricular

glands of the male pronghorn (*Antilocapra americana*) are believed to function as possible precursors and/or diluents for the active volatile components, 2-methylbutyric and isovaleric acids (Müller-Schwarze et al., 1974). Unlike the subauricular scent mark of the male pronghorn, however, free *n*-butyric acid is not present in the marmoset scent mark.

On the other hand it is conceivable that an "ester profile" from a given individual serves to distinguish that individual from other animals (Nicolaides, 1974). In addition, it is possible that information on sex is communicated by the ester profiles. However, for information of this type to be communicated, both sexes would require a specific set of butyrates present in a diagnostic ratio. Studies to explore these possibilities are continuing in our laboratories. Finally, the function of squalene in the scent secretion of the marmoset monkey is unknown, although it is present in the epidermal surface of many mammals (Nicolaides et al., 1968).

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FIELD EVIDENCE OF SYNERGISM AND INHIBITION IN THE SESIIDAE[1] SEX PHEROMONE SYSTEM[2]

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Abstract—The E,Z, Z,Z, and Z,E geometric isomers of 3,13 octadecadien-1-ol acetate were used singly and in binary combinations to trap sesiids in Wisconsin cherry orchards. The Z,E isomer alone did not capture any sesiids. A synergistic effect, however, of Z,E on *Synanthedon pictipes* response to its pheromone E,Z was demonstrated for the first time. Strong inhibitory effects of the Z,Z isomer on *S. pictipes* response to E,Z, and of the E,Z isomer on *S. scitula* response to Z,Z, were found. As little as 0.5% of Z,Z in E,Z completely inhibited the *S. pictipes* response. This species' response was also reduced by 85% when Z,Z was evaporated at 4 points, each ca. 6 m from the pheromone trap. Consistencies and discrepancies of the data with relevant experimental results from other geographical areas are briefly discussed.

Key Words—Sesiidae, *Synanthedon*, sex pheromones, inhibition, synergism.

INTRODUCTION

Several species of Sesiidae (clearwing moths) utilize pheromones in their sex communication (Girault, 1907; Cleveland and Murdock, 1964; Nielsen

[1] Lepidoptera: Sesiidae (=Aegeriidae), the clearwing moths.

[2] Research supported by the College of Agriculture and Life Sciences, University of Wisconsin, Madison, and by the Wisconsin Red Tart Cherry Ind. Market. Order.

and Balderston, 1973; Yonce et al., 1974; Nielsen et al., 1975). The sex pheromone of *Synanthedon pictipes* (Grote and Robinson) (lesser peachtree borer) was identified by Tumlinson et al. (1974) as E,Z-3,13-octadecadien-1-ol acetate (E,Z-3,13-ODDA). The same investigators found that its Z,Z isomer was the sex pheromone of another sesiid, *Sanninoidea exitiosa* (Say) (peachtree borer). These two pest species were not attracted to each other's pheromone in Georgia and Ohio (Tumlinson et al., 1974; Nielsen et al., 1975).

Nielsen et al. (1975) recorded two other sesiid species which responded to *S. pictipes* female ether extracts and additional species which responded to the Z,Z isomer. Moreover, *S. exitiosa* males were attracted to caged *Podosesia syringae* (Harris) (ash and lilac borer) females of late flight in Ohio (Nielsen and Balderston, 1973). In another study, traps baited with a Z,Z Farchan formulation (93% of Z,Z and 7% of E,Z and Z,E) captured more *S. exitiosa* males than traps baited with pure Z,Z (Nielsen et al., 1975).

Tumlinson et al. (1974) demonstrated that *S. pictipes* attraction to E,Z was strongly inhibited when the Z,Z isomer was evaporated simultaneously from the same locus. Similarly, the *S. exitiosa* attraction to Z,Z was inhibited when a mixture containing 10% or more of E,Z was used. Disruption of *S. pictipes* and *S. exitiosa* sex communication by permeation of the atmosphere with either E,Z or Z,Z isomers has been reported recently (McLaughlin et al., 1975).

Such phenomena of inhibition and synergism in the sex pheromone systems of several insect species probably contribute to the reproductive isolation of closely related species (Roelofs and Cardé, 1974), as well as to the community structure of some taxa. Hence, the ecological relevance of such interactions is obvious. Furthermore, their importance to the development of insect pest population suppression strategy cannot be overlooked (Mitchell, 1975).

Distant geographic populations of the same insect species often respond maximally to different pheromone blending (Klun et al., 1973; Klun and Cooperators, 1975). This necessitates studies at several areas within the species range (Lanier et al., 1972).

Our quantitative study attempts to evaluate sesiid response to three geometric isomers (E,Z, Z,Z, Z,E) of 3,13-ODDA (singly and in various combinations) in Wisconsin.

METHODS AND MATERIALS

Experiments were conducted in commercial cherry orchards (ca. 10 acres each) in Door County, Wisconsin, during 1974 and 1975. Because sesiids are strong fliers and *S. pictipes* males can be attracted to a pheromone source

several hundred meters upwind (Karandinos, 1974), the experimental orchards were selected to be at least 1.5 km apart from each other. Sticky traps were hung on trees 1.7 m above ground. The isomers were synthesized and purified by liquid chromatography on silver nitrate-coated silica gel (Tumlinson et al., 1974). The purified isomers were 99+% pure when analyzed by gas–liquid chromatography on OV-101 Carbowax 20 M, and a GE cyano-silicone stationary phase. Sesiids captured in the sticky traps were cleaned with hexane and stored in 75% alcohol for laboratory identification.

RESULTS

Sex Pheromone Inhibition[6] *and Synergism*[6] *with Isomers Evaporated at the Same Locus*

1974 Experiments with E,Z and Z,Z Isomers, Used Singly and In Combination. A sequence of 6 experiments was conducted between July 17 and August 15, 1974. Three traps (treatments) per orchard (replication) were used in the first four experiments and two in the last two. The number of replications varied from three to six. Table 1 lists the isomers used to bait the three traps, which were placed at the corners of a triangle ca. 70 m apart from each other. The traps were made of cylindrical ice cream cartons (17 cm diameter, 18 cm long), coated inside with Stickem® and with the ends removed.

The isomers, diluted in hexane, were applied either to rubber septa (the E,Z) or to polyethylene caps (the Z,Z) which were suspended ca. 2 cm above the trap's floor. Sesiids were removed daily. The summarized results are given in Table 1.

A large number (561) of *S. pictipes* males were captured in the E,Z traps during the first two experimental periods, but not a single specimen was captured in the Z,Z or in the mixture traps. Thus, an inhibitory effect of Z,Z was suggested. Yet other contributing factors, such as trap placement, location, etc., could probably be postulated. Thus, a "self-controlled" experiment (#3) was conducted in which the Z,Z dispensers were simply removed from the mixture traps between August 3–5. The large number of *S. pictipes* captured further supported the notion of inhibitory effects of the Z,Z isomer.

In the subsequent experiments, the Z,Z dispensers were used again in the mixture traps, but the amount of Z,Z was reduced to 10, 2, and finally

[6] In this paper "inhibition" simply means a reduction in the "treatment" trap catches compared to catches in the "control" traps. The same term is used, regardless of whether the inhibitory compound was evaporated at the same locus or at a distance from the pheromone source. Likewise, the term "synergism" means an increase in catches in two-compound traps.

TABLE 1. SESIIDAE MALES CAPTURED IN WISCONSIN CHERRY ORCHARDS WITH E,Z AND Z,Z ISOMERS OF 3,13-OCTADECADIEN-1-OL ACETATE SINGLY AND IN VARIOUS MIXTURES (1974 EXPERIMENTS)

Experiments	Attractants	*S. pictipes*	*S. scitula*	*S. fatifera*	*S. viburni*	*S. exitiosa*
Exp. 1. July 17–26	100 μg E,Z	447	0	0	0	0
(four orchards)	100 μg Z,Z	0	66	1	0	2
	100 μg E,Z+100 μ,g Z,Z	0	0	0	0	1
Exp. 2. July 27–Aug. 2	100 μg E,Z	114	0	0	0	0
(same four orchards)	100 μg Z,Z	0	19	0	0	3
	100 μg E,Z+50 μg Z,Z	0	0	0	0	0
Exp. 3. Aug. 3–5	100 μg E,Z	18	0	0	0	0
(same four orchards)	100 μg Z,Z	0	0	0	0	1
	100 μg E,Z	77	0	0	0	0
Exp. 4. Aug. 1–9	100 μg E,Z	174	0	0	0	0
(three orchards)	100 μg Z,Z	0	0	0	0	3
	100 μg E,Z+10 μg Z,Z	0	0	0	1	0
Exp. 5 Aug. 4–15	100 μg E,Z	156	0	0	0	0
(six orchards)	100 μg E,Z+2 μg Z,Z	0	0	0	0	0
Exp. 6. Aug. 8–15	100 μg E,Z	62	0	0	0	0
(five orchards)	100 μg E,Z+0.5 μg Z,Z	0	0	0	0	0
Total		1048	85	1	1	10

0.5 μg. No *S. pictipes* specimens were captured, even when the Z,Z was only 0.5% of the pheromone. Of course, the amounts of the two isomers placed in the traps are not necessarily proportional to the release rates.

A substantial number (85) of another sesiid, *Synanthedon scitula* (Harris)[7] (dogwood borer) were captured with Z,Z, but not with the E,Z or the mixture of the two isomers. Thus, an inhibitory effect of E,Z on *S. scitula* response to Z,Z is suggested.

There was no previous record of *Synanthedon viburni* (Englh.) captured in sex attraction traps and no previous record of *S. fatifera* Hodges having been collected in Wisconsin, both of which are *Viburnum* borers. *S. fatifera* was also captured in Mazomanie Wildlife Refuge of the Wisconsin River (unpublished records), in Ohio (Nielsen et al., 1975), and in South Carolina (personal communication) with the Z,Z isomer.

1975 Experiments with E,Z, Z,Z, and Z,E Isomers Used Singly and in Binary Combinations of Equal Amounts. Six cherry orchards (replications) were sampled between July 21 and August 20, 1975, with six Pherocon 1 C® (Zoecon) traps (treatments) per orchard. Rubber septa dispensers were each charged with 100 μg of one isomer. Two septa, each charged with a different isomer, were placed in a trap when mixtures were tested. The six traps were 14–20 m apart from each other and formed a circle in the centre of the orchard. The wind direction, which, if constant, could have introduced a bias in the allocation of males, varied considerably during the experiment.

TABLE 2. SESIIDAE MALES CAPTURED IN WISCONSIN CHERRY ORCHARDS WITH THE INDICATED COMBINATIONS OF 3,13-ODDA ISOMERS (100 μg). (July 21–August 20, 1975 Experiment)

Orchards (replications)	Treatments						Total
	E,Z	E,Z+Z,E	E,Z+Z,Z	Z,E	Z,Z	Z,Z+Z,E	
I	15–0[a]	56–0	0–0	0–0	0–14	0–15	71–29
II	23–0	57–0	4–0	0–0	0–19	0–29	84–48
III	0–0	68–0	0–0	0–0	0–9	0–3	68–12
IV	11–0	30–0	0–0	0–0	0–2	0–3	41–5
V	4–0	20–0	0–0	0–0	0–0	0–4	24–4
VI	6–0	13–0	0–0	0–0	0–2	0–1	19–3
Total	59–0	244–0	4–0	0–0	0–46	0–55	307–101

[a] The first number in each pair refers to *S. pictipes* and the second to *S. scitula* specimens.

[7] Previously placed in the genus *Thamnosphecia* Spuler by Engelhardt (1946). It exhibits a great adaptability to different unrelated food plants. Found normally on oaks, hickory, dogwood. Occasional records from cherry, apple, mountain ash, willow, birch, beech, *Crataegus* spp., wax myrtle, quince, hazelnut, bayberry and wisteria. Serious damage is caused to pecan in the southern states by the form "*corusca* Edw."

In order to further reduce the bias due to trap location, we rerandomized them twice during the course of the experiment.

The Sesiidae specimens were removed from the traps on July 23, 25, 28, 30, and August 4, 7, 11, 13, and 20. The results are given in Table 2. No specimens were captured in traps baited with Z,E alone. Similar results were obtained in Byron, Georgia (Tumlinson et al., 1974). However, when the Z,E isomer was combined with E,Z, a significantly[8] greater number of *S. pictipes* were captured than with the E,Z alone. This is the first report of such a synergistic effect for this species.

The inhibitory effects of Z,Z on *S. pictipes* and of E,Z on *S. scitula* found in this experiment (Table 2) are consistent with our 1974 results (Table 1).

Sex Pheromone Inhibition of S. pictipes *with Z,Z Evaporated at a Distance from the E,Z Traps*

In these experiments we compared the *S. pictipes* catches in pairs of traps, ca. 70 m apart from each other, baited with 100 μg E,Z in rubber septa. The traps were hung on trees 1.7 m above ground. One of the traps

TABLE 3. *Synanthedon pictipes* MALES CAPTURED IN WISCONSIN CHERRY ORCHARDS IN PAIRS OF TRAPS (CA. 70 M APART) BAITED WITH E,Z-3,13-OCTADECADIEN-1-OL ACETATE. ONE TRAP IN EACH PAIR WAS SURROUNDED BY 4 Z,Z DISPENSERS AT EA 6 M. (1974 AND 1975 EXPERIMENTS)

Orchard	100 μg E,Z	100 μg E,Z+4 (500 μg) Z,Z	Inhibition (%)
	August 6–15, 1974 *experiment*		
I	24	3	87
II	16	1	94
III	46	9	80
IV	54	8	85
Total	140	21	85
	June 19–*July* 14, 1975, *experiment*		
I	114	21	82
II	48	16	67
III	74	21	72
IV	70	11	84
V	113	9	92
VI	211	23	89
Total	630	101	84

[8] At 0.99 level. Due to the proximity of the traps the counts could not be considered independent. Thus, we used the binomial model testing $H_0: p = 0.5$ against $H_1: p \neq 0.5$.

was surrounded by 4 dispensers each at ca. 6 m from the trap and charged with 500 μg Z,Z. These dispensers were attached to trees at 1.7 m above ground. One pair (replication) was used per orchard. Four orchards were used in 1974 and six in 1975. Ice cream carton traps were used in 1974 and Pherocon 1 C® in 1975. The specimens were collected daily. The results are given in Table 3.

The traps surrounded by the Z,Z dispensers captured a significantly ($P = 0.99$) smaller number of *S. pictipes* than did the "control" traps. In 1974 and 1975 the disruptions were 85% and 84%, respectively.

DISCUSSION

Two cases of inhibition and one case of synergism have been established in the present experiments.

The inhibitory effect of Z,Z on *S. pictipes* male response found in this study is consistent with previous results (Tumlinson et al., 1974). However, the behavioral mechanism of the inhibition is not known. We also obtained the first evidence of an inhibitory effect of E,Z on *S. scitula* male response.

Contrary to previous results (Tumlinson et al., 1974) the present data indicate a synergistic effect of Z,E on *S. pictipes* response to E,Z. The discrepancy may be due to differences in the two populations of *S. pictipes* in Georgia and Wisconsin or to different experimental procedures.

A small number of *S. pictipes* males responded to a mixture of E,Z + Z,Z (Table 2). Such an "abnormal" phenomenon has also been observed by Nielsen et al. (1975). They reported three *S. pictipes* males that responded to a mixture of 93%, Z,Z and 7% of E,Z and Z,E.

In all six orchards sampled (Table 2), the number of *S. pictipes* captured was three times greater than that of *S. scitula*. This does not necessarily imply there were more *S. pictipes* than *S. scitula* in the orchards, because the attractive power of our set of compounds to each species is not known.

On the other hand, it can be shown mathematically that the correlation coefficient between trap counts of two species (in various orchards) is equal to the correlation coefficient between the abundances of the two species, as long as the relationship between trap counts and abundance is linear. Whether the relationship is increasing or decreasing is immaterial. The correlation coefficient between trap counts (Table 2) of *S. pictipes* and *S. scitula* is 0.86 (sign 5%).

The data in Table 3 show that under our experimental conditions the Z,Z isomer, when evaporated at four points and at a distance of 6 m from the pheromone source, disrupted the sex communication of *S. pictipes* by 85% and 84% in our 1974 and 1975 experiments, respectively. By increasing the

number of evaporators to 36 (over an 18 × 23-m area) and the total amount of Z,Z to 7200 μg, McLaughlin et al. (1975) achieved 99% disruption. The sex communication of several species of insects (Mitchell, 1975) has been experimentally disrupted by the permeation technique. In most of these cases, the disrupting compound was the sex pheromone or a sex attractant of the target species, while in our case, it is a sex pheromone of another species.

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SEX ATTRACTANT OF THE ALFALFA LOOPER *Autographa californica* AND THE CELERY LOOPER *Anagrapha falcifera* (Lepidoptera: Noctuidae)

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Abstract—In field tests, traps baited with a combination of (Z)-7-dodecen-1-ol acetate (previously proposed to be the sex pheromone of *A. californica*) and (Z)-7-dodecen-1-ol formate caught about 100 times as many males as (Z)-7-dodecen-1-ol acetate did alone. Highest catches of males were obtained with traps baited with 0.5 mg of (Z)-7-dodecen-1-ol acetate and 0.1 mg of (Z)-7-dodecen-1-ol formate impregnated on red rubber sleeve stoppers. The celery looper, *Anagrapha falcifera*, was also caught in traps baited with a combination of these two chemicals.

Key Words—sex pheromone, sex attractant, *Autographa californica*, *Anagrapha falcifera*, alfalfa looper, celery looper, attractant synergist, Z-7-dodecen-1-ol acetate, Z-7-dodecen-1-ol formate.

INTRODUCTION

A systematic investigation of compounds that increase or decrease trap catches with sex pheromones of Lepidoptera was carried out by Roelofs and Comeau (1971) in their study of the redbanded leafroller, *Argyrotaenia velutinana*. Since then such investigations have resulted in the discovery of inhibitors or synergists of the pheromones of several moth species (e.g., Klun and Robinson, 1972; Roelofs et al., 1973; Voerman and Minks, 1973; Voerman et al., 1974). Recently, at this laboratory (George et al., 1975), ester and ether derivatives of the sex pheromone of the codling moth, *Laspeyresia pomonella*, that extended the length of the pheromone molecule,

provided several potent inhibitors. Consequently, this type of derivative was investigated for synergistic or inhibiting effects on moth species that are attracted to (Z)-7-dodecen-1-ol acetate (Z7-12:Ac), previously proposed to be the sex pheromone of the alfalfa looper, *Autographa californica* by Kaae et al., 1973. Z7–12:Ac was first described as the sex pheromone of the cabbage looper, *Trichoplusia ni* (Berger, 1966).

METHODS AND MATERIALS

The derivatives were prepared from (Z)-7-dodecen-1-ol (Z7–12:OH), which was obtained by saponifying Z7–12:Ac (Farchan Chemical Co.) in methanolic sodium hydroxide. The formate of Z7–12:OH was prepared by allowing a solution of 0.1 g of Z7–12:OH, 0.5 g of 1,1′-carbonyldiimidazole, and 0.15 ml of 90% formic acid in 12 ml of tetrahydrofuran to stand for 2 hr. Other esters were prepared by refluxing the appropriate anhydride or acid chloride in pyridine with Z7–12:OH.

The esters were separated from possible traces of Z7–12:OH by high-pressure liquid chromatography with a silica gel column and elution with 1.5% ether in hexane or by liquid chromatography on alumina and elution with 2% ether in hexane. The structure of each ester from each preparation was verified by infrared spectra and comparisons of experimentally determined gas chromatographic retention indexes (columns: Carbowax 20M and SE-30, each at 160°C) with retention indexes calculated from the data of McReynolds (1966).

For the field tests, various amounts of candidate compounds and/or the pheromone in 200 μl of methylene chloride were impregnated on red rubber sleeve stoppers (No. 1F-66F, West Co., Phoenixville, Pennsylvania) held in the trap by a dispenser. The dispenser, a 3.5-cm diameter × 7-cm long, snap-cap polystyrene vial with nine 3-mm diameter holes bored through the cap was inverted and suspended from the top front of a trap so that the cap openings were 32–45 cm from the one-way entrance baffles around the bottom sides of the trap (Figure 1) which is a modification of the trap of Killinen and Ost (1971). The trap was covered by 0.01-cm-thick polyethylene film supported by 16-mesh aluminium screening so the pheromone and candidate compound were emitted only through the 8-mesh hardware cloth baffles.

In earlier unpublished work (J.E. Halfhill), a 2-mm (ID) capillary tube containing 0.1 g of Z7–12:Ac and a cotton thread wick extending 2 cm beyond the 5-cm capillary tube was used to survey for cabbage and alfalfa loopers. This lure, referred to as the wick, was used in the present tests for comparison.

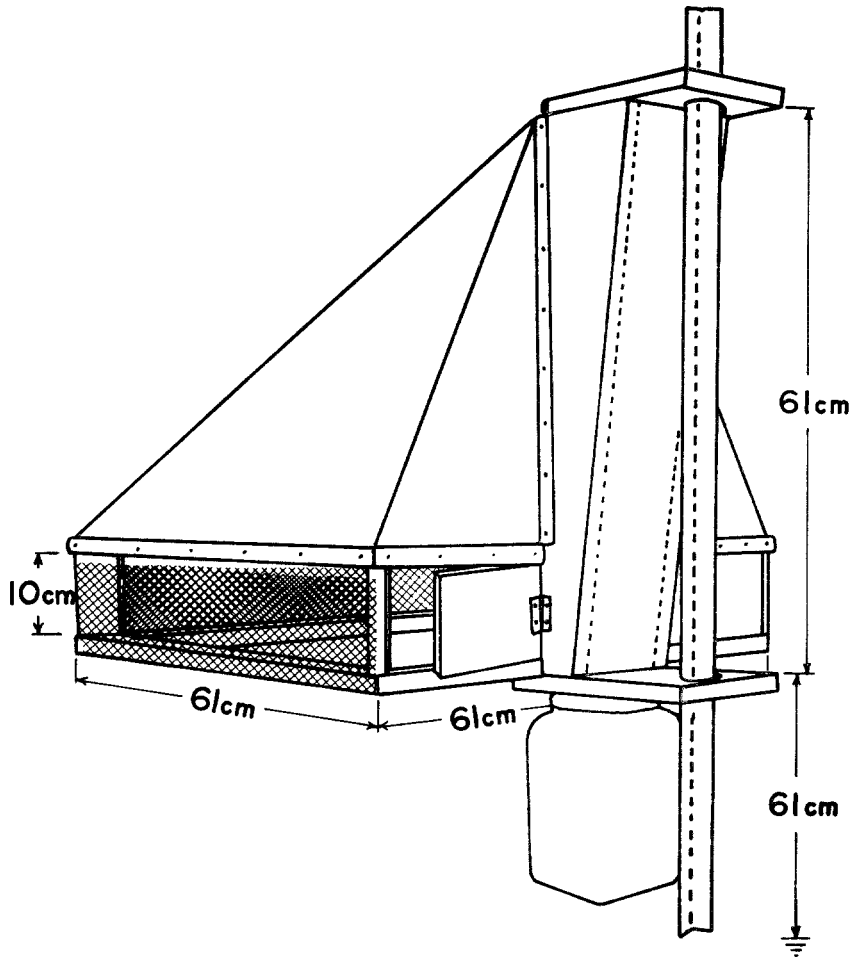

FIG. 1. Sex attractant trap for monitoring alfalfa looper activity.

The test site was a 1200-m long ditch bank situated in the middle of an arid area supporting an *Artemisia–Agropyron* biome (a specific complex of plants, mainly sagebrush and cheatgrass) that was bounded 150 m to the east by an alfalfa field and 750 m to the west by the Columbia River. The traps were placed 30 m apart along the ditch bank. The prevailing wind was from the west. The treatments were randomized at 3-day intervals by rotating the dispensers among the traps. In some tests, the pheromone and candidate compound were impregnated on separate sleeve stoppers, then when the pheromone was used alone for the control, a blank sleeve stopper was added.

RESULTS AND DISCUSSION

In the first test, the effect of Z7–12:OH or its esters on the attractiveness of Z7–12:Ac was determined by exposing two rubber stoppers in a trap, one impregnated with 2 mg of Z7–12:Ac and the other impregnated with 2 mg of the test compound. Single traps were used for each test and replication was obtained by rotating baits 5 times during the test period (Aug. 26–Sept. 8, 1975). The total catches of males for the indicated test compounds with Z7–12:Ac were as follows: Z7–12:OH, 8; formate (Z7–12:Fo), 700; propionate, 26; butyrate, 25; valerate, 37. The catch with Z7–12:Ac alone was 9. On the basis of Duncan's new multiple range test (5% level), only the

TABLE 1. DETERMINATION OF THE OPTIMUM RATIO OF (Z)-7-DODECEN-1-ol ACETATE TO (Z)-7-DODECEN-1-ol FORMATE (4 REPLICATES EACH, RANDOMIZED 3 TIMES)[a]

Z7–12:Ac (mg)	Z7–12:Fo (mg)	Ratio of Z7–12:Ac to Z7–12:Fo	Average catch/trap[b] Alfalfa looper	Celery looper
		Sept. 8–19, 1975		
0	2		1.0 b	0 c
0.2	0		1.3 b	0 c
2	0		0 b	0 c
2	0.4	5:1	30.5 a	0.3 b,c
2	2[c]	1:1	29.5 a	0.5 b,c
2	2[d]	1:1	25.9 a	2.4 a
2	10	1:5	1.0 b	1.5 a,b
100 (wick)	0		0 b	0 c
		Sept. 20–Oct. 1, 1975		
2	0		0.3 d	0 a
2	0.02	100:1	5.7 c,d	0 a
2	0.05	40:1	18.5 b,c,d	0.5 a
2	0.1	20:1	20.6 a,b,c,d	1.8 a
2	0.2	10:1	39.0 a,b	1.7 a
2	0.4	5:1	47.3 a	1.7 a
2	2	1:1	32.4 a,b,c	2.9 a
100 (wick)	0		0.08 d	0 a

[a] Each material was on separate rubber stoppers.
[b] Number caught followed by the same letter were not significantly different based on Duncan's new multiple range test (5% level).
[c] Aged 4 weeks in the field.
[d] Newly prepared.

catches with the combination of Z7–12:Fo and Z7–12:Ac were significantly different from the catches by Z7–12:Ac alone. This combination resulted in an almost 100-fold increase in catch over Z7–12:Ac alone (to the nearest order of magnitude).

Further experiments were designed to determine the possible attractiveness of Z7–12:Fo alone, the optimum ratio of Z7–12:Ac to Z7–12:Fo, and the optimum dose level. As Table 1 shows, Z7–12:Fo alone did not produce significant catches; it therefore appears to depend on the presence of Z7–12:Ac for attraction. It should be noted, however, that, on a statistical basis, the catches in traps baited with Z7–12:Ac were also not significant.

During the test period Sept. 8–19, 1975 (Table 1), ratios of Z7–12:Ac to Z7–12:Fo of 5:1 and 1:1 were equivalent, while a ratio of 1:5 produced significantly lower catches of alfalfa looper. However, ratios of 1:1 and 1:5 produced significant catches of the celery looper, *Anagrapha falcifera.* During the test period of Sept. 20–Oct. 1, 1975, ratios of 1:1, 5:1, 10:1, and 20:1 were not significantly different, while ratios of 40:1 and 100:1 were significantly less attractive than the 5:1 ratio for the alfalfa looper. Again, the effect of the 5:1 ratio was approximately 100 times that for non-synergized Z7–12:Ac.

The 5:1 ratio was chosen for the tests made to optimize the dose level for the alfalfa looper because it is within the extremes of the 1:1 and 20:1 ratios between which maximum attractiveness occurred. The data are

TABLE 2. DETERMINATION OF THE OPTIMUM DOSE LEVEL OF THE 5:1 RATIO OF (Z)-7-DODECEN-1-OL ACETATE TO (Z)-7-DODECEN-1-OL FORMATE FOR THE ALFALFA LOOPER, OCTOBER 6–15, 1975 (4 REPLICATES EACH, RANDOMIZED 3 TIMES)

Z7–12:Ac[a] (mg)	Z7–12:Fo (mg)	Average catch/trap[b]
0.03	0.00625	10.9 b
0.125	0.025	43.3 b
0.5	0.1	90.5 a
2.0	0.4	37.8 b
8.0	1.6	7.4 b
2.0	2.0	37.7 b
2.0	0.4[c]	10.3 b
83	17 (wick)	0.7 b

[a] Both materials on same stopper except where noted.
[b] Number caught followed by the same letter were not significantly different based on Duncan's multiple range test (5% level).
[c] Each material on separate stoppers.

summarized in Table 2. Only the traps containing stoppers impregnated with 0.5 mg of Z7–12:Ac and 0.1 mg of Z7–12:Fo caught significantly more male alfalfa loopers than the other dose levels.

Because the synergistic effect of Z7–12:Fo is so large, it could be that it is either a component of the pheromone or is simulating the effect of some other previously unidentified pheromone component. Kaae et al. (1973) showed that prevention of interspecies attraction between the alfalfa looper and the cabbage looper could be achieved by differences in emission rates. The cabbage looper is attracted to higher rates than the alfalfa looper. However, if the cabbage looper catches are not also increased by Z7–12:Fo, then Z7–12:Fo or a pheromone component it is mimicking would contribute to species isolation.

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SEX PHEROMONE SPECIFICITY AS A REPRODUCTIVE ISOLATING MECHANISM AMONG THE SIBLING SPECIES *Archips argyrospilus* and *A. mortuanus* AND OTHER SYMPATRIC TORTRICINE MOTHS (Lepidoptera: Tortricidae)

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Abstract—Male *Archips argyrospilus* (Walker) were maximally attracted to a 60:40:4:200 admixture of (Z)-11-tetradecenyl, (E)-11-tetradecenyl, (Z)-9-tetradecenyl and dodecyl acetates. The identification of (Z)-9-tetradecenyl acetate, in addition to the other 3 components reported previously, is the first report of a 4-component pheromone system in moths. *Archips mortuanus* Kearfoot, a sibling species, was maximally attracted to a blend of the same components, but in a 90:10:1 mix of the tetradecenyl acetates. Among a number of tortricine moths that co-occur on apples and other hosts in New York, British Columbia, and elsewhere, differences in diel rhythms of attraction and seasonal distribution are insufficient to maintain reproductive isolation. Although the attractant systems of these species often possess components in common, males are maximally attracted to a species-specific blend. In tortricine moths attraction specificity appears to be a paramount mechanism for species partitioning.

Key Words—*Archips argyrospilus*, *Archips mortuanus*, pheromone, attractant, Tortricidae, (Z)-11-tetradecenyl acetate, (E)-11-tetradecenyl acetate, (Z)-9-tetradecenyl acetate, dodecyl acetate.

INTRODUCTION

The species statuses of *Archips argyrospilus* (Walker) (fruittree leafroller) and of *Archips mortuanus* Kearfoot have been debated inasmuch as these

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taxa are morphologically indistinguishable. Powell (1964) considered *A. mortuanus* to be merely a color variant of *A. argyrospilus*. Roelofs and Comeau (1969) demonstrated that crude female abdominal-tip extracts of these sibling species were maximally attractive to conspecific males, implying sex pheromone specificity and reproductive isolation. In sympatric New York populations, traps baited with *A. argyrospilus* extract captured 203 conspecific and no *A. mortuanus* males, whereas traps baited with *A. mortuanus* extract lured 82 conspecific and 10 *A. argyrospilus* males. Roelofs et al. (1974) characterized the sex pheromone of *A. argyrospilus* as a blend of (Z)- and (E)-11-tetradecenyl and dodecyl acetates (Z11–14:Ac, E11–14:Ac and 12:Ac) with a 7:3:40 ratio being the most attractive. We report here the identification of a fourth pheromone component of *A. argyrospilus* and field tests defining the optimum specific attractant blends for *A. argyrospilus* and *A. mortuanus*.

Since the pheromones of tortricine moths generally have at least one component that is either a 14-carbon chain acetate, alcohol, or aldehyde with unsaturation in the C_{11-12} position, many sympatric tortricines possess overlapping chemical communication systems. This paper assesses species recognition of distinctive pheromone bouquets and nonpheromonal reproductive isolating mechanisms such as differential mating rhythmicity and habitat preferences.

MATERIALS AND METHODS

Archips argyrospilus and *A. mortuanus* for chemical analyses were collected either as overwintering eggs in the Okanagan Valley of British Columbia and reared on fava bean plants or as penultimate and last instar larvae in the Hudson Valley of New York in late May and reared on apple leaves. Pupae were segregated by sex and emergent females were held for 2–3 days prior to excision of the abdominal tips into methylene chloride.

The GLC columns (1.8 × 4 mm) were 3% OV-1 (methyl silicone on 100–120 mesh Gas-chrom Q), 10% XF-1150 (50% cyanoethyl methyl silicone on 100–120 mesh Gas-chrom Q), and 3% PDEAS (phenyldiethanolamine succinate on 100–120 mesh Chromosorb W-AW-DMCS). A flame ionization detector was used. The TLC silica-gel plates (30% $AgNO_3$) were washed by development with 10% ether in benzene and dried. Components were separated by development with spectrograde benzene. Microozonolyses were conducted according to the methods of Beroza and Bierl (1967).

Electroantennograms (EAG) for assaying male response to GLC collections of female abdominal tip extract and for determining a response profile to monounsaturated acetates, alcohols, and aldehydes were recorded as described elsewhere (Roelofs and Comeau, 1971a, b; Roelofs, 1976).

Field tests were conducted in abandoned apple orchards in Highland and Dresden, New York, and in Summerland, British Columbia. Test chemicals were placed in polyethylene closures (OS-6, Scientific Products) and these dispensers were positioned in the bottom center of Pherotrap® 1C traps (Zoecon Corp). The Z11–14:Ac and the E11–14:Ac were obtained from Farchan Chemical and the 12:Ac from Eastman Chem. The (Z)-9-tetradecenyl acetate (Z9–14:Ac) was synthesized in our laboratories and purified by $AgNo_3$-silica gel high-pressure liquid chromatography. All mixtures were analyzed for component ratios by GLC on either XF-1150 or OV-1 columns.

Traps were positioned in apple trees at a height of 1.5 m and separated by ca. 12–14 m. All tests utilized a randomized complete block design. Data were transformed to $\sqrt{(x+0.5)}$ and, except in Table 3, submitted to analyses of variance. Throughout, treatment means followed by the same letter are not significantly different at the 5% level according to Duncan's new multiple-range test.

RESULTS

Analysis of New York Archips mortuanus Female Extract

Crude female extract was collected from the OV-1 GLC column (171°C), and EAG assay of the fractions showed activity of 3.0 mV from 10.5 to 13 min (background responses <1.0 mV; standard 14:Ac at 11.95 min). Injection of the active fraction on the PDEAS column produced two peaks in a 9:1 ratio. The larger peak had a retention time identical to that of Z11–14:Ac (8.9 min), and the smaller peak had a retention time (8.4 min) that corresponded to both E11–14:Ac and Z9–14:Ac.

Male *A. mortuanus* EAG responses to 12-, 14-, and 16-carbon chain acetates, alcohols, and aldehydes showed that the 14-carbon acetates elicited the greatest responses of the chemicals tested. The response profile of the 14-carbon acetates (Figure 1) indicates that Z11–14:Ac evokes the maximum antennal depolarization, with E11–14:Ac and Z9–14:Ac also eliciting good responses. The EAG response elicited by Z9–14:Ac relative to that of Z11–14:Ac is greater than that obtained with species such as *Argyrotaenia velutinana* (Walker) that utilize Z11–14:Ac and E11–14:Ac, but not Z9–14:Ac, in their attractant blends (Roelofs and Comeau, 1971a, b); Roelofs et al., 1975), but is similar to the EAG response profile of *Clepsis spectrana* (Minks et al., 1973) and *Pandemis limitata* (Roelofs et al., 1976a), which utilize Z9–14:Ac as the minor component of a blend with Z11–14:Ac. This suggested that Z9–14:Ac and E11–14:Ac both might be components of the *A. mortuanus* pheromone blend. Also, just Z11–14:Ac and E11–14:Ac in a

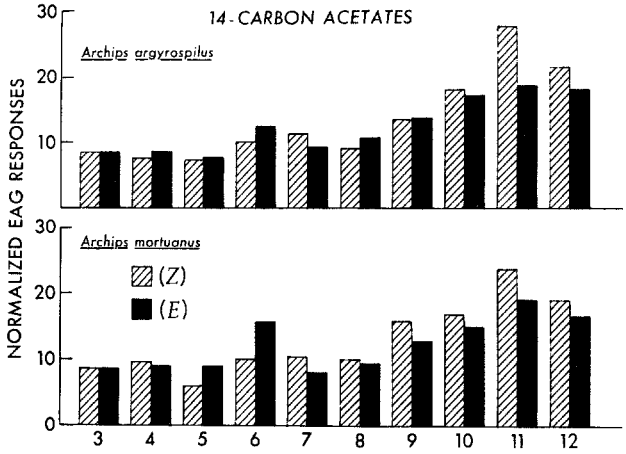

FIG. 1. Normalized electroantennogram responses of *Archips argyrospilus* and *Archips mortuanus* to a series of tetradecenyl acetates.

TABLE 1. CAPTURES OF MALE *Archips mortuanus* IN ORCHARDS IN HIGHLAND, NEW YORK, JUNE 17–28, 1973. EACH DISPENSER CONTAINED 5 MG OF CHEMICAL

Test 1	
Z11–14:Ac to E11–14:Ac	$\bar{X}$ males/trap[1]
98:2	12.8 a
95:5	13.6 a
91:9	16.4 a
80:20	2.4 b
70:30	0.4 b
60:40	0.0 b
Unbaited	0.0 b
Test 2	
Z11–14:Ac (2% E) to Z9–14:Ac	$\bar{X}$ males/trap
100:0	10.6 b
95:5	31.6 a
90:10	23.6 a
80.20	9.8 b
65:35	4.2 c
20:80	1.0 c
10:90	0.4 c
5:95	0.0 c
Unbaited	0.0 c

[1] Means followed by the same letter are not significantly different at the 5% level.

9:1 blend have rarely lured *A. mortuanus* males in field tests in low populations.

Field Tests for Archips mortuanus and Archips argyrospilus Attraction

In 1973, captures (Table 1, test 1) of *A. mortuanus* in New York during a population flush by various binary combinations of Z11–14:Ac and E11–14:Ac were greatest with ca. 98:2 to 91:9. Admixture of these two compounds (98:2) with varying ratios of Z9–14:Ac in a concurrent trial (Table 1, test 2)

TABLE 2. FIELD TRAPPING OF *Archips argyrospilus* and *A. mortuanus* IN HIGHLAND, NEW YORK, JUNE 15 TO JULY 5, 1975. SEVEN REPLICATES, RERANDOMIZED TWICE

Treatment 5 mg [11–14:Ac's+Z9–14:Ac (<0.2% E9–14:Ac)]	*A. Argyrospilus* males/trap	*A. mortuanus* males/trap
Z11–14:Ac (0.4% E11–14:Ac)	0.0 c	1.0 c,d
Z11–14:Ac (0.4% E11–14:Ac)+1% Z9–14:Ac	0.0 c	1.4 c,d
Z11–14:Ac (0.4% E11–14:Ac)+4% Z9–14:Ac	0.0 c	1.1 c,d
Z11–14:Ac (0.4% E11–14:Ac)+6% Z9–14:Ac	0.1 c	0.6 d
Z11–14:Ac (0.4% E11–14:Ac)+12% Z9–14:Ac	0.0 c	0.1 d
Z11–14:Ac (0.4% E11–14:Ac)+20% Z9–14:Ac	0.0 c	0.1 d
Z11–14:Ac (6% E11–14:Ac)	0.0 c	1.9 c,d
Z11–14:Ac (6% E11–14:Ac)+1% Z9–14:Ac	0.0 c	14.7 b
Z11–14:Ac (6% E11–14:Ac)+4% Z9–14:Ac	0.0 c	4.1 c
Z11–14:Ac (6% E11–14:Ac)+6% Z9–14:Ac	0.0 c	1.7 c,d
Z11–14:Ac (6% E11–14:Ac)+12% Z9–14:Ac	0.0 c	0.0 d
Z11–14:Ac (6% E11–14:Ac)+20% Z9–14:Ac	0.0 c	1.0 c,d
Z11–14:Ac (11% E11–14:Ac)	0.6 c	1.0 c,d
Z11–14:Ac (11% E11–14:Ac)+1% Z9–14:Ac	0.0 c	23.1 a
Z11–14:Ac (11% E11–14:Ac)+4% Z9–14:Ac	0.1 c	10.4 b
Z11–14:Ac (11% E11–14:Ac)+6% Z9–14:Ac	0.3 c	5.9 c
Z11–14:Ac (11% E11–14:Ac)+12% Z9–14:Ac	0.0 c	0.3 d
Z11–14:Ac (11% E11–14:Ac)+20% Z9–14:Ac	0.0 c	0.3 d
Z11–14:Ac (0.4% E11–14:Ac)+6% Z9–14:Ac+ 10 mg 12:Ac	0.1 c	0.7 d
Z11–14:Ac (6% E11–14:Ac)+6% Z9–14:Ac+ 10 mg 12:Ac	0.0 c	10.0 b
Z11–14:Ac (40% E11–14:Ac)	0.0 c	1.0 c,d
Z11–14:Ac (40% E11–14:Ac)+1% Z9–14:Ac	1.3 b	0.7 d
Z11–14:Ac (40% E11–14:Ac)+4% Z9–14:Ac	5.1 a	0.7 d
Z11–14:Ac (40% E11–14:Ac)+8% Z9–14:Ac	2.0 b	0.3 d
Z11–14:Ac (40% E11–14:Ac)+14% Z9–14:Ac	0.0 c	0.1 d
Z11–14:Ac (40% E11–14:Ac)+10 mg 12:Ac	2.6 b	1.6 c,d
Unbaited	0.0 c	0.3 d

TABLE 3. POOLED 1974 CAPTURES IN NEW YORK OF MALE *Archips agyrospilus* IN HIGHLAND (5 TRAPS FROM JUNE 9 TO JULY 3) AND DRESDEN (10 TRAPS FROM JULY 9 TO JULY 22). MEANS COMPARED WITH STUDENT'S ONE-TAILED t TEST: $P < 0.02$. BOTH TREATMENTS DIFFER FROM UNBAITED TRAPS AT $P < 0.001$.

Treatment	$\bar{X}$ males/trap
5 mg Z11–14:Ac and E11–14:Ac (60:40)+20 mg 12:Ac	3.9
5 mg Z11–14:Ac, E11–14:Ac, and Z9–14:Ac (56:38:6)+20 mg 12:Ac	7.8

showed that 5–10% of this third compound produced the highest catches. A more extensive range of ratio comparisons in 1975 (Table 2) indicated that an 88:11:1 blend produced a trap catch greater than any other binary or trinary mixture tested. Addition of 12:Ac (one of the previously reported components of the *A. argyrospilus* pheromone bouquet) also was found to increase *A. mortuanus* trap catch. Although the optimum blend was not used, addition of 10 mg of 12:Ac to the 5 mg of 88:6:6 ratio caught significantly more males than did the 88:6:6 treatment without 12:Ac.

Since Z9–14:Ac had not been evaluated previously for modulation of *A. argyrospilus* attraction, this compound was added (Table 3) to the 60:40:200 Z11–14:Ac, E11–14:Ac, and 12:Ac pheromone blend for this species (Roelofs et al., 1974). In New York the presence of Z9–14:Ac as 6% of the tetradecenyl acetates increased male captures twofold over the mixture lacking this component. Although the optimal 60:40 Z11–14:Ac and E11–

TABLE 4. CAPTURES OF *Archips argyrospilus* MALES IN SUMMERLAND, BRITISH COLUMBIA. TEST CONDUCTED FROM JUNE 18 TO JULY 30, 1975. FIVE REPLICATES RERANDOMIZED WEEKLY. FIVE MG Δ^{11}–14:Ac's (Z11–14:Ac AND E11–14:Ac 67:33) PLUS Z9–14:Ac (<0.2% E9–14:Ac)

Treatment	$\bar{X}$ males/trap
Δ^{11}–14:Ac's	7.6 c
Δ^{11}–14:Ac's+Z9–14:Ac (6%)	29.6 b
Δ^{11}–14:Ac's+Z9–14:Ac (3%)+20 mg 12:Ac	51.6 a,b
Δ^{11}–14:Ac's+Z9–14:Ac (6%)+20 mg 12:Ac	35.4 b
Δ^{11}–14:Ac's+Z9–14:Ac (12%)+20 mg 12:Ac	30.4 b
Δ^{11}–14:Ac's+20 mg 12:Ac	61.8 a

14:Ac mixture alone was not attractive in New York (Roelofs et al., 1974), addition of Z9–14:Ac as a third component, particularly as 4% of the 11-position acetates, yielded good attractancy (Table 2). From these findings, the 4-component attractant systems of *A. argyrospilus* and *A. mortuanus* in New York appeared to be species specific as a result of the difference in the ratios of Z11–14:Ac to E11–14:Ac (90:10 for *A. mortuanus* and 60:40 for *A. argyrospilus*).

In British Columbia, Roelofs et al. (1974) also found *A. argyrospilus* males attracted to a 60:40:400 blend of Z11–14:Ac, E11–14:Ac, and 12:Ac. But unlike New York *A. argyrospilus*, males in British Columbia also were lured to mixtures that were low in either E11–14:Ac or 12:Ac (e.g., 98:2:400 or 60:40:0). In 1975 tests in British Columbia (Table 4), addition of 6% of Z9–14:Ac to a 67:33:0 mixture greatly increased trap catch. The 4-component mixture tested, Z11–14:Ac, E11–14:Ac, Z9–14:Ac, and 12:Ac at 67:33:3:400, was as attractive as the 67:33:0:400 blend. The effect of Z9–14:Ac on trap catch in New York and British Columbia suggested that this compound could be a constituent of the natural pheromone system of this tortricine. Consequently, we investigated female abdominal tip extract for the presence of Z9–14:Ac.

Chemical Analysis of British Columbia Archips argyrospilus Extract

Female abdominal tip extract of *A. argyrospilus* and an internal standard of (Z)-11-tridecenyl acetate (Z11–13:Ac) were fractionated on TLC. The (Z) and (E) regions were removed together, extracted with methylene chloride and rechromatographed on TLC. The (Z) region was removed and extracted with methylene chloride. Injection of an aliquot on OV-1 (150°C) showed peaks at 23.7 and 25.3 min, corresponding to the retention times of synthetic Z9–14:Ac and Z11–14:Ac, respectively, at 23.6 and 25.0 min. An aliquot was collected from OV-1 (150°C) from 22.5 to 27.0 min and ozonized. The ozonolysis products were injected onto OV-1 (150°C). Peaks present at 6.3 and 15.8 min corresponded to the ozonolysis products of standard Z9–14:Ac and Z11–14:Ac at 6.4 and 15.9 min, respectively. These findings confirm that Z9–14:Ac is present in the female abdominal tip. The Z9–14:Ac comprised only 1.2% of the quantity of the Z11–14:Ac, based on the ratios both of the ozonolysis products and the components on OV-1.

The (E) TLC region also was removed, extracted, and an aliquot injected on OV-1 (150°C). No peak was evident at 17.0 min, the retention time of the internal standard, Z11–13:Ac, indicating the complete separation of the (Z) and (E) isomers by TLC. A prominent peak at 24.8 min corresponded to E11–14:Ac but there was no visible component at 23.8 min, the retention time of E9–14:Ac.

DISCUSSION

Multiple Component Pheromone Blends

The 4-component bouquets maximally attractive to males of *A. argyrospilus* and *A. mortuanus* represent the most complex male attractant systems uncovered to date in the Lepidoptera. The present identification of Z9–14:Ac in the female abdominal tips of *A. agryospilus*, in addition to the previously characterized Z11–14:Ac, E11–14:Ac, and 12:Ac (Roelofs et al., 1974), is the first report of a 4-component pheromone system in moths. The presence of these same acetates in either the female abdominal tip or effluvium of *A. mortuanus* remains to be rigorously demonstrated, although the field data indicate that all four are used.

The effect of Z9–14:Ac on trap catch, when present as a minor constituent of an attractant medley, appears similar to the field effect of (E)-11-tetradecen-1-ol (E11–14:OH) at ca. 1% of the attractant blend of *Platynota stultana* (Walsingham) (Hill and Roelofs, 1975; Baker et al., 1975) and (Z)-8-dodecen-1-ol (Z8–12:OH) as ca 1% of the attractant blend of *Grapholitha molesta* (Busck) (Cardé et al., 1975c). In the former species the E11–14:OH was found in the female abdominal tip, whereas Z8–12:OH has not been characterized as yet from the latter insect. If the occurrence of pheromone components at such low ratios is frequent in moth communication systems, their complete elucidation will assume added complexity.

In both *Archips* species the precise behavioral effect of each component has not been determined. To increase trap catch, compounds need not mediate long-range anemotaxis (attraction), but instead some components could elicit either close-range orientation or landing near the chemical source, thereby elevating the trap effectiveness (Cardé et al., 1975a, b; Baker et al., 1976). Thus, the term attractant "synergist" may be inappropriate for the behavioral effects evoked by Z9–14:Ac and 12:Ac in these two *Archips* species.

Constraints Upon Blends in Chemical Communication

One interesting feature of the tortricid multichemical attractant systems described to date is the dissimilarity among species in the effect of modifications of the optimum blend ratio. In many species relatively subtle alterations of the optimum blend for field trapping greatly decreases the trap catches. For example, a 5% alteration in the optimum 91:9 ratio of Z11–14:Ac to E11–14:Ac drastically lowers the male catch for *Argyrotaenia velutinana* (Walker) (Klun et al., 1973, Roelofs et al., 1975).

In other species maximum trap catch is effected by a combination of either a single compound or a blend, plus a modifier which increases trap

TABLE 5. ATTRACTANT BLENDS OF SYMPATRIC, APPLE-FEEDING TORTRICINE MOTHS OCCURRING IN NEW YORK

Species	Attractant blend[a]	Ratio[b]	Attractant inhibitors[c]	Adult seasonal distribution[d]	Regression equation for attraction time[e,f]	"Typical" activity time[f,g]	References
Archips argyrospilus (Walker)	Z11–14:Ac*† E11–14:Ac*† Z9–14:Ac*† 12:Ac*†	**60:40:4:200**	Z11–14:OH E11–14:OH	June to early July	$H = 11.45+0.63T_{22.5}$	22.2	Roelofs et al., 1974; this paper
Archips mortuanus Kearfoot	Z11–14:Ac† E11–14:Ac† Z9–14:Ac† 12:Ac	**90:10:1:200**		June to early July	$H = 14.98+0.43T_{21.0}$	22.3	This paper
Archips semiferanus (Walker)	Z11–14:Ac*† E11–14:Ac*†	**30:70**		June to early July	$H = 18.18+0.25T_{24.0}$	22.3	Miller et al., 1976
Argyrotaenia velutinana (Walker)	Z11–14:Ac*† E11–14:Ac*† 12:Ac*	**9:1:15**	Z9–14:Ac Z11–14:OH	April; late June to mid July; August to early Sept.	$H = 17.45+0.22T_{20.5}$	21.5	Roelofs and Arn, 1968; Roelofs and Comeau, 1969; Klun et al., 1973; Roelofs et al., 1975
Choristoneura rosaceana (Harris)	Z11–14:Ac* E11–14:Ac*	**9:1**	Z11–14:OH Z9–14:Ac 12:Ac	June to early July; August	$H = 16.74+0.40T_{24.0}$	23.3	Roelofs and Tette, 1970; Roelofs, unpublished
Choristoneura fractivittana (Clemens)	Z11–14:OH		Z11–14:Ac	June	$H = 17.65+0.25T_{21.0}$	23.4	Roelofs and Comeau, 1970
Pandemis limitata (Robinson)	Z11–14:Ac* Z9–14:Ac*	**9:1**	E11–14:Ac 12:Ac	June			Roelofs et al., 1976b
Platynota idaeusalis (Walker)	E11–14:OH* E11–14:Ac*	1:1	Z11–14:OH Z11–14:Ac	June, August			Hill et al., 1974
Platynota flavedana (Clemens)	Z11–14:OH* E11–14:OH*	**15:85**	Z11–14:Ac	June; August			Hill et al., 1977

[a] Components asterisked have been identified as being present in either the female abdominal tip or the effluvium. Other compounds have been determined by empirical field screening and some supporting data from EAG and gland analyses. Compounds daggered are obligatory for field attractancy.
[b] Ratios for optimum field trap catch. Ratio components in boldface cannot be varied without greatly reducing trap catch. In excess such components could be considered inhibitory.
[c] Compounds (other than pheromone components) that reduce trap catch when emitted with the attractant blend.
[d] Flight periods in the lower Hudson Valley of New York. Additional information in Chapman and Lienk (1971).
[e] Regression equation for H, the mean hour of activity, including the effect of temperature (T) near the mean time of attraction (see Comeau, 1971; Comeau et al., 1976; Cardé et al., 1975).
[f] All times Eastern Daylight Time.
[g] The mean hour of activity for a "typical" June evening where the temperature is at 1800 = 23°C, 2000 = 19°C, 2200 = 17°C, 2400 = 16.5°C, and 0200 = 15.5°C.

catch. Such modifiers are neither intrinsically attractive nor are they requisite to attraction. In *A. velutinana* 12:Ac alone does not lure males to traps (Roelofs et al., 1975), although at extremely high dosages in the laboratory 12:Ac evokes upwind anemotaxis (Baker et al., 1976). However, the addition of 12:Ac in widely varying proportions (1:5 to 2:1) to the 91:9 Z11–14:Ac, E11–14:Ac attractant combination can increase the trap catch 10-fold over the attractant alone (Roelofs et al., 1975). Similar cases of noncritical ratios are given in Table 5 and in addition include *Argyrotaenia citrana* (Fernald) (Hill et al., 1975), as well as other moths.

The *raison d'être* of these divergent communication blends may relate to volatility of the various components. Those compounds that differ either in the position or geometrical configuration of the double bond but are similar in carbon-chain length and functionality will possess essentially similar vapor pressures and diffusion rates at different environmental temperatures. Such components would emanate from a pheromone gland and diffuse within the active space (Wilson and Bossert, 1963) at nearly the same rates. Hence, the ratios would remain constant throughout the active space, provided that the components are transported at equal rates to the surface of the pheromone gland.

Blend components with either different functional moieties or chain lengths would possess differing relative vapor pressures (even at the minor alterations of ambient temperature near calling females, e.g., from ca. 16 to 30°C). Hence, the ratio of components emitted from the gland could vary sufficiently with temperature to preclude the use of such compounds in precise ratios.

Role of Pheromones in Premating Reproductive Isolation

Although the importance of pheromones in reproductive isolation was reviewed by Roelofs and Cardé (1974), the recent characterizations of numerous pheromone systems utilizing two or more components suggest the desirability of reappraising their role in species partitioning. Since many moth species differ (and hence are isolated) by disjunct geographical, seasonal, or habitat distributions, the assessment of the pheromonal specificity for these species may be largely theoretical since both cross-communication and communication interference are eliminated by spatial and temporal isolation of the populations.

However, the tortricids listed in Table 5, which include several closely related congeners, co-occur in time and space in the lower Hudson Valley region of New York. Their temporal distributions in June are essentially synchronous except for *A. velutinana*, which commences adult eclosion somewhat after the peak of emergence of the other species. Their larval

food-plant ranges include apple as a primary host, and these species often coexist on the same trees (Chapman and Lienk, 1971).

Although apple was naturalized into North America in the 1600s, this tortricid guild association has an older and more extensive interrelationship, since these species coexist on a number of additional hosts (Chapman and Lienk, 1971). At any time an individual apple leaf will rarely support more than a single tortricid larva, and, except in infrequent population flushes, the majority of leaves will not have any larvae. Since not all of the available food plant is utilized, either inter- or intraspecies larval competition for food is not an obvious feature of this feeding guild. The exact importance of larval competition in regulating population density in these tortricids is unknown, but availability of food does not appear to limit density (Hairston et al., 1960) and direct competition may be lessened by parasitoids. As a consequence, females of several different tortricids may routinely emerge and mate within very close proximity.

Some of the same species associations given in Table 5 also co-occur widely in other geographical regions. In the Okanagan Valley of British Columbia *A. argyrospilus*, *P. limitata*, *P. idaeusalis*, and *C. rosaceana* also have been recorded as co-occurring on apple as well as other hosts (Prentice, 1965; Mayer and Beirne, 1974a, b). Another tortricine *Archips rosanus* (L.), is also common on apple, and this species utilizes Z11–14:Ac and Z11–14:OH in a 9:1 blend as its attractant pheromone (Roelofs et al., 1976b).

Partitioning by exclusive daily mating rhythms could be an effective reproductive isolating mechanism (Roelofs and Cardé, 1974). The diel mating rhythms of several of the tortricines of Table 5 do not occur in constant daily intervals, since their attraction periodicities can be shifted by changes in ambient temperature. Comparison of the regression equations for the mean hour of activity as modified by the ambient temperature (Table 5) reveals that under average June conditions there were only subtle differences in the times of male attraction, since the mean time of attraction would occur between 2100 and 2400 hours for those species investigated. The particular daily temperature regime modifies the degree of partitioning, but in all cases the temporal isolation attributable to mating rhythmicity will be inadequate to isolate these tortricids effectively (Comeau, 1971; Comeau et al., 1976; Cardé et al., 1975c).

It is important to note that besides attraction (upwind anemotaxis) specificity, additional isolation could be effected in the behavioral patterns evoked by the pheromone blend. The sequence of male and female behavior involved in mating, even among closely related species, may be a crucial premating reproductive isolating device (Grant and Brady, 1975; Grant et al., 1975). Additionally, the rate of pheromone emission could confer attraction specificity (Kaae et al., 1973; Cardé et al., 1975b).

Attractant blends that are highly species specific in elicitation of attraction to the chemical source, despite some overlap in utilization of some common components, appear to be the paramount premating reproductive isolating mechanism among the Tortricidae considered here. Sticky traps baited with the appropriate synthetic attractant bouquet lure conspecific males nearly exclusively, even when the traps are positioned only ca. 2 m apart. The capacity either of certain compounds or of ratio alterations to depress trap catches does not seem to extend beyond the immediate vicinity of the trap, and thus would not suppress or eliminate the catches of nearby traps. Similarly, females of the apple-feeding tortricids considered here could call in close proximity on the same host tree without causing interspecies communication interference.

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FACTORS INFLUENCING MALE SEXUAL RESPONSE IN THE AMERICAN COCKROACH *Periplaneta americana*

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Abstract—Male sexual response in *Periplaneta americana* is influenced by the presence or absence of sex pheromone, the length of time since last exposure to pheromone, the length of that exposure, and the photocycle. Females begin emission of sex pheromone 9 or more days after the molt to adult. The quantity emitted does not appear to be influenced by the photocycle. During the light phase of the photocycle, males only respond to high concentrations of pheromone. They recover quickly after brief exposures, but after prolonged exposures, a second exposure 24 h later elicited responses of lower intensity.

Key Words—sex pheromone, *Periplaneta americana*, photocycle, behavior, habituation.

INTRODUCTION

The adult female of the American cockroach, *Periplaneta americana*, produces a pheromone which attracts adult males but neither nymphs nor other females. The emission of this sex pheromone appears to be closely related to mating, mated females being considerably less attractive than virgins (Roth and Willis, 1952). Wharton and Wharton (1957) stated that production of the pheromone begins about one week after the adult molt but failed to investigate exactly when emission began or whether some periodicity of emission might exist.

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Recent studies of the orientational responses of adult males of *P. americana* to sex pheromone have shown that the males orient to the source by chemotaxis (Rust et al., 1976) and also orient positively to air currents containing sex pheromone by "chemo-anemotaxis" (Rust and Bell, 1976). Rust and Bell (1976) demonstrated that the sex pheromone also elicits an increase in locomotion which is independent of spatial chemolocation.

A great many physiological and environmental factors may influence male sexual response. Of these, the effects of the following four will be considered:

1. emission of sex pheromone by virgin females;
2. pheromone concentration and the photocycle;
3. irregular distribution of the pheromone;
4. prolonged exposure to the pheromone.

METHODS AND MATERIALS

Adult males were selected from laboratory colonies and placed in clear plastic containers ($29 \times 18.5 \times 12.5$ cm) which were marked on the bottom with a black line that divided the long axis of the cage into two halves. Each container served as a housing cage and testing arena for five male cockroaches. The experimental animals were maintained under a 12-hr light, 12-hr dark cycle at $24 \pm 2°C$ and 60% relative humidity in a room totally devoid of females.

A group of late instar female nymphs was selected from the colonies and placed in a similar-sized cage in an incubator under the same light, temperature, and humidity conditions as the males. The nymphs were checked daily, and newly emerged adult females were transferred to individual 250-ml plastic beakers supplied with food and water. Cardboard lids were used to assure isolation from males.

Bioassays of male response to sex pheromone followed the basic design of Block and Bell (1974). The number of times that males crossed the center line was used as an indicator of the level of overall activity. Prior to testing with sex pheromone, a clean 5.5-cm disk of Whatman No. 1 filter paper was introduced, and one of the authors recorded the level of activity for 5 min to obtain a basal rate. Sex pheromone extract was then introduced on an identical disk. After a 1-min delay to allow the pheromone to permeate the air in the cage, the activity was measured for another 5 min. Observations during the dark cycle were aided by GE Ruby photographic lamps (590–680 nm).

In the bioassay to determine the age at which females begin to emit sex pheromone, disks of filter paper were placed in the bottoms of the beakers

containing females. These disks were changed daily. The pheromone-impregnated disks were then presented to caged males, and the level of activity elicited by a given filter paper was taken as representative of a female's pheromone emission during the preceding 24-hr period. In the remainder of the experiments, extracts of sex pheromone were prepared as described by Rust (1976).

In determining the effect of the photocycle on male responsiveness, 5 cages of 5 males each were exposed to the two concentrations of sex pheromone every hour of the photocycle. Each cage was tested only once within a 7-day period to preclude diminished activity due to habituation or sensory adaptation. Control levels of activity for males were determined by measuring the activity of 20 cages in each hour of the photocycle.

RESULTS

Emission of Sex Pheromone

Bioassays were conducted on filter papers from virgin females 1–42 days old, i.e. 1–42 days after the molt to adult. From 9–15 females were tested for each of the first 17 days of adult life. Beyond the 17th day, periodic sampling was done to determine if sex pheromone emission remained reasonably constant.

Papers from females 1–8 days old elicited no change in the activity levels of the males. By day 9, 4 of 14 females (28%) were emitting sufficient amounts of sex pheromone to elicit a strong increase in male activity. The increase in activity above the basal level elicited by exposure to papers from 9-day-old virgin females was significant as determined by Wilcoxon's signed ranks test ($P < 0.05$). The responses to filter papers from 10-day-old females was very similar. Thirty-three percent elicited marked increases in male activity, and the group as a whole was significantly different from the controls ($P < 0.05$). The percentage of females emitting sex pheromone in sufficient quantities to elicit significant increases in male activity continued to increase from 60% on day 11 to 90% on day 16. Some females apparently never initiated emission, however.

Figure 1 shows the mean male response to 24-hr filter papers from cages of virgin females 1–20 days old. The graph represents the average increase in male activity, i.e. treatment activity counts minus basal or control activity. Periodic checks beyond 20 days were performed. Papers from cages containing females up to 42 days old elicited male responses comparable to those elicited by the same females at 15–20 days. This suggests that there is no drastic change in the level of pheromone emission from its onset at 9–15 days until at least 40 days beyond the adult molt. The sharp decline in male

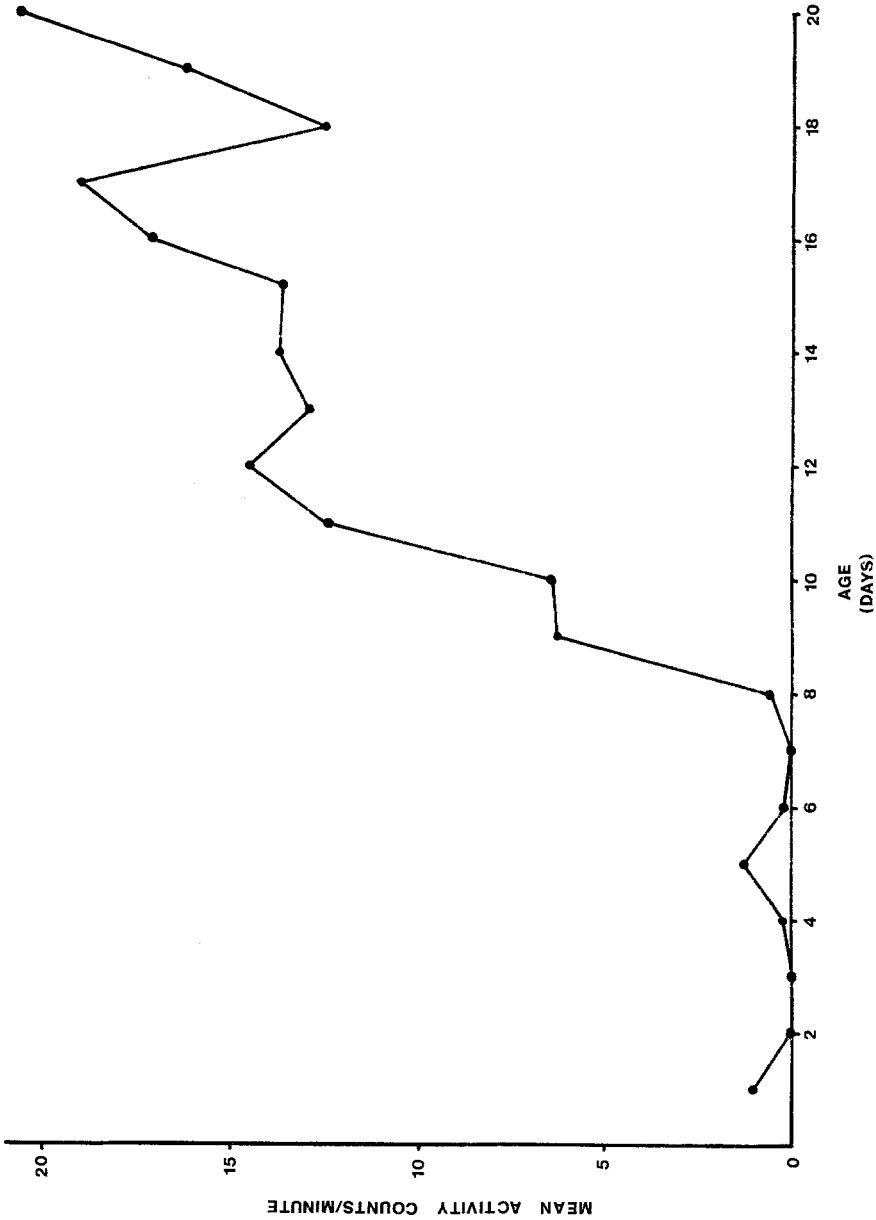

FIG. 1. Male activity elicited by filter papers from cages containing virgin females 1–20 days old as an indicator of sex pheromone emission. One group of 5 males/female (N = 9–15 females/age class).

TABLE 1. MEAN MALE RESPONSE TO 24-HR SEX PHEROMONE EMISSION OF A TYPICAL VIRGIN FEMALE AMERICAN COCKROACH

Age (days)	Control[a]	Treatment[b]	Difference[c]
3	0.6	2.4	1.8
4	7.8	6.2	−1.6
5	2.0	2.4	0.4
6	5.8	3.4	−2.4
7	4.4	3.8	−0.6
8	5.4	7.6	2.2
9	1.0	26.2	25.2
10	0.6	21.8	21.2
11	1.8	13.2	11.4
12	1.8	30.4	28.6
13	4.8	28.2	23.4
14	0.8	26.2	25.4
15	5.4	36.2	30.8
16	5.4	28.8	23.4
17	0.2	15.8	15.6
20	2.2	21.4	19.2
33	2.6	35.6	33.0

[a] Clean 5.5-cm disks of Whatman No. 1 filter paper.
[b] Similar disk removed from cage containing one female.
[c] Treatment minus control.

response at day 18 may be related in some way to the egg maturation cycle, but may only reflect the small sample size ($N = 9$). No attempt was made to correlate emission levels with ovarian development or production of oöthecae.

Table 1 shows the level of male activity elicited by papers from a typical female. Note that fluctuations in the basal activity rates are reflected to some degree by correspondingly higher or lower activity in the treatment and that the sum of the treatment minus control figures for days 3–8 is close to zero (0.2). This implies that no sex pheromone is present or that the level is very low and suggests that the order in which the filter papers are presented is not a major source of bias. A series of experiments in which the usual control was followed by a second blank filter paper instead of a disk impregnated with pheromone extract showed that the order of presentation made no significant difference.

It is evident from the data that pheromone emission is initiated by some females as early as the 9th or 10th day after the adult molt and that over

90% of the females tested had begun emission by day 16. The onset of emission is generally quite abrupt and marked by a sharp increase in male activity. Thus, males encountering virgin females less than 9 days old are not likely to be stimulated to a level sufficient to result in the full courtship display. The likelihood that a given female will be attractive to males increases until about 20 days after the molt.

In another series of experiments, 24 virgin females 10–42 days old were tested for cyclic emission of sex pheromone associated with the photocycle. The filter papers in the beakers housing the females were changed every 12 hr at the end of the light and dark phases. Male response to the two papers for each female was compared using Wilcoxon's signed ranks test. Although there appears to be a great deal of variation in pheromone emission between females and between the light and dark phases for a given female, there was no evidence of cyclic emission, and the overall differences were not significant.

Pheromone Concentrations and the Photocycle

Butz and Aronoff (1970) reported that responses of the male *P. americana* to sex pheromone correlated positively with the circadian rhythm of activity, but they tested only one concentration of the pheromone. In order to determine if various concentrations elicit different locomotory responses and courtship behavior during different portions of the photocycle, males were tested with undiluted sex pheromone extract and with a fraction diluted to 10^{-3}. The extract is a mixture of Periplanone A and Periplanone B as described by Persoons et al. (1974).

Males exposed to 5 μl of the undiluted extract (6.2×10^{-4} g) showed a significant increase in locomotory activity during both phases of the photocycle ($P < 0.05$; Mann-Whitney U test). Running activity was 20–30 times higher than the activity in the controls, with no significant difference between the light and dark phases (Figure 2). Exposure to the undiluted extract also resulted in wing-raising, abdominal extensions, and backing during both phases. These courting displays occurred in 35–100% of the males with no apparent difference between the light and dark phases. Backing and abdominal extensions are probably due in part to tactile stimuli provided by the other males in the group, but these behaviors do not occur at low concentrations. Males were aroused by sex pheromone and performed complete behavioral displays, even during the light phase (Figure 3), but neither the incidence of terminal courting displays nor the increase in locomotory activity is correlated with the circadian rhythm of activity.

Males exposed to 5 μl of pheromone extract diluted to 10^{-3} (6.2×10^{-7} g) showed no significant increase in activity during the light phase or

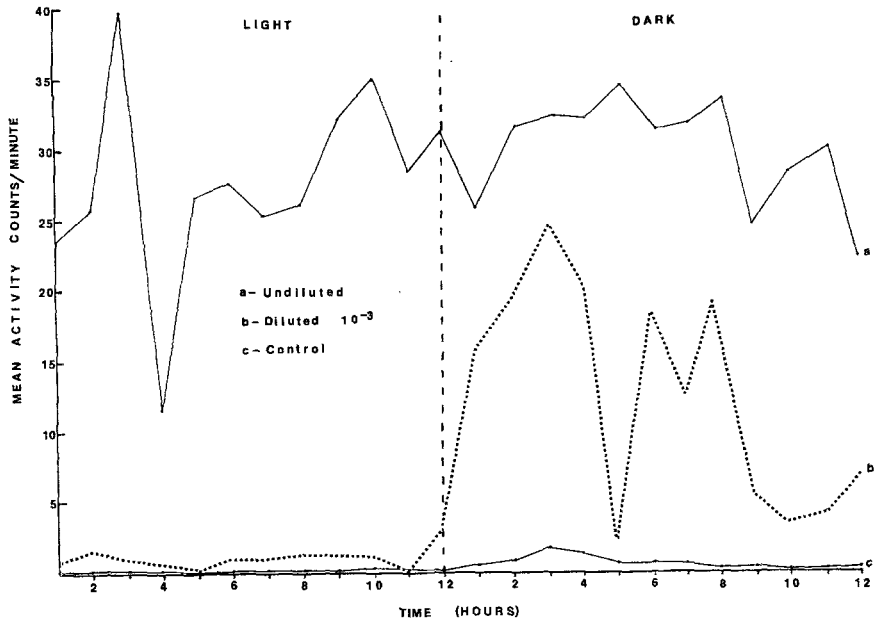

FIG. 2. The relationship between the photocycle and the level of male activity elicited by two different concentrations of sex pheromone extract. Five groups of 5 males/hr for a and b; 20 groups of 5 males/hr for c.

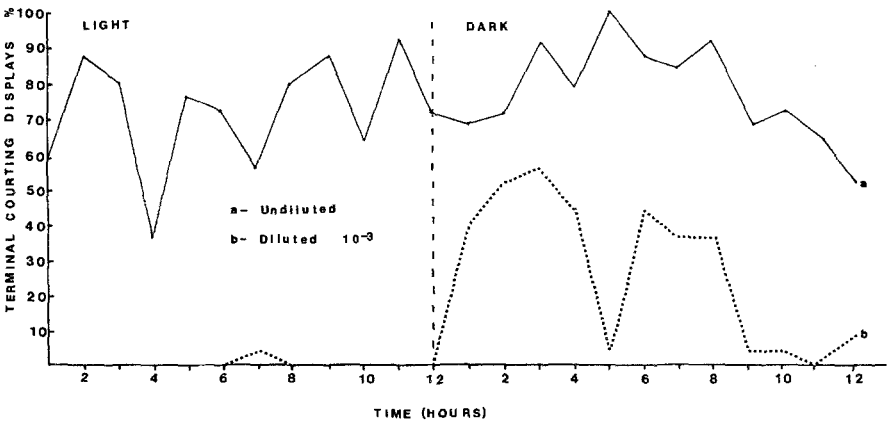

FIG. 3. The relationship between the photocycle and the percentage of males exhibiting terminal courtship displays in response to two different concentrations of sex pheromone extract. Five groups of 5 males/hr for each concentration.

during hours 5 and 10 of the dark phase. At the onset of the dark cycle, activity increased very rapidly and remained rather high through the first 8 hr, except during hour 5 in which the males failed to respond (Figure 2). Male activity throughout the dark phase was significantly higher than basal levels ($P < 0.05$; Wilcoxon's signed ranks test). The percentage of males exhibiting wing-raising, abdominal extension, and backing movements increased rapidly at the onset of the dark phase. These displays were most frequent during the third hour of the dark phase when almost 60% of the males responded. With the exception of hour 5, the frequency remained above 30% for the first 8 hr and dropped off quickly after that. Terminal courting displays rarely occurred during the light phase (Figure 3).

Irregular Distribution of Sex Pheromone

It is unrealistic to assume that sex pheromone is transported in a continuous gradient over long distances under natural conditions. Air movements may drastically alter the diffusion of the pheromone, creating a mosaic pattern of varying concentrations. As males pass through such a nonuniform field, they are likely to encounter pockets of air carrying sex pheromone. If they are to extract any useful information from this mosaic, they must be able to respond and then recover quickly to permit responsiveness upon their next contact with the pheromone.

To determine the rapidity of response and the rate of recovery, groups of 5 males were exposed to a filter paper treated with 6.2×10^{-4} g of sex pheromone extract for 30 sec and the rate of activity measured for 32 min. The sex pheromone released from the filter paper during this period was not evacuated from the cage. Tests were conducted during hours 3 and 4 of the dark phase of the photocycle. After the 30-sec exposure, locomotory activity was 8–9 times higher than the basal rate through the first 8 min (Figure 4). It gradually declined until it was only 2–3 times higher than the basal rate 20 min into the test. Initially, males responded to the pheromone with increased, frenzied locomotion, but this response diminished quickly within the first 16 min.

To simulate conditions in which males encounter pheromone in nonuniform patterns, males were exposed to the extract in periodic pulses. After an initial 30-sec exposure as explained above, activity was recorded for 16 min. The males were then given another 30-sec exposure and the activity recorded for another 16 min. After the first exposure, the males showed an initial high rate of activity which gradually decreased to near the basal level by the end of the 16 min. After the second exposure, running activity increased to a level not significantly different from that observed immediately after the first. The level of activity did not decrease as rapidly, however. The

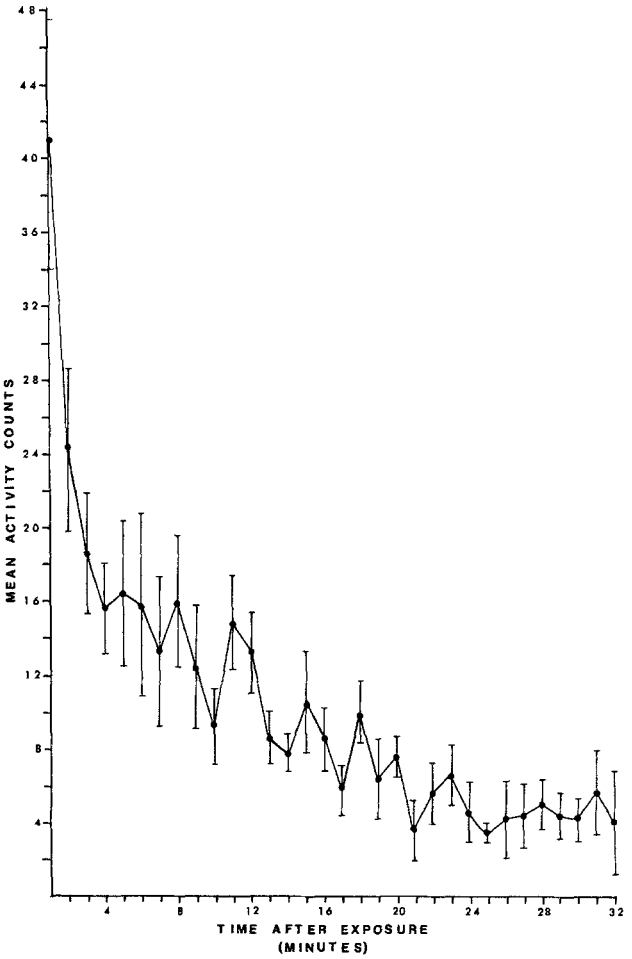

FIG. 4. Male activity elicited by a 30-sec pulse of sex pheromone extract. Dark dots represent means; vertical lines represent standard errors of the means ($N = 5$ groups of 5 males).

activity level 8 min after the second exposure was significantly higher than that at the same point after the first ($P < 0.05$; Mann-Whitney U test). After each of the two exposures to pheromone, males responded with rapid increases in locomotion followed by a period of rapid decrease in activity. The level of activity during the last 8 min of the test remained higher after the second exposure (Figure 5).

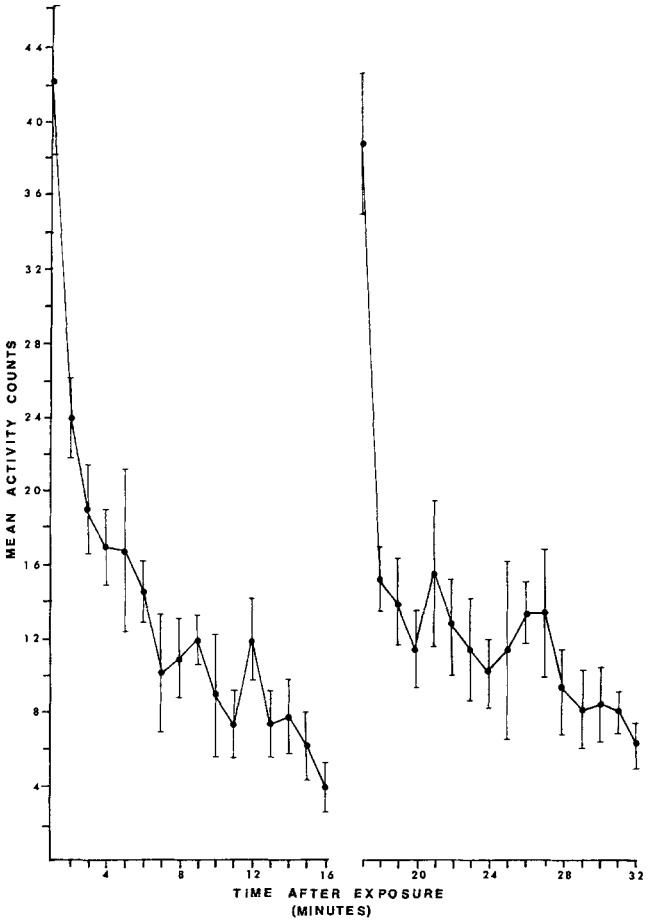

FIG. 5. Male activity elicited by two consecutive 30 sec pulses of sex pheromone extract ($N = 5$ groups of 5 males). Refer to Figure 4 for explanation of symbols.

In a similar experiment, males were exposed to four 30-sec pulses of sex pheromone separated by 8-min recovery periods. The males responded with a rapid increase in locomotion after each pulse (Figure 6). Apparently, *P. americana* males are able to respond quickly and then recover in order to be ready to respond to the next stimulus—even if it is presented as soon as 8 min afterwards. This brief recovery period is in marked contrast to the longer times required by other animals. For example, the cockroach *Byrsotria fumigata* requires 4–5 days (Bell et al., 1974).

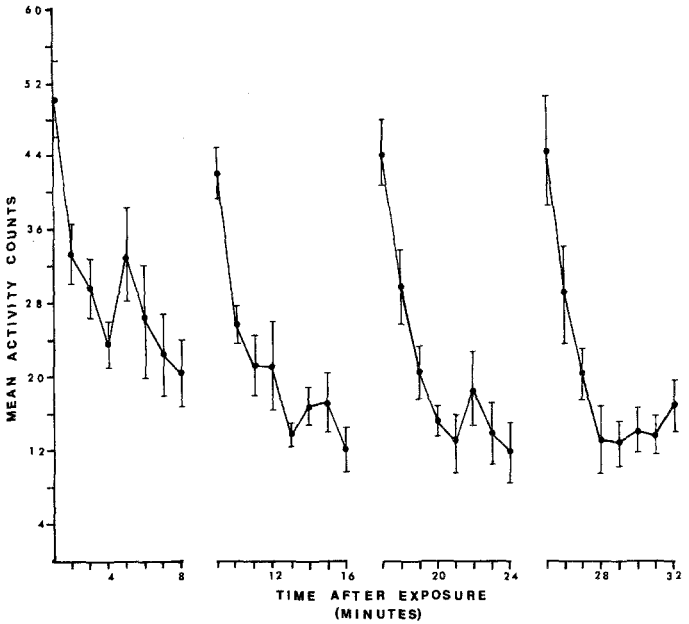

FIG. 6. Male activity elicited by four consecutive 30 sec pulses of sex pheromone extract ($N = 5$ groups of 5 males). Refer to Figure 4 for explanation of symbols.

Prolonged Exposure to Sex Pheromone

In order to determine the extent to which continuous exposure causes decreases in responsiveness, 5 groups of 5 males each were exposed to sex pheromone extract (6.2×10^{-4} g) for a period of 32 min. Another 5 groups were exposed for 16 min after which the amount of pheromone was doubled, and the males were exposed for another 16 min.

Males that were exposed continuously to one concentration had a very high rate of locomotory activity initially, 15–20 times higher than the basal rate. The rate of activity declined through the first 10 min of exposure but remained fairly constant thereafter. The activity in the final 22 min was still significantly greater than the basal rate ($P<0.05$). The activity curve is similar to that for males exposed to a single 30-sec pulse of sex pheromone except that the rate of activity is considerably higher (Figure 4).

In the second group, the initial response was similar, but when the amount of pheromone was doubled, the level of activity increased slightly, but only for the first minute. This increase was not significant, and the absence of an increase suggests that habituation (or sensory adaptation) had occurred (Figure 7).

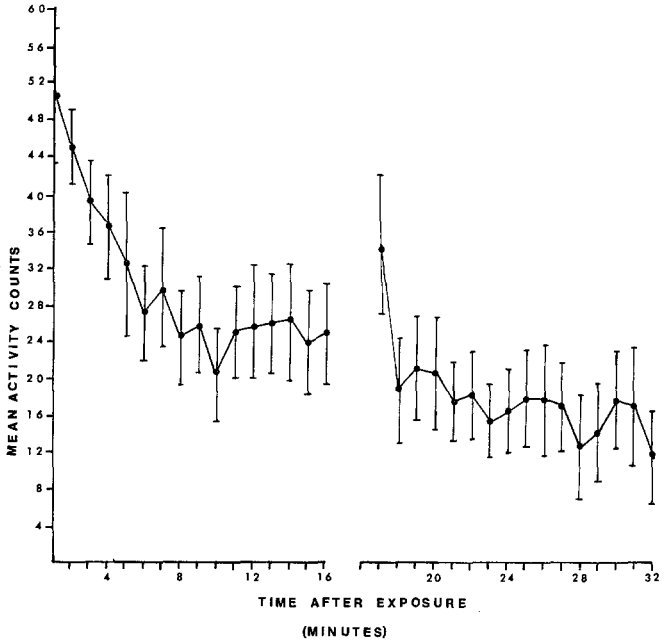

FIG. 7. Male activity elicited by continuous exposure to sex pheromone extract, doubling the amount of pheromone after 16 min (N = 5 groups of 5 males). Refer to Figure 4 for explanation of symbols.

To determine the long-term effects on male responsiveness, males were continuously exposed to sex pheromone for period of 32, 64, 96, or 120 min. Twenty-four hours later, they were exposed to the same concentration of pheromone, and the activity was recorded for 5 min. Males that had been unexposed for 120 hr served as controls. The males that had been exposed 24 hr earlier were significantly less active than the controls ($P < 0.005$; Kruskal-Wallis test). The actual length of prior exposure had little apparent effect. Although the level of activity for previously exposed males was lower than that in the controls, it was still 4–5 times higher than the basal rate (Figure 8).

DISCUSSION

In *Periplaneta americana*, the way in which a male responds to a given female is influenced to a large degree by her physiological state. Recently emerged adult females do not emit sex pheromone and are known to be

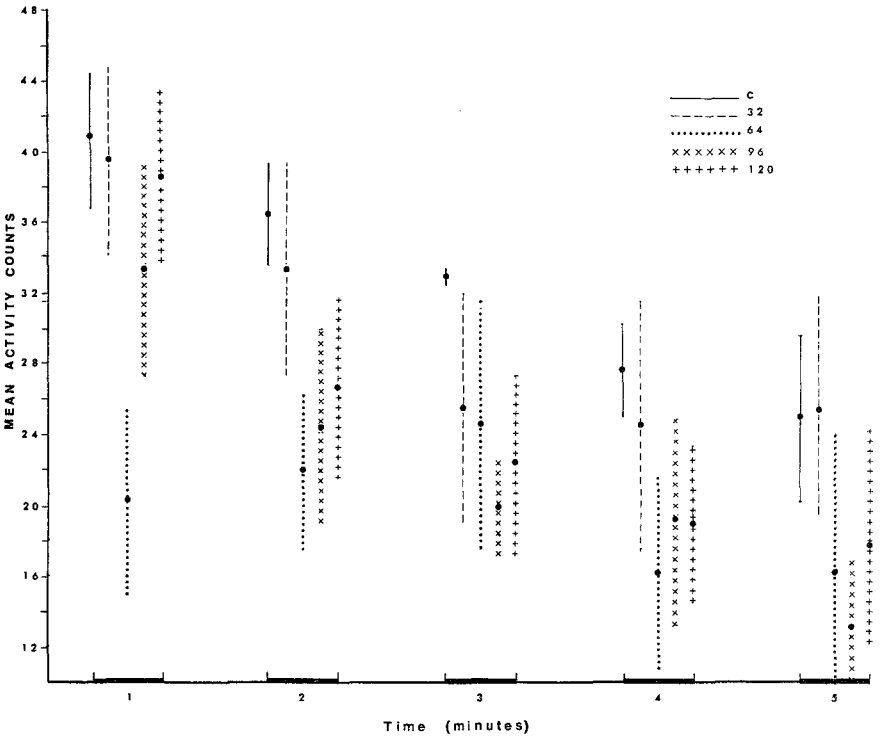

FIG. 8. Male activity elicited by exposure to sex pheromone extract 24 hr after exposure for 32, 64, 96, and 120 min. Dark dots represent means; vertical lines and symbols represent standard errors of the means ($N = 5$ groups of 5 males/exposure period).

unreceptive; mated females emit little or no pheromone and rarely mate a second time (Wharton and Wharton, 1957). Only adult virgin females 9 or more days old emit sex pheromone in sufficient quantities to elicit terminal courting displays. Thus, males encountering very young or previously mated females or virgin females whose pheromone emission is very low are unlikely to perform a complete courtship display.

The influences of the photocycle on male responsiveness to sex pheromone may limit mating encounters to the dark phase. Lipton and Sutherland (1970) reported that the activity of *P. americana* females appeared to be influenced by the reproductive cycle and did not display a detectable circadian rhythm. Males do have a well-defined circadian rhythm of activity and will not respond to sex pheromone during the light phase unless high concentrations are present. Additionally, Rust (1976) has shown that their

sequential behavioral responses are organized by increasing concentrations of sex pheromone. Therefore, matings are most likely to occur during the dark phase.

P. americana sex pheromone is transported by air currents and by diffusion. Numerous obstacles in the cockroach's habitat deflect air currents, preventing the formation of stable concentration gradients and resulting in increasingly irregular spatial distributions of the pheromone as the distance from the source increases. Therefore, males are likely to periodically encounter pockets of air carrying pheromone that will yield no useful directional information but will elicit the characteristic increase in locomotory activity. Males thus stimulated will soon run out of this air pocket and must recover quickly to permit response the next time sex pheromone is encountered. Otherwise, they may pass through an undisturbed concentration gradient which could lead them to a receptive female and fail to respond. Our tests indicate that males are able to respond to a second pheromone stimulus within 8 min after initial stimulation. This is an exceptionally brief recovery period.

If male cockroaches were often exposed to sex pheromone for prolonged periods of time and such an exposure produced a major long-term decline in responsiveness, they might not respond to a receptive female encountered soon afterward. Males tested 24 hours after 32-, 64-, 96-, or 120-min exposure to sex pheromone showed significantly lower activity than did control animals. While males are able to recover very quickly from the effects of brief exposures, prolonged exposures to sex pheromone result in habituation/sensory adaptation which substantially lowers male response for at least 24 hr. This suggests that prolonged exposures are not common in nature and that there has been little selective pressure against this effect.

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BENZOYL CYANIDE AND MANDELONITRILE BENZOATE IN THE DEFENSIVE SECRETIONS OF MILLIPEDES

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Abstract—Analyses of the defensive secretions of 17 species of polydesmoid millipedes show that other chemicals besides HCN and benzaldehyde are liberated during cyanogenesis. Several members of the families Polydesmidae, Paradoxosomatidae, and Euryuridae are shown to secrete both phenol and guaiacol, with one paradoxosomatid also producing ethyl benzoate and benzoic acid. Also, members of the family Xystodesmidae commonly produce the three following compounds: benozoic acid, mandelonitrile benzoate, and benzoyl cyanide. Benzoyl cyanide has not been found previously as a natural product. The defensive role of these additional natural products as antipredator and antibiotic agents is discussed. For certain predators benzoyl cyanide in particular seems to possess anaesthetic properties. Our studies provide an initial chemotaxonomic basis for distinguishing between various polydesmoid taxa.

Key Words—cyanogenesis, polydesmoid millipedes, phenol, guaiacol, benzoyl cyanide, mandelonitrile benzoate, ethyl benzoate, defense, antibiosis.

INTRODUCTION

The chemical nature of the defensive secretions of the various orders of millipedes has been relatively unexplored compared to the incomplete but

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more detailed knowledge of the defensive chemistry of insects (Eisner, 1970; Roth and Eisner, 1962; Weatherston and Percy, 1970). Besides the most commonly encountered defenses—HCN and benzaldehyde (Eisner, 1970; Eisner et al., 1975)—very few additional compounds accompanying cyanogenesis in polydesmids have been identified. Several millipedes such as *Orthomorpha coarctata* (De Saussure) and *Euryurus leachii* (Gray) have been shown to contain phenol and guaiacol as well (Duffield et al., 1974). In isolated incidences *p*-isopropylmandelonitrile glucoside (Pallares, 1946), benzoic acid, and mandelonitrile benzoate (Casnati et al., 1963) have been shown to occur in the defensive secretions of *Polydesmus collaris collaris* (Koch) and *P. vicinus* L., respectively.

In this paper we document the occurrence of several unique millipede natural products, as well as demonstrate that benzoic acid and mandelonitrile benzoate (Casnati et al., 1963) are common features of the family Xystodesmidae. The chemotaxonomic significance of our findings is discussed. Polydesmoid millipedes are thus shown to have a considerably greater diversity of defensive chemicals than has been previously documented.

METHODS AND MATERIALS

Acquisition and Maintenance of Live Specimens

The majority of the millipedes were collected in the vicinity of Athens, Georgia, or Georgian and North Carolinian Appalachians. Larval phengodid beetles (*Phenogodes* sp.) were collected at Athens, Georgia. The specimens of the polydesmoid genus *Motyxia* and the phengodid larvae *Zarhipis integripennis* (LeConte) were collected in Kern County, California. All living specimens were kept as previously described (Duffey et al., 1974).

Collection and Analysis of Defensive Secretions

Live millipedes (2–30 individuals of a species) were milked of their defensive secretions by persistently rubbing their phlanges with small balls of Kim-Wip® held by forceps. Contamination with fecal material was thus avoided. These wipes were stored in a minimal volume of mass spectrographic grade methylene dichloride, containing anhydrous sodium sulfate, until analysis. The secretions were analyzed by a GLC-MS interface using 10% SP-1000 as the liquid phase with a temperature program (70–250°C at 10°C min).

The presence of HCN as a defensive secretion was detected by several qualitative means (Eisner et al., 1963b; Towers et al., 1972). Also the presence of phenol and guaiacol in the defensive secretions was monitored by

thin-layer chromatography on silica gel using petroleum ether–acetone (50:1) as the solvent. These phenolics were detected by spraying the developed chromatogram with an ethanolic solution of 2,6-dichloroquinone-4-chloroimide followed by base (Booth and Boyland, 1958). Phenol turns turquoise and guaiacol blue with this reagent. Various other phenolics also give distinctive colors with this detective system.

Chemical Synthesis

Benzoyl cyanide was purchased from Aldrich Chemical Co. In order to verify the existence of this compound in millipede secretions, both the commercial and the natural compounds were treated with methoxyamine hydrochloride in the presence of pyridine. This process converted benzoyl cyanide to *N*-methoxylenzamide.

Mandelonitrile benzoate was synthesized from a mixture of benzaldehyde, KCN, and benzoyl chloride as outlined by Holm (1965).

Repellency Studies

The reaction of two ant species (*Pogonomyrmex badius* and *Formica rufa*) to the presence of millipede defensive compounds was determined by presenting different concentrations of the test chemicals as suspensions in drops of 50% aqueous honey solution. These baits were placed on the foraging platforms of the ants, and the reactions of the workers were observed for up to 30 min. Six replicates were tested for each concentration, and no colony was tested more than once during each 24-hr period.

RESULTS AND DISCUSSION

Fewer than 20 species of Polydesmidae have been investigated for cyanogenic capabilities, and in most instances the only products to be identified were HCN and benzaldehyde (Eisner et al., 1963a, 1975; Blum and Woodring, 1962; Duffey et al., 1974). However, some studies have shown the existence of additional compounds in the defensive secretions; *p*-isopropylmandelonitrile glucoside (Pallares, 1946), mandelonitrile benzoate (Casnati et al., 1963), and phenol and/or guaiacol (Monteiro, 1961; Blum et al., 1973; Duffield et al., 1974). Unfortunately, the studies on *p*-isopropylmandelonitrile and mandelonitrile benzoate do not indicate whether these components are major or minor contributors to cyanogenesis. In the case of phenol and guaiacol, these compounds are present up to 2% of the total secretion. With the above observations as precedent, it seems strange that

TABLE 1. DISTRIBUTION OF DEFENSIVE COMPOUNDS IN VARIOUS CYANOGENIC MILLIPEDES

Millipede—Family, species	φCHO[a,b]	φCOCN[a]	φCOOH[a]	φMANDBZ[c]	ETOHBZ[c]	φOH[c]	GUAI[d]
Polydesmidae							
Pseudopolydesmus erasus (Loomis)	++++			++		+[b,e]	+[b]
Paradoxosomatidae							
Orthomorpha coarctata (De Saussure)	P[g]						P
Oxidus gracilis (Koch)	++++		+	+[b]	+	+	Tr[b]
Euryuridae							
Euryurus australis (De Saussure)	P						P
Euryurus leachii (Gray)	P						P
Euryurus maculatus (Koch)	++++			++[b]		+	+
Xystodesmidae							
Brachoria sp.	++++	++	+	++			
Cherokia georgiana ducilla (Chamberlin)	++++	++	++	+[b]			
Cherokia georgiana georgiana (Bollman)	++++[b]	++[b]	+[b]	+[b]			
Cherokia georgiana latassa Hoffman	++++	++	++++	+			
Cleptoria rileyi (Bollman)	++++	+	+	++			
Motyxia tiemanni Causay	++	++	+++	++			
Motyxia tularea (Chamberlin)	++++	+[b]		+++			
Motyxia sequoiae (Loomis & Davenport)	++++	+[b]	+[b]	++			
Paimoikia sp.	++++	o[f]					
Sigiria sp.	++++	++					
Sigmoria nantahalae Hoffman	++++	++++	++++				
Stelgipus agrestis Loomis	+	+[b]					

[a] φCHO = benzaldehyde; φCOOH = benzoic acid; φCOCN = benzoyl cyanide.
[b] Presence detected only by GLC on 3% OV-25 at 80°C/min temperature program.
[c] MANDBZ = mandelonitrile benzoate; ETOHBZ = ethyl benzoate; φOH = phenol.
[d] GUAI = guaiacol; phenol and guaiacol also detected by TLC on silica gel, Solvent petroleum ether–acetone (50:1), sprayed with methanolic 2,6-dichloroquinone-4-chloroimide followed by aqueous NaOH; phenol turns turquoise, guaiacol turns blue. Analysis based on pooled samples of 10–30 specimens per species except for *S. nantahalae* and *S. agrestis* where 2 specimens each used. ++++ ≳50%, +++, 25–50%, ++, 5–25%, + ≲5%.
[e] = presence of compound analyzed by GLC–MS interface using 10% SP-1000 initially at 70°C with 10°C/min. temperature program.
[f] o = odor of benzoyl cyanide perceptible; tr = trace.
[g] P = presence determined by other authorities cited in text.

the presence of the above compounds or additional chemicals is not found to be more prevalent. In fact, such millipedes as *Oxidus gracilis* (Gudensteeden-Egeling, 1882; Eisner et al., 1963a) and *Cherokia georgiana* (Bollman) (Eisner et al., 1963a) are described as smelling like bitter almonds (HCN and benzaldehyde); however, while collecting endemic and introduced millipedes of the Appalachian regions, we noted that many of the forms had initial odors which overpowered the bitter almond odor. In the case of *Oxidus gracilis*, the additional odorous component was identified as phenol (Blum et al., 1973). However, in the case of a xystodesmid like *Cherokia georgiana*, the presence of compounds besides benzaldehyde and HCN was clearly indicated. Furthermore, since the odors of many members of the families Paradoxosomatidae, Xystodesmidae, Polydesmidae, and Euryuridae were often distinctive, we decided to investigate the nature of these odorous components.

All millipedes investigated (Table 1) produced HCN and benzaldehyde. *Oxidus gracilis*, *Cherokia georgiana* (subsp. ?) (Eisner et al., 1963a), and *Motyxia* (= *Luminodesmus*) *sequoiae* (Loomis and Davenport) (Davenport et al., 1952) have been previously studied for cyanogenesis. Benzaldehyde was confirmed by GLC–MS analyses to be a major component of the defensive secretion of all millipedes, as indicated by the semiquantitative indices of $+++\geq 50\%$, $+++ = 25$–50%, $++ = 5$–25%, $+ \geq 5\%$ (Table 1). All millipedes, except *Paimoikia*, were found to produce either traces or significant levels of other compounds in their defensive secretions.

For example, in species of the family Xystodesmidae, the presence of benzoyl cyanide has been found to be a uniform chemical character (Table 1), representing up to 30% of the secretion in forms like *Sigmoria nantahalae* Hoffman and *Cherokia georgiana georgiana* (Bollman). In most Xystodesmid millipedes it constituted about 10% of the total secretion, but even in the secretion of *Motyxia sequioae*, where the level of this compound was about 1%, a very distinctive and irritating odor was imparted to the defensive secretion. On the other hand, benzoyl cyanide was never detected in any of the polydesmids, euryurids, or paradoxosomatids investigated.

The identification of benzoyl cyanide, previously undocumented as a biological product, was based on GLC–MS comparison with the synthetic chemical. The retention times and mass spectra of the natural and synthetic products were found to be identical. The ion (m/e = 131, 53%) gave rise, by loss of CN, to $C_6H_5CO^+$ (m/e = 105, 100%). This ion underwent the expected losses of CO (metastable at m/e 65.6), followed by acetylene (metastable at m/e 33.8) to produce the ion at m/e 51 ($C_4H_3^+$). Further characterization of benzoyl cyanide was derived by treating either an authentic sample or the millipede secretion with methoxyamine hydrochloride in the presence

of pyridine. This resulted in the conversion of the nitrile to *N*-methoxybenzamide (M^+ = *m/e* 151, 20%) as expected for α-ketonitriles (Dornow and Theidel, 1954).

It was also found that *O. gracilis*, and two thirds of the species of Xystodesmidae investigated, had benzoic acid in their secretions. In many cases, such as *C. g. latassa* (Table 1), benzoic acid represented about 40% of the secretion, and was clearly a prominent component (+) in the secretions of the other millipedes. Benzoic acid was probably not observed in the exudates of *P. erasus*, *E. maculatus* (Koch), *Sigiria* sp., *Paimoikia* sp., and *M. tularea* (Chamberlin) because of the limited amount of sample available in each case. Benzoic acid definitely does not arise from the oxidation of benzaldehyde during cyanogenesis, or during storage of the defensive material prior to analysis. Fresh secretions of various millipedes analyzed immediately after cyanogenesis by GLC revealed that similar proportions of benzoic acid and benzaldehyde were present as those found in stored samples which were later analyzed by GLC–MS. In fact, by carefully dissecting out several storage vestibules (void of the lyase present in the reaction chamber) of *C. g. georgiana*, it could be shown by GLC analysis that a large quantity of benzoic acid was present even before mandelonitrile degradation. The presence of benzoic acid in the defensive secretion has been documented in only two other millipedes, *Orthomorpha coarctata* (Monteiro, 1961) and *Gomphodesmus pavani* (Casnati et al., 1963).

The isolation of benzoic acid is also biochemically consistent with the presence of the benzoic acid ester of mandelonitrile in the secretions of 75% of the millipedes. Again, it was found that mandelonitrile benzoate was a prominent constituent of the defensive secretion, approximately 35% in *M. tularea*. In other millipedes it occurred at the level of 10% or less. In some of the Xystodesmidae this chemical was not detected; we feel this is because sufficient amounts of defensive secretion were not available to detect its presence. Identification of mandelonitrile benzoate was based upon congruent GLC retention time comparisons and mass spectral comparisons with a synthetic sample. Also, the mass spectrum was in excellent agreement with that presented in the original identification of this chemical in *Polydesmus collaris collaris* (Casnati et al., 1963). Since the time of the original identification, this chemical has remained undetected. In fact, the validity of the natural occurrence of mandelonitrile benzoate in the secretion was retracted (Barbetta et al., 1966) because it was considered to be an artifact of the isolation techniques. This is not a possibility with our analytical method since fresh glandular secretion was examined.

Only one millipede, *O. gracilis*, was found to contain ethyl benzoate (M^+ = 150, 32%) previously undetected in arthropod secretions. Its identification was based on GLC–MS congruency with a synthetic standard. The

lack of this chemical in all other millipedes may point to this being a unique character of *O. gracilis*.

Phenol (ϕOH) and/or guaiacol (GUAI) have been previously identified in the defensive secretions of the following millipedes (Table 1): *O. gracilis* (ϕOH), *Euryurus leachii* (Gray), *E. australis* (De Saussure) (GUAI), and *Orthomorpha coarctata* (ϕOH, GUAI) (Blum et al., 1973; Duffield et al., 1974). By our present analyses, employing both GLC–MS and TLC (silica gel, petroleum ether–acetone (50:1) sprayed with 2,6-dichloroquinone-4-chloroimide, followed by base), we have demonstrated that *O. gracilis*, *P. erasus* (Loomis), and *E. maculatus* (Koch) secrete both phenol and guaiacol. Reliance strictly on conventional GLC methods often makes it difficult to demonstrate the existence of trace components such as guaiacol, unless a large number of animals is available. Analyses of the secretions of *C. g. georgiana* and *C. rileyi* failed to show the existence of any components similar to phenol and guaiacol. This suggests that the presence of phenol and/or guaiacol may be eventually valuable as a chemotaxonomic character of the polydesmid, euryurid, paradoxosomatid, and related families (this does not imply these particular families are related).

The distribution data of the occurrence of benzoyl cyanide, ethyl benzoate, phenol, and guaiacol (Figure 1) in the four Appalachian diplopod families (Table 1) are suggestive of a possible basis for chemotaxonomic distinctions. Our data show that the major products of the cyanogenic gland in Xystodesmids are distinguishable from those of the other three families investigated. However, we have only investigated a few members from 4 of

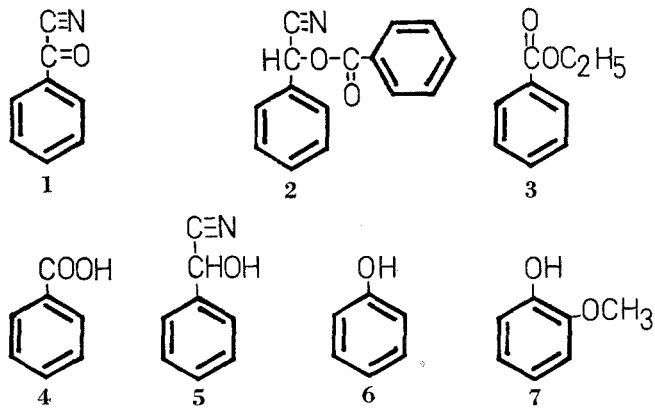

FIG. 1. Defensive compounds of polydesmid millipedes: 1, benzoyl cyanide; 2, mandelonitrile benzoate; 3, ethyl benzoate; 4, benzoic acid; 5, mandelonitrile; 6, phenol, 7, guaiacol.

the some 20 polydesmoid families in the world. On the other hand, chemical differences or similarities may in future work be observed with no apparent taxonomic relevance. This appears to be the case for our data in which the paradoxosomatids, euryurids, and polydesmids are chemically most similar but taxonomically very distinct and unrelated (Hoffman, 1976). In this case, the chemical similarities probably reflect some environmental selection factors producing chemical congruence. This brings to question the various roles of the multitude of chemicals present in the defensive secretions of these various millipedes.

The most obvious role, invariably invoked for most defensive secretions, is that of deterring predators such as ants, lizards, birds, etc. (Blum and Woodring, 1962; Blum et al., 1973; Eisner, 1970). In our own somewhat artificial trials it was found that synthetic mandelonitrile, which degrades rapidly to HCN and benzaldehyde, was an effective deterrent against ants (*Pogonomyrmex badius* and *Formica rufa*) when placed on mealworm larvae. Benzaldehyde alone was less effective. When live specimens of *O. gracilis*, *C. georgiana* subsp., and *Cleptoria rileyi* (Bollman) were presented to these ants, the secretion of the cyanogenic glands was a major factor in blunting the formicid attack and enabling the millipedes to escape. The presence of benzoyl cyanide in the defensive secretion of xystodesmid millipedes adds a new dimension to chemical resistance to predators because benzoyl cyanide has the ability to initially highly excite the ants and derange the normal attack behavior of these insects. Several minutes exposure to the fumes (1 ml saturated air diluted to 1 liter) causes a knockdown response in ant workers and/or a dysfunction of the antennae. The olfactory sensitivity of the ants appears to be reduced in that the ability of the formicids to respond properly to their alarm pheromones (formic acid for *F. rufa*, and 4-methyl-3-heptanone for *P. badius*) is impaired. Also, after several minutes of exposure, the ants carry their antennae crossed above their heads, a highly abnormal response. If given longer exposures to the fumes (3–5 min), the ants collapse; eventually, after several hours in the absence of benzoyl cyanide, they recover from the apparent anesthetic effect. Workers of *F. rufa* are also highly sensitive to this chemical, and since they occur in the same areas as many of the millipedes investigated, this chemical may be highly adaptive in deterring attacks of these ants. Benzoyl cyanide is also a highly reactive molecule chemically; it has the ability to benzoylate OH, NH, and presumably SH groups (Dornow and Theidel, 1954). It may partially exert its effect by interacting chemically or physically with the predator's receptive mechanisms.

The addition of either benzoyl cyanide, guaiacol, or phenol to droplets of aqueous honey solution resulted in repellency of ants at molar concentrations ranging from 10^{-7} to 10^{-8}. Benzoyl cyanide was a very effective repellent (10^{-7}–10^{-8}) for workers of *Pogonomyrmex badius* and at higher

concentrations the ants exhibited frequent cleansing behavior while moving rapidly and erratically without orienting to the bait source. Both of the phenolics repelled the ants at a molar concentration of 10^{-7} and, as in the case of benzoyl cyanide, the excited workers performed frequent cleansing movements. Essentially the same behavioral repertoire was observed by workers of *Formica rufa*, which avoided the fortified baits at a molar concentration of 10^{-7} for all three compounds.

The physical properties of phenol, guaiacol, and benzoyl cyanide are highly suited for defensive purposes, for all compounds are moderately volatile, liquid in the biological state, and amphiphilic so as to be able to dissolve readily in tissues. This ability to associate readily with biological fluids or tissues is presumably an important asset in their effectiveness as deterrents. We feel that the basis of their defensive effectiveness lies in their potential to interact physically with the olfactory and/or gustatory organs of the predator with the result being a general disturbance of the acuity of perception of the predator. This is similar to the "jamming response" suggested by Blum (1974). Inducing a perceptive blockage in the predator would permit the prey more time to escape. If a predator's attack is primarily based on tactile and olfactory cues (ants), then an encounter with a component like benzoyl cyanide could essentially "blind" the antagonist. To the human nose, eyes, and mouth, benzoyl cyanide is extremely irritating (highly reactive and lachrymatory like benzoyl chloride), and it is easy to envisage a bird, lizard, or small mammal highly repulsed by this chemical.

Large predators are not the only environmental stresses placed upon millipedes. Millipedes live within the top soil and litter layers of the forest floor, and presumably must be subject to attack by fungi and bacteria. Preliminary results (unpublished data) on the antibiotic properties of phenol, guaiacol, benzoic acid, and benzoyl cyanide indicate that these compounds have the ability to inhibit spore germination and radial growth of several types of fungi (*Penicillium*, *Trichoderma*, *Cladosporium*) and bacteria at a concentration of about 1×10^{-4} mole/liter. This evidence strongly implicates these compounds as being involved in defense of the external cuticle and particularly the enzyme solution in the reaction chamber against microorganisms.

The question arises as to why some millipedes like *O. gracilis* have high concentrations of phenolics, phenol, and/or guaiacol, in the reaction chamber. It is to be recalled that the cyanogenic gland of millipedes is comprised of two chambers: the storage chamber which holds mandelonitrile and additives, and a reaction chamber which contains an aqueous enzyme solution responsible for the degradation of mandelonitrile (Blum and Woodring, 1962; Eisner et al., 1963b). We propose the following two hypotheses: (1) These chemicals will be effective as antibiotics to prevent growth of bacteria or

fungi in the enzyme solution, and probably on the cuticular surface as well, and (2) these chemicals will provide an effusion (vapor) pressure in the open pore of the reaction chamber such that the entry of small spores or fragments of organic matter contaminated with microorganisms will be somewhat prevented. This hypothesis is feasible considering the small size of the cyanogenic glands. In *O. gracilis*, the pore canal, approximately 30×60 μm, essentially focuses the vapor pressure of the phenol escaping to an infinitely diluting environment. Small particles then will be bombarded from the pore area by a Brownian motion phenomenon. The vapor pressure arising from H_2O is not a likely candidate for this physical antibiosis; since the millipede lives in a virtually saturated environment, no gradient exists for the water vapor to flux from the pore. This hypothesis is applicable to other larger polydesmids as well, since we have found that larger millipedes restrict their

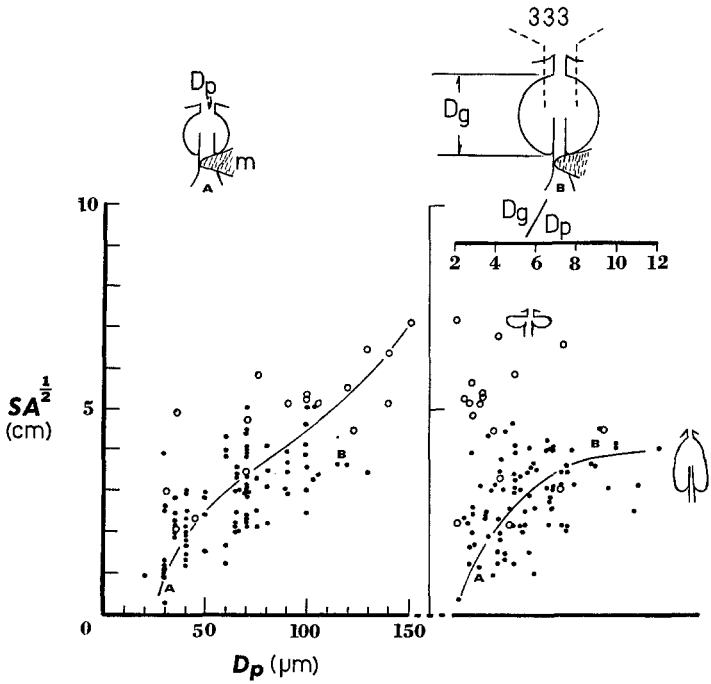

FIG. 2. The relationship of pore diameter to reaction chamber of the cyanogenic glandular reaction in various sized polydesmoid millipedes. $SA^{1/2}$ = surface area of millipede, Dp = diameter of external pore of reaction chamber; Dg = diameter of reaction chamber. (●) = members of Xystodesmidae, Euryuridae, Polydesmidae, and Paradoxosomatidae; (○) = members of Platyrhacidae, Oxydesmidae, Chelodesmidae, and Gomphodesmidae.

external pore diameter. By measuring the diameter of the pore (Dp) and comparing this with the diameter of the reaction chamber (Dg) (Figure 2), we have found that a small millipede like *O. gracilis* (1.5 × 0.25 cm, Dp = 30 μm, Dg = 100 μm) has Dg/Dp ratio equal to 3.3. Progressing through a series of larger species of millipedes to one like *Barydesmus dasypus*, the ratio becomes larger, Dg/Dp = 10 (Dg = 1000 μm and Dp = 100 μm). These observations (Figure 2) indicate that as the millipede species becomes larger, the pore size is restricted. Limiting the size of the pore diameter would be an effective means of maintaining a sufficiently high effusion pressure in even the largest millipedes to prevent entry of foreign microorganisms. If millipedes did not restrict the pore, it can be seen (Figure 2) that the diameter would be approximately 333 μm, 3 times larger in diameter, and considerably less effective in its bombardment potential.

It is noted (Figure 2) that species of the families Xystodesmidae, Polydesmidae, Euryuridae, and Paradoxosomatidae (●) are not generally as large as members of the Platyrhacidae, Oxydesmidae, Chelodesmidae, and Gomphodesmidae (○). Yet, these larger millipedes (○), although they have smaller Dg/Dp ratios, do not have excessively large external pores. This last group of millipedes tends to have glands that are flattened, hence lower Dg values; the former groups tend in the larger forms to have glands that are discoidal, hence large Dg values. In both cases the value of Dg can be considered to be limited for purposes of protecting the enzymic contents of the reaction chamber. In order for this mechanism to work in other millipedes which do not produce phenol and store it in the reaction chamber, a volatile chemical is required. Since the pH of the reaction-chamber fluid in several millipedes [*Harpaphe haydeniana* (Wood), *Cherokia georgiana georgiana; Cleptoria rileyi*, and *Sigmoria nantahalae*] has been found to be 4, we suggest that low-molecular-weight fatty acids could provide both the pH and the effusion potential. This possibility remains to be determined. However, Casanti et al. (1963) reported that the defensive secretion of *Polydesmus collaris collaris* contained formic, acetic, and isovaleric acids.

One of the more interesting aspects of polydesmid millipede biology is predation by phengodid beetle larvae. The larval phengodid is a voracious predator of both polydesmoid and spirobolid types of millipedes (Rivers, 1886; Tiemann, 1967). We observed larvae of both *Zarhipis integripennis* and *Phenogodes longicollis* to be effective predators upon *Atopetholus michelbacheri, Narceus americanus, N. annularis, Sigmoria nantahalae, Cherokia georgiana* subspp., and *Dicellarius atlanta*. Despite the liberation of copious amounts of benzoquinones, mandelonitrile, benzoyl cyanide, and other defensive attributes by the millipedes, the phengodids are undeterred. Perhaps, much of the ability of the beetle larva to persist in attack, while its whole exterior is coated in the millipede's defensive secretions, lies in the

extremely smooth surface of the beetle's cuticle. This cuticular surface has very low wettability by water or the defensive secretions. In addition, the beetle larvae might also keep their spiracles closed during the time of the attack. One might also suspect that the beetle larvae have some biochemical adaptations for feeding upon millipedes since they externally digest their prey (Tiemann, 1967). This digestion process often results in the rupture of the defensive storage organs, liberating either HCN and benzaldehyde or benzoquinones into the liquid food. This occurrence appears not to affect the phengodids. Indeed, besides starlings and toads (Cloudsley-Thompson, 1968), the phengodids appear to be one of the few obvious and well-described predators that have been able to overcome and prosper in the advent of two normally exceptionally effective deterrent mechanisms of millipedes, the liberation of either HCN–benzaldehyde (plus additives) or benzoquinones (plus additives).

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SEX PHEROMONE COMPONENTS AND CALLING BEHAVIOR OF THE FEMALE DERMESTID BEETLE, *Trogoderma variabile* BALLION (COLEOPTERA: DERMESTIDAE)[1]

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Abstract—Females of the dermestid beetle, *Trogoderma variabile* Ballion, exhibit a diel pattern of calling behavior. A potent sexual excitant and attractant for the male beetles can be collected on Porapak Q during aeration of female beetles, but cannot be detected in extracts of macerated females. The attractant has been identified as (Z)-14-methyl-8-hexadecenal. Of the five additional compounds previously identified as attractants in other *Trogoderma* species, only two were found; (Z)-14-methyl-8-hexadecen-1-ol is present in extracts of macerated female beetles, but not in extracts of Porapak Q, and γ-caprolactone is present in extracts of Porapak Q. Three collection procedures were necessary to ensure that all the pheromone components had been isolated. Synthetic (Z)-14-methyl-8-hexadecenal elicits attraction and sexual arousal in *T. variabile* males.

Key Words—Coleoptera, Dermestidae, *Trogoderma variabile*, pheromone, isolation, identification, (Z)-14-methyl-8-hexadecenal, aeration, Porapak Q, sex attractant.

[1] Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the USDA.
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INTRODUCTION

Trogoderma variabile Ballion (= *parabile*) was given the common name "warehouse beetle" by Okumura (1972), who regarded it as the next most serious dermestid pest after the khapra beetle, *T. granarium* Everts. *T. variabile* is often of major importance as a pest of stored foods, especially packaged, processed foods.

Adult *T. variabile* females produce a pheromone that excites and attracts the males (Burkholder, 1970). We describe here a study of the sex pheromone components that were collected on Porapak Q as female beetles were aerated. Although this technique led to the discovery of a potent new *Trogoderma* pheromone component (Cross et al., 1976), three of the five pheromone components previously found in other *Trogoderma* species (Rodin et al., 1969; Yarger et al., 1975) could not be detected in a Porapak extract. We therefore compared the Porapak extract with extracts of whole females and of the filter paper on which female beetles were held.

METHODS AND MATERIALS

Insects were reared according to the procedures of Hammack et al. (1973). The calling frequency of female adults was recorded by the procedure of Hammack et al. (1976). A total of 100 females (25 ♀/dish) 7–8 days old was used. To assay circadian fluctuation in whole-body-female pheromone titer, female extracts were prepared (Hammack et al., 1973) at 2-hr intervals over a 24-hr period, with extra hourly collections during the peak calling period. The extracts were diluted to 1×10^{-5} female equivalents (FE)/μl, and 10-μl amounts (1×10^{-4} FE) were bioassayed by the method of Vick et al. (1970). Each extract was tested with 50 8-10-day-old males; a hexane blank tested with 50 males served as the control in each extract series.

Groups of 1000–2000 female beetles, 1–5 days old, were aerated at an airflow rate of 2 liters/min for 15 days in a glass apparatus loosely filled with filter paper in which the beetles hid; the air then passed through a tube of Porapak Q, a porous polymer absorbent that removes organic chemicals, but not water, from a stream of air. The house vacuum pulled room air into the apparatus through a charcoal filter (Cross et al., 1976). A total of 27,250 beetles was used in the course of several studies.

The Porapak Q, 15 g 60/80 mesh, was preextracted in a Soxhlet extractor with 500 ml redistilled reagent-grade pentane or hexane for 24 hr, dried overnight in a N_2 stream at 50°C, used in the aeration, extracted in the same manner, and reused in subsequent aerations. The extract that contained the pheromone (Porapak extract) was reduced in volume to 2 ml by distillation

TABLE 1. DISTRIBUTION OF PHEROMONE ACTIVITY DURING AERATION AND COLLECTION

	Level to achieve 50% response[a]
Porapak extract	8×10^{-6} beetle/day equivalents
Beetle-contaminated filter paper	1×10^{-3} beetle/day equivalents
Macerated beetles	9×10^{-3} female equivalents

[a] One beetle/day equivalent is defined as the total quantity of a substance in the extract divided by the product of the number of beetles aerated and the number of days of aeration. One female equivalent is the average quantity of pheromone extractable from one female.

through a 20-cm column packed with glass beads. Beetle-contaminated filter paper was similarly extracted.

To determine the distribution of pheromone activity between Porapak, filter paper, and beetles (Table 1), one group of approximately 2000 females was aerated as described above. The Porapak and the filter paper were extracted in the standard manner. After aeration, the females were macerated and extracted with hexane as previously described. The three extracts were assayed by the method of Vick et al. (1970).

For chemical analysis of pheromone components in whole females, 2500 female beetles about 14 days old, unaerated and held on filter paper in closed containers, were extracted with hexane in a Waring blender, and the hexane was distilled as above. The oily residue was subjected to short-path distillation (180°/0.5 mm Hg, 4 hr), and the distillate was dissolved in 2 ml of hexane.

Pure compounds were isolated for identification by fractionation of the Porapak extract on the following gas-liquid chromatography (GLC) columns: column A, 10% Carbowax 20 M on Chromosorb W 60/80 mesh, 2.4 m × 6 mm (OD) glass, 60 ml/min He flow rate at 125° initial temperature for 6 min, then temperature programmed at 4°/min to 200° and held there for 50 min; column B, 5% SE-30 on Chromosorb G 60/80 mesh, 5 m × 6 mm (OD) glass, 60 ml/min He flow rate at 190°; column C, diethylene glycol succinate (DEGS) support-coated open column (SCOT), 30.5 m × 0.5 mm stainless steel, 6 ml/min He flow at 140°; column D, 10% Apolar 10C on Gas Chrom Q 100/120 mesh, 5 m × 6 mm (OD) glass, 45 ml/min He flow rate at 185°; column E, 5% DEGS on Chromosorb G 60/80 mesh, 5 m × 6 mm (OD) glass, He flow rate 60 ml/min at 187°. All glass columns and solid supports were acid washed and treated with dichlorodimethylsilane. Fractions from the Varian Model 204B gas chromatograph were collected in glass

capillary tubes (30 cm × 2 mm OD) in a the mal gradient collector (Brownlee and Silverstein, 1968).

Nuclear magnetic resonance and mass spectra were obtained as reported elsewhere (Cross et al., 1976). The high-resolution mass spectrum was obtained by Shrader Analytical, Detroit, Michigan. Microozonolyses were performed in hexane at −65°C, and the ozonides were decomposed with triphenylphosphine (Beroza and Bierl, 1966). Preliminary bioassays were performed by a modification of the method reported by Burkholder and Dicke (1966). The method of Greenblatt et al. (1976) was used as a quantitative bioassay to determine the activity of the aldehyde component of the pheromone.

The aldehyde was synthesized by oxidation with Collins' reagent (Ratcliffe and Rodehorst, 1970) of (Z)-14-methyl-8-hexadecen-1-ol (Rodin et al., 1969; Farchan Division, Story Chemical Co., Willoughby, Ohio), and purified by preparative gas chromatography.

The quantity of a particular component from the Porapak extract could be estimated by comparing the area under its peak on column A with that of a known quantity of the synthetic compound.

RESULTS

Calling Behavior

Calling activity (Figure 1) was largely restricted to a 7-hr interval with a maximum at 2–4 hr after light onset (Figure 2). The maximum percentage of calling females observed at any one time was 44%. The highest pheromone titer, based on male response, was found in females extracted during the time of peak calling activity (Figure 2).

Isolation and Identification of (Z)-14-Methyl-8-hexadecenal

The biologically active concentrate of the Porapak extract (Table 1) was separated into nine fractions on column A. Although several fractions showed biological activity, the one with a retention time of 41 min (methyl palmitate retention time = 40 min) was by far the most active. Chromatography of this fraction on column B resulted in seven fractions, one of which (retention time = 74 min, methyl pentadecenoate retention time = 65 min) was more active than the other six fractions combined. Since this fraction gave only a single peak on column C, it was assumed to be a pure compound. This compound was a major component of the Porapak extract with an average production of 35.66 ng/beetle/day.

The NMR, IR, and mass spectra are shown elsewhere (Cross et al.,

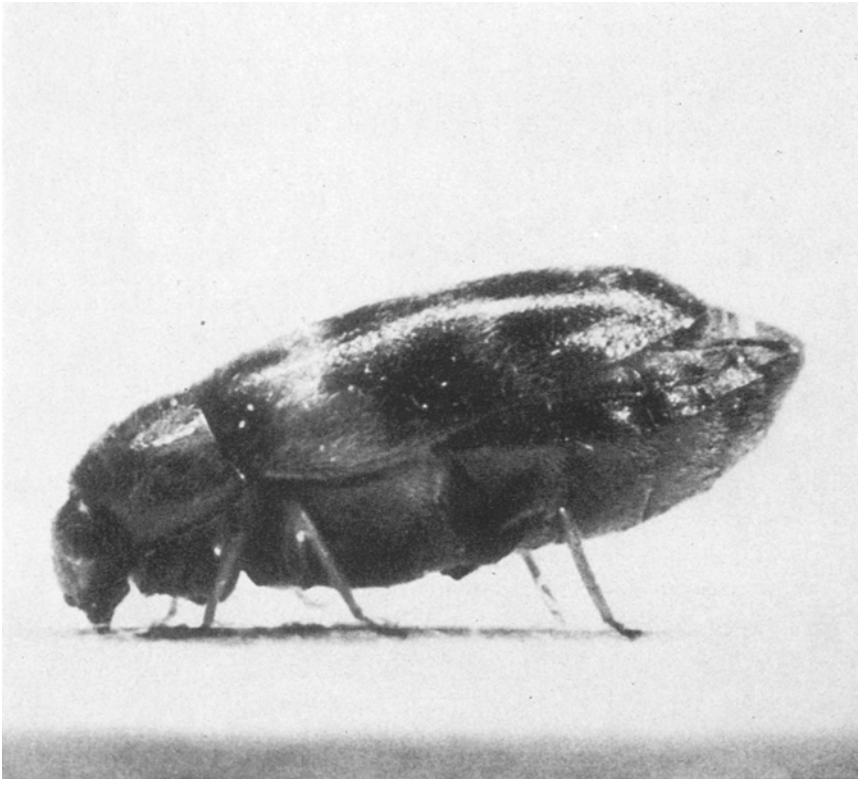

FIG. 1. Female *Trogoderma variabile* in calling position.

1976). Microozonolysis yielded 1,8-octanedial and 6-methyloctanal. Chromatography on column D showed that only the Z isomer was present. Insufficient material remained for determination of the optical rotation.

Other Pheromone Components

The GLC fractions of the Porapak extract were examined for the presence of the five presumptive pheromone components previously identified from *T. inclusum* (Rodin et al., 1969) and *T. glabrum* (Yarger et al., 1975). These results are tabulated in Table 2. Caproic acid was not detectable, but methyl-(Z)-7-hexadecenoate (separated from the aldehyde on column B) was present in small amounts. γ-Caprolactone was present in a fraction from column A that showed some activity in the bioassay. Methyl-(Z)-14-methyl-8-hexadecenoate and (Z)-14-methyl-8-hexadecen-1-ol were both

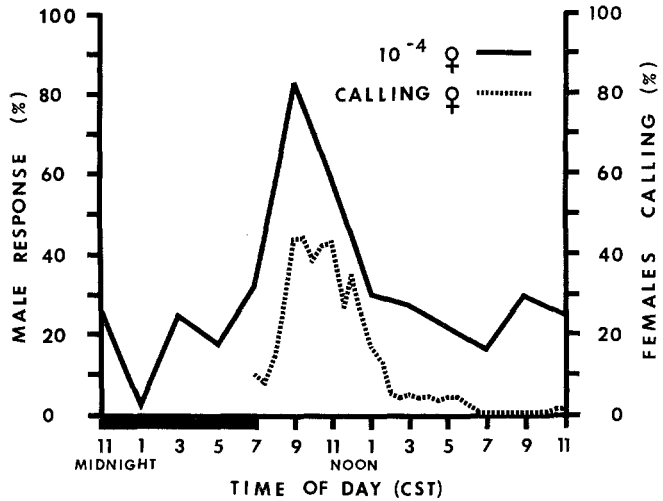

FIG. 2. *Trogoderma variabile* female calling frequency and circadian fluctuation in female whole body pheromone titer based on male response to a dilution of whole body female extracts equal to 1×10^{-4} female equivalents.

undetectable in the Porapak extract; appropriate fractions, which also contained extraneous compounds, were collected from column A and were rechromatographed on column B. No peaks appeared at the retention times of the authentic alcohol or ester. Thinking these compounds were not volatile enough to collect by aeration, we extracted 2500 macerated female beetles, which had been kept in closed containers, and the filter paper on which they had been maintained. Although the aldehyde was readily detectable in the filter paper extracts (Table 3), the filter paper did not contain detectable quantities of the branched-chain ester or alcohol; i.e., less than 25 ng of the

TABLE 2. PHEROMONE COMPONENTS OF *T. variabile* IN PORAPAK EXTRACT

Components	Amounts (ng/beetle/day equivalent)
Caproic acid	Not detected
γ-Caprolactone	15.15
(Z)-14-methyl-8-hexadecenal	35.66
Methyl (Z)-7-hexadecenoate	0.022
Methyl (Z)-14-methyl-8-hexadecenoate	Not detected
(Z)-14-methyl-8-hexadecen-1-ol	Not detected

TABLE 3. DISTRIBUTION OF *Trogoderma* ATTRACTANTS IN *T. variabile*

	(Z)-14-methyl-8-hexadecenal	(Z)-14-methyl-8-hexadecen-1-ol	Methyl (Z)-14-methyl-8-hexadecenoate
Porapak	Present	Not detected	Not detected
Filter paper	Present	Not detected	Not detected
Macerated beetles	Not detected	Present	Not detected

compound was present in a single gas chromatographic injection, which amounted to about 20% of the total quantity of extract. The beetle extracts contained no detectable quantity of the aldehyde or branched-chain ester even though the beetles were killed during the peak of their daily calling period. Isolation of the alcohol by chromatography on column A followed by chromatography of its acetate derivative (pyridine, acetic anhydride) on column D established the presence of the Z isomer of the alcohol in the beetle extracts. None of the E isomer was detected.

Bioassays

The total Porapak extract and the aldehyde from it elicited 50% arousal responses at 8×10^{-5} (95% confidence level: 4×10^{-5}–1×10^{-4}) and 2×10^{-5} (95% confidence level: 1×10^{-5}–4×10^{-5}) beetle/day equivalents, respectively; the Porapak extract minus the aldehyde elicited a 50% arousal response at 8×10^{-3} (95% confidence levels: 4×10^{-3}–2×10^{-2}) beetle/day equivalents. The insects were more sensitive to the Z isomer of the aldehyde than to the E isomer. Four ng (95% confidence level: 2–9 ng) of the synthetic E isomer, but only 50 femtograms (95% confidence level: 30–100 fg) of the synthetic Z isomer were required to achieve a 50% arousal response.

Isolation and Identification of 1-(*Ethylphenyl*)-1-*phenylethane* (*an Artifact from the Absorbent*)

The fraction from column A with retention time 30.6–34.5 min showed some biological activity. Fractionation on column B gave six fractions, four of which showed some activity. The largest component (retention time = 33 min, methyl myristate retention time = 40.2), purified on columns E and D, was identified as 1-(ethylphenyl)-1-phenylethane by its NMR and high-resolution mass spectra (HRMS). The HRMS showed the molecular formula was $C_{16}H_{18}$ (mol wt 210.1419, 44% of base). The base peak was at mass 195.1173 ($C_{15}H_{15}$). Other prominent ions were at masses 181.1017

($C_{14}H_{13}$, 14%), 167.0860 ($C_{13}H_{11}$, 17%), 166.0771 ($C_{13}H_{10}$, 12%), and 165.0703 ($C_{13}H_9$, 17.5%). No other peaks greater than 8% of the base peak were present. The NMR absorptions were assigned as follows: 1.210 δ (3H, t, J = 7.6±0.125 Hz, CH_3CH_2—Ar); 1.631 δ (3H, d, J = 7.3 Hz, CH_3—C—H); 2.611 δ (2H, quartet, J = 7.6 Hz, CH_3CH_2—Ar); 4.124 δ (1H, quartet, J = 7.3 Hz, CH_3C—*H*); 7.048 δ–7.12 δ (8H, m, Ar). The substitution pattern of the ethylphenyl ring was not determined.

DISCUSSION

The results of the female calling and female pheromone content experiments support our hypothesis that abdominal elevation by *T. variabile* females can be considered part of a pattern of postural activities accompanying sex pheromone release. A similar behavior has been described by Hammack and Burkholder (1976) and Hammack et al. (1976) for *T. glabrum*. There is a dramatic difference in the time of day activity for the two species. The peak pheromone activity of *T. variabile* occurs approximately 7 hr earlier than that of *T. glabrum* during a 16:8 L:D cycle. This could be a factor in the reproductive isolation of these two species.

The most important component of the *T. variabile* pheromone, present in the Porapak extract in relatively large amounts, is (Z)-14-methyl-8-hexadecenal. The 50% arousal response of *T. variabile* males to 8×10^{-5} beetle/day equivalents of the Porapak extract is due solely to this aldehyde. The remaining components of the extract elicit the 50% arousal response at 8×10^{-3} beetle/day equivalents, although this may be due in part to contamination by small quantities of aldehyde. No apparent synergistic interaction exists between the aldehyde and the remaining components since the aldehyde alone is as active as the entire mixture in eliciting arousal. Larger quantities of the aldehyde elicit arousal responses from all the male beetles, but mating responses were not observed as they were in *T. glabrum* males (Greenblatt et al., 1976). Mating in *T. variabile* may be mediated by contact chemoreception of chemicals dissolved in the cuticular wax of the females, but which are not major components of the airborne pheromone, or by nonchemical means. In a preliminary laboratory bioassay, the aldehyde attracted 50% of released, nonflying males over a distance of more than 1 m in less than 2 min.

It is obvious that important insect pheromone components may be missed if only a single isolation procedure is used. Yarger et al. (1975) identified five pheromone components from macerated *T. glabrum* females, but the most important component for this species, (E)-14-methyl-8-hexadecenal, was found later by aeration of live beetles and trapping the volatile

components on Porapak Q (Cross et al., 1976; Silverstein, 1976). Two possible reasons may be offered. Maceration may have led to enzymatic degradation of the aldehyde, or the beetles may synthesize and release the aldehyde only "on demand," presumably during their calling period; the experiences reported by Hill et al. (1975) and by Pearce et al. (1975) lend support to the latter. On the other hand, (Z)-14-methyl-8-hexadecen-1-ol was found in macerated *T. variabile* females, but not in the Porapak extract nor on the filter paper. Failure of the insect to release this compound would account for these results; the low volatility of the alcohol would also account for its absence in the Porapak extract. However, the synthetic alcohol elicits arousal responses from 50% of the male beetles tested at a level of 1 ng, a quantity we could not detect (Greenblatt et al., 1977). Thus, the alcohol may well be emitted and be present in the extracts of filter paper and Porapak, but it can be isolated in identifiable quantities only from extracts of macerated beetles.

The presence of the artifact, 1-(ethylphenyl)-1-phenylethane, from two of the six batches of Porapak Q, a copolymer of ethylvinylbenzene and divinylbenzene, shows the need for careful preextraction of the absorbent. Strangely enough, this artifact, or an inseparable impurity, elicited a weak response from male beetles.

Like other *Trogoderma* pheromones, that of *T. variabile* apparently has several components. Whether or not (Z)-14-methyl-8-hexadecen-1-ol is emitted is unclear for the reasons discussed above. The corresponding ester, methyl (Z)-14-methyl-8-hexadecenoate, was not detected in any of our extracts but does elicit 50% arousal responses in quantities of 10^{-1} μg (Greenblatt et al., 1977). We have searched for three other possible components that have been identified in other *Trogoderma* species (Yarger et al., 1975). Caproic acid was not detectable. γ-Caprolactone was present, but its biological role is not understood. Methyl (Z)-7-hexadecenoate was present in such small quantities that it is unlikely to be involved in the attraction process (see Table 2).

The total pheromone may well include additional compounds with low activity or with activity in mediating behavior other than that for which we have assayed. Greenblatt et al. (1976) report that caproic acid may increase the frequency of mating responses from male *T. glabrum* beetles elicited by (E)-14-methyl-8-hexadecenal. Further comparative studies of the components are in progress.

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ATTRACTIVENESS OF TOBACCO BUDWORM FEMALES ALTERED BY ORAL CHEMOSTERILANTS AND DIETARY ADDITIVES[1,2]

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Abstract—Female *Heliothis virescens* (F.) moths reared from larvae on diet treated (0.1%) with experimental chemosterilants or the dietary additive, DL-leucine, were used as bait in sex lure traps in field cages when they were 2–4 nights old. Catches of untreated released males were used to determine relative attractiveness of the chemically treated females. The catch (indicating quantity or production frequency of pheromone) was significantly increased when DL-leucine had been fed in the larval diet, and sulfanilamide caused a slight increase in female attractiveness. The catch of males was significantly reduced when either reserpine or quercitin had been added to the diet. The other chemicals, bisdicumarol, 2,4-dichlorophenoxyacetic acid, β-sitosterol, and dihydrocholesterol, did not significantly affect the catch.

Key Words—Sex pheromone production, *Heliothis virescens* (F.), attractiveness, chemosterilants.

INTRODUCTION

The tobacco budworm, *Heliothis virescens* (F.), is a major pest of tobacco, cotton, and several vegetable crops in the southern United States, and it has

[1] This paper reports the results of research only. Mention of a pesticide in this paper does not constitute recommendation of use by the U.S. Department of Agriculture nor does it imply registration under FIFRA as amended.

[2] In cooperation with the Texas Agricultural Experiment Station, Texas A&M University, College Station 77843.

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devastated cotton in more southerly countries. During the 1970s, insect-resistant strains of the tobacco budworm has spread from Mexico and Texas eastward throughout Louisiana, Mississippi, and into Alabama. Therefore, these resistant budworms are now a target for alternate control methods involving release of sterilized insects for large-area suppression.

Presently two methods are used to sterilize tobacco budworms, exposure to gamma irradiation from radioactive cobalt-^{60} or cesium-^{137} isotopes (Flint and Kressin, 1967; El Sayed and Graves, 1969) or feeding of chemosterilants in the larval diet (Guerra, 1970). However, the ability of the reared insects (either sex) to compete with native tobacco budworms in the field may be altered or reduced by either of these sterilizing techniques. For example, Hendricks (1974) found that ^{60}Co irradiation of pupae significantly reduced the attractiveness of the resulting females in simulated field tests. The effect of feeding chemosterilants in the larval diet on the attractiveness of females has not been reported. However, they do affect budworm pupation, eclosion, fecundity, and egg hatch (Guerra, 1975). Such data were needed to determine which chemical agents would yield females with the ability to produce sufficient sex pheromone to attract fertile males.

METHODS AND MATERIALS

Tobacco budworm larvae in the first stage were placed singly in 3/4-oz plastic cups of diet containing the experimental chemosterilants (0.1%, w/v) and held at 28 ± 2°C with a photoperiod of 14 hr light:10 hr dark until pupation. Larvae were also reared on untreated diet so the resulting females could be used as checks. Sexes were separated in the pupal stage, and females were held in moistened vermiculite until eclosion. (The males were discarded). The adult females were fed a 10% sucrose solution during the first day of their life and used as bait in saucer-type pheromone traps for 2–4 days to test their attractiveness to males.

The tests were made in $1.8 \times 1.8 \times 5.5$-m screened field cages where pheromone traps baited with treated or untreated females (Hendricks et al., 1973) were suspended 1.2 m above ground and 1.2 m apart in the test cage. In each cage (replicate), five traps were set up, one baited with an untreated (check) female, one left empty, and the other three traps each baited with a single female from diet treated with a different chemosterilant. As soon as the traps were baited, 50 newly eclosed male tobacco budworms were released in the cage. Catches of males that were attracted to the traps were tabulated daily for 3 days and used to determine the relative attractiveness of the treated females.

Five cages (replicates) were stocked identically and used simultaneously.

Two cages were set on a line perpendicular to the other three cages, and none was exposed to bright artificial light to which males might be attracted. Treatments (female moths) within each cage were randomized and rotated between replicates to eliminate bias due to trap location.

The females used as bait in test 1 had been reared as larvae on diet treated with sulfanilamide, bisdicumarol, or reserpine, or were untreated. Those used in test 2 had been reared on diet with DL-leucine (a dietary amino acid), 2,4-dichlorophenoxyacetic acid, or β-sitosterol, or were untreated. Those females used in test 3 had been fed diet with dihydrocholesterol, quercetin, reserpine, or were untreated. During the 8 months required for the entire series of tests, temperature ranged from 5 to 39°C, and relative humidity ranged from 45 to 98%. However, the test cages were not set up when the temperature dropped below 15°C.

RESULTS AND DISCUSSION

Attractiveness of females from larvae reared on diet treated with reserpine was significantly reduced (Table 1, test 1); attractiveness of females from larvae reared on diet treated with sulfanilamide was increased (15.4%); and attractiveness of females from larvae reared on diet treated with bisdicumarol was slightly reduced (−2.3%).

DL-Leucine did significantly increase the average catch (28.7%), perhaps by increasing the quantity of pheromone or production frequency from the females (test 2), but 2,4-dichlorophenoxyacetic acid and β-sitosterol did not significantly alter the total catch of males. In test 3, reserpine again significantly reduced (−39.3%) catches of males, and quercitin caused an even greater reduction (−43.8%). Dihydrocholesterol had no statistically significant effect, but these females did not catch as many males as untreated females.

Daily catches of males (percentage trapped per night) in tests 1 and 2 showed no obvious shift in time of peak female attractiveness due to the treatments; the majority of males were caught the first night. However, in test 3, most males attracted by females from larvae treated with reserpine were caught the second night. During the first night, the percentages of males attracted to all females were substantially reduced compared with those for tests 1 and 2. Perhaps this shift of relatively greater catches to the second night occurred because test 3 was made in September and October when the nighttime temperatures were relatively low (47–62°F), and the duration of peak pheromone production and male response were shortened (Hendricks and Tumlinson, 1974).

Chemosterilants are added to larval diets or applied topically to

TABLE 1. ATTRACTIVENESS OF FEMALE TOBACCO BUDWORMS REARED FROM LARVAE FED DIET TREATED WITH A CHEMOSTERILANT (0.1% w/v)

Treatment	Average no. males caught/5 replicates[a]	Males trapped on indicated night (%)[b]			Total no. males trapped	Difference from catch of untreated females (%)
		1st	2nd	3rd		
	Test 1 (35 Replicates)					
Sulfanilamide	46.7 (a)	86.6	12.7	0.7	299	+15.4
♀ (untreated)	41.0 (a)	78.0	17.4	4.6	259	0
Bisdicumarol	40.1 (ab)	89.7	8.7	1.6	253	−2.3
Reserpine	33.1 (b)	71.1	17.3	2.0	204	−21.2
Empty trap (check)	4.0 (c)	adjustment factor				
	Test 2 (40 Replicates)					
DL-Leucine	42.4 (d)	87.1	12.6	0.3	318	+28.7
♀ (untreated)	33.5 (e)	88.3	12.1	−0.4	247	0
2,4-Dichlorophenoxy-acetic acid	30.4 (e)	87.8	12.2	0	222	−10.1
β-Sitosterol	29.7 (e)	87.1	10.6	2.3	217	−12.2
Empty trap (check)	2.6 (f)	adjustment factor				
	Test 3 (25 Replicates)					
♀ (untreated)	24.6 (g)	55.4	40.2	4.4	112	0
Dihydrocholesterol	22.0 (g)	50.5	44.4	5.1	99	−11.6
Reserpine	15.8 (h)	38.2	54.4	7.4	68	−39.3
Quercitin	14.8 (h)	61.9	33.3	4.8	63	−43.8
Empty trap (check)	2.2 (j)	adjustment factor				

[a] Means followed by the same letters are not significantly different ($P = 0.05$) by modified t test.

[b] Adjusted to compensate for chance catch by subtracting numbers caught per replicate in unbaited traps.

produce some degree of sterility (Flint et al., 1968; Guerra et al., 1972; Wolfenbarger et al., 1974). For example, sulfanilamide and dicumarol (Guerra, 1975) and also reserpine (Guerra et al., 1972), fed in the diet of the tobacco budworm were all found to increase mortality of the pupal stage, 10, 7, and 41%, respectively. Nevertheless, these three experimental compounds did cause female sterility and subsequent reduction in egg hatch to some degree. Also, β-sitosterol reduced egg production 24% but had little effect on egg hatch (Guerra, 1975). However, the results of our tests show that dietary additives such as DL-leucine and some chemicals used for experimental sterilization purposes can affect overall female attractiveness in the

field. This altered attraction, and its relation with mating behavior, would directly influence the competitiveness of chemosterilized females with untreated native females. For example, diet treated with sulfanilamide, β-sitosterol, or bisdicumarol (as sterilants) may be used effectively to produce sexually attractive females. Also, DL-leucine might be used as a dietary additive to enhance attractiveness and consequently the competitiveness of laboratory-reared female tobacco budworms. Reserpine and quercitin significantly reduce attractiveness, so are not suitable sterilants when highly competitive females are required for mass-release operations to suppress large native populations.

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EFFECTS OF SLOW-RELEASE FORMULATION OF SYNTHETIC *endo*- AND *exo*-BREVICOMIN ON SOUTHERN PINE BEETLE FLIGHT AND LANDING BEHAVIOR[1]

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Abstract—A slow release formulation of the bark beetle pheromones, *endo*- and *exo*-brevicomin, significantly reduced landing of southern pine beetles on host trees. Beetle flight activity within the infestation was not affected by the pheromones. The pheromones were released at approximately 600 mg/ha/day via Conrel® hollow fiber controlled-release dispensers.

Key Words—pheromone, inhibitor, bark beetle, *Dendroctonus*, southern pine beetle, slow-release formulations.

INTRODUCTION

The southern pine beetle (*Dendroctonus frontalis* Zimm.) is a chronic pest of southern pines. In eastern Texas, the beetle has caused large timber losses regularly since 1957. Conventional insecticides (BHC and lindane) were

[1] *Dendroctonus frontalis* Zimm. (Coleoptera: Scolytidae). The work was funded in part by McIntire-Stennis project 1525 TAES and TEXY-00011 (SFASU), the U.S. Department of Agriculture sponsored program entitled "The Expanded Southern Pine Beetle Research and Applications Program" through TAES-CSRS grant 680-15-10 and SFASU-CSRS grant 680-15-13, and U.S. Forest Service Coop. Agreement USDA-SFES 19-145 to TAES and SFASU. The findings, opinions, and recommendations reported herein are those of the authors and not necessarily those of the U.S. Department of Agriculture. Texas Agricultural Experiment Station paper No. 12731.

used as the primary means of southern pine beetle control until 1969. Their use was greatly reduced for various reasons, one of which was the lack of proven effectiveness of the insecticides on total populations of the insect (Williamson and Vité, 1971).

Research on alternative methods of southern pine beetle control utilizing pheromones was begun in 1962 and resulted in the formulation of the attractant frontalure (Vité, 1971), which consists of the insect-produced pheromone, frontalin (Kinzer et al., 1969), and a compound from the pine host, α-pinene. Subsequently, a control method was tested using frontalure in combination with the silvicide cacodylic acid (Vité, 1971; Capony and Morris, 1972). The method has not been adopted on an operational basis.

In addition to attractants, other types of behavioral chemicals have been implicated as having a role in southern pine beetle attack behavior. The compounds, *endo*- and *exo*-brevicomin (Silverstein et al., 1968), the former found in small amounts in the beetles, were reported to reduce the attractiveness of frontalure when each compound was combined with the attractant mixture (Vité and Renwick, 1971). Similarly, in 1974 and 1975 field tests using sticky traps, *endo*- plus *exo*-brevicomin reduced the attractiveness of frontalin plus loblolly turpentine (containing α-pinene) by approximately 80% (Payne et al., unpublished).

These and the earlier results prompted us to field-test the effect of the continual presence of both *endo*- and *exo*-brevicomin on the flight and landing activity of southern pine beetles in a natural infestation. We also evaluated a controlled-release formulation of the compounds that may have application in the operational deployment of these behavioral chemicals.

METHODS AND MATERIALS

Study Area

The study site was located in a 500-tree, southern pine beetle infestation in a loblolly pine (*Pinus taeda*)–mixed hardwood forest, on International Paper Company land at Bald Hill, south of Lufkin, Texas. The infestation consisted of trees in all stages of beetle attack. Tests were carried out over a 24-day period from October 9 to November 2, 1975.

Monitoring Flight and Landing Activity

Southern pine beetle activity in the infestation was monitored with the use of two types of unbaited traps coated with Stickem Special®. Nine large wing traps (4 vanes, 0.9×2.1 m/vane) were used to monitor distribution of flying beetles (flight traps). The traps were constructed of 1/3-cm mesh

fiberglass screen and positioned on standards with the top edge 3.5 m above the ground. The second type of trap consisted of a single 15 × 60-cm piece of 1/3-cm hardware cloth and was used to monitor landing activity (landing traps). Individual landing traps were nailed at 2–2.4 m to selected host trees in the infestation. All flight and landing traps were picked clean of trapped southern pine beetles by 10:00 AM (CDST) each day. In cases where individual traps caught such high numbers that field counting was impractical, the traps were removed from their standards, replaced with clean traps, and taken to the laboratory for cleaning. In all cases, the southern pine beetles from each trap were placed in separate vials along with a label containing trap type, trap number, and date. All beetles were sexed. Where large catches occurred, a sequential sampling procedure was used (Johnson, unpublished) to determine sex ratios.

Compound Formulation and Handling

Endo- and *exo*-brevicomin (>99% pure by GLC) were dispensed from Conrel ® hollow fiber controlled release strips prepared by the FRL Corporation (Albany International Co., Norwood, Massachusetts) (Fig. 1). Each dispenser consisted of 30 polyester fibers, each 3 cm × ca. 0.5 mm (length × ID). Alternative fibers contained *endo*- and *exo*-brevicomin, and each dispenser contained a total of ca. 90 mg of the compounds. The release rate per dispenser was determined cathetometrically as ca. 3.0 mg/day (1.5 mg/day per compound) at constant temperature (21°C) and humidity (65%) (D.W. Swenson, FRL, personal communication). At that rate, each dispenser was estimated to provide ca. 30 days of continuous release.

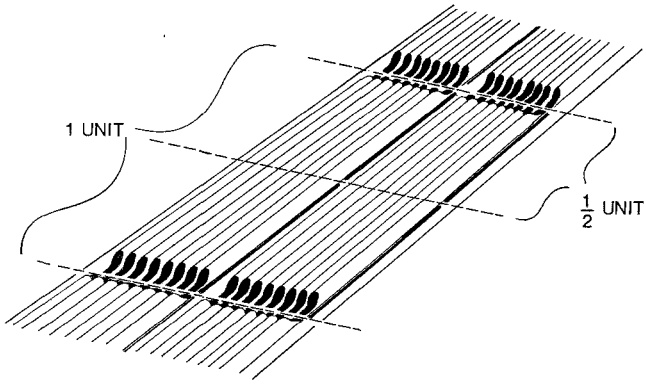

FIG. 1. Schematic of Conrel® hollow-fiber controlled-release unit.

Each dispenser was cut in half (Figure 1) to provide dispensers with ca. 15 days continuous release capacity. Thirty-six half dispensers were taped 1/card, to 7.5×12.5 cm cards which were attached to host trees at selected release points in the plot. Purity was determined for 1 dispenser each, exposed under field conditions for 4, 8, or 12 days. These performance-trial dispensers were exposed simultaneously with other dispensers in the same manner only 0.8 km from the plot. Compound purity was determined by GLC. Ten dispensers were measured at the end of the test as an estimate of the release rate under field conditions. The amount of compound per fiber was measured in mm and converted to the approximate amount in mg. When not exposed in the field, all dispensers were held at $-60°C$.

Experimental Design

Check and treatment tests were conducted sequentially in the same infestation. Check data were taken for 4 consecutive days without synthetic behavioral chemicals in the plot. Then synthetic behavioral chemicals were introduced and treatment data were gathered for four consecutive dates, i.e., days 5–8 inclusive. At the beginning of the ninth day, the chemicals were removed and the second 4-day check period began. The procedure was repeated three times, providing three replicates each of the treatment and check tests.

The plot consisted of a 3×3 grid of adjacent 15-m^2 blocks (Figure 2). The grid did not encompass the entire infestation but was limited to that area containing primarily fresh attacked and unattacked trees. The grid was repositioned after each complete treatment–check replicate to include the leading edge of the infestation. We did not use a larger grid because of the unavailability of sufficient amounts of the compounds to obtain the desired release rate over a larger area. A flight trap was located at the center of each block within the grid. Sixteen of the landing traps were located at 15-m centers corresponding to the corners of each 15-m block. In addition, 18 landing traps were positioned at 7.5-m centers within the grid, with 2 traps per block, one each in the northeast and southwest corners.

A total of 36 dispensers were used in the plot during each treatment replicate. Four dispensers were positioned in each block on host trees at ca. 7.5-m centers. The dispensers were nailed to the north side of the trees at 1.8–2.4 m above ground level. A landing trap was attached at two dispenser positions per block. The infestation was cruised daily, and newly attacked trees were flagged and mapped.

Temperature, relative humidity, and precipitation were monitored throughout the tests using a Weathermeasure Corp. H311 hygrothermograph and P501 remote-recording rain gauge situated within the forest stand.

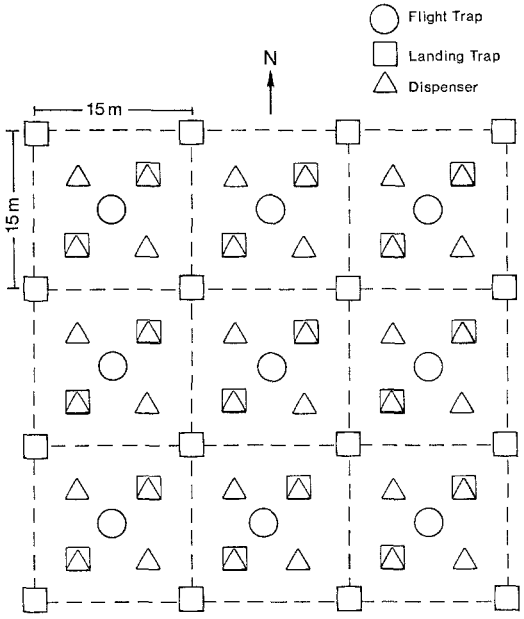

FIG. 2. Schematic of plot design to evaluate effects of a slow-release formulation of synthetic *endo*- and *exo*-brevicomin on flight and landing activity by the southern pine beetle.

Landing trap catches for the treatment replicates were examined with *t* tests to check for possible positional effects due to experimental design. Trap catches were compared as follows: peripheral traps without dispensers vs. all traps with dispensers, interior traps without dispensers vs. all traps with dispensers, and traps surrounded by dispenser positions vs. all other traps. Significant differences were not found at the 5% level. Therefore, landing trap catches were summed in subsequent analyses of variance tests.

Two evels of analysis of variance were utilized for landing trap catches. The first included all 3 replicates (plots) and tested for significant treatment, plot location, and sex effects. The second tested treatment and sex effects within each replicate. Landing trap catches were summed for each day. The *t* tests were used to compare overall landing trap catches for treatment vs. checks for each sex on individual trap catch per day.

A single three-factor analysis of variance was used to test for significant treatment, plot location, and sex effects for the flight trap catches. Flight trap catches were summed over each day.

RESULTS AND DISCUSSION

Mean total landing trap catch was significantly less during the periods when *endo*- and *exo*-brevicomin were dispensed (treatment) as compared to periods when the compounds were not present (check) (Table 1). Landing activity was significantly less for treatment vs. check comparisons within each of the three replicates of the test (Table 1). This reduction was significant for both sexes (Table 2). However, sex ratios were not significantly different for treatment vs. check comparisons. There was no significant difference in trap catch for treatment replicates between landing trap positions with dispensers and those without, or trap positions surrounded and not surrounded by dispensers.

Flight trap catch showed no significant difference between treatment and check periods (Table 3), and no significant differences in sex ratio occurred. There was, however, a significant difference in beetle catch due to plot location which indicated differences in flying beetle density among the three replicates.

The landing trap catches showed that the treatment did inhibit or interrupt the landing of southern pine beetles on host trees on an area basis, even though 16 of the 34 landing traps were not in direct association with the dispensers, and only 12 traps were surrounded by dispensers. Flight trap catches showed

TABLE 1. TOTAL AND MEAN DAILY LANDING TRAP CATCHES OF SOUTHERN PINE BEETLES IN SYNTHETIC *endo*- AND *exo*-BREVICOMIN-TREATED VS. CHECK TESTS FOR EACH 4-DAY REPLICATION

	Numbers		
	Total	Mean[a]	Significance
Replicate 1			
Treatment	114	29 ± 5.20	$\alpha \leq 0.005$
Check	458	115 ± 30.34	
Replicate 2			
Treatment	438	110 ± 30.14	$\alpha \leq 0.05$
Check	980	245 ± 78.33	
Replicate 3			
Treatment	199	50 ± 22.55	$\alpha \leq 0.05$
Check	1088	272 ± 110.22	
Totals			
Treatment	751	63 ± 15.43	$\alpha \leq 0.005$
Check	2526	211 ± 46.46	

[a] Means $\pm$ SE-.

TABLE 2. TOTAL AND MEAN DAILY LANDING TRAP CATCHES OF MALE AND FEMALE SOUTHERN PINE BEETLES IN SYNTHETIC *endo*- AND *exo*-BREVICOMIN-TREATED VS. CHECK TESTS

	Numbers		
	Total	Mean[a]	Significance
Males			
Treatment	486	41 ± 10.33	$\alpha \leq 0.001$
Check	1466	122 ± 29.88	
Females			
Treatment	265	22.08 ± 5.28	$\alpha \leq 0.001$
Check	1060	88.33 ± 18.69	

[a] Means $\pm$ $SE_{\bar{x}}$ for three replicates of 4 days each.

that intraplot flight activity was not significantly affected by the presence of the compounds.

Compound purity remained >99% throughout the life of the units as determined by the analysis of the three field conditioned dispensers. For the 10 dispensers measured, a mean of ca. 7 ± 1.0 mg of the compounds per dispenser (ca. 0.2 ± 0.03 mg/fiber) remained at the end of the test. This amounted to a release rate of ca. 3.3 mg/day/dispenser or ca. 600 mg/ha/day (300 mg/ha/day/compound). There were no significant differences in precipitation, temperature, and relative humidity between the treatment and check days of the test to suggest the results were biased by climatic factors.

The point source effect of the antiattractant, methylcyclohexenone (MCH) (Kinzer et al., 1971) has been reported from tests for at least two

TABLE 3. TOTAL AND MEAN DAILY FLIGHT TRAP CATCHES OF THE SOUTHERN PINE BEETLE IN SYNTHETIC *endo*- AND *exo*-BREVICOMIN-TREATED AND CHECK TESTS

	Numbers		
	Total	Mean[a]	Significance
Treatment	24,528	2044 ± 410	N.S.
Check	24,904	2075 ± 755	

[a] Means $\pm$ $SE_{\bar{x}}$ for three replicates of 4 days each.

other *Dendroctonus* species. MCH was shown to reduce the catch of Douglas-fir beetles (*D. pseudotsugae* Hopk.) on traps baited with an attractant (Rudinsky et al., 1972). In addition, MCH was effective in reducing the number of attacks on attractant-baited and freshly-felled host trees (Furniss et al., 1972, 1974). The pheromone was also found effective against the spruce beetle, *D. rufipennis* (Kirby) (Rudinsky et al., 1974) by essentially preventing attack on felled host trees and by nearly nullifying response to synthetic attractants (Kline et al., 1974).

The results from the test reported here suggest the potential for development of these behavioral chemicals for southern pine beetle population control on an area basis. However, additional controlled field tests using different release rates and spacing, over extended periods of time, will be needed before these or any other behavioral chemicals can be adequately evaluated for use in southern pine beetle pest management. To our knowledge, the test reported here is the first demonstration of an area-wide influence of synthetic inhibitors on the landing behavior of southern pine beetles in a natural infestation.

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ATTRACTION OF REDBANDED LEAFROLLER MOTHS, *Argyrotaenia velutinana*,[1] TO BLENDS OF (Z)- AND (E)-11-TRIDECENYL ACETATES

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Abstract—The pheromone of *Argyrotaenia velutinana* (Walker) is a 92:8:150 mixture of (Z)-11-, (E)-11-tetradecenyl and dodecyl acetates. An 85:15 blend of (Z)-11- and (E)-11-tridecenyl acetates produces a trap catch equivalent to that with the pheromone blend. The sensory input elicited by (Z)-11-tridecenyl acetate appears to substitute for the sensory input of both (Z)-11-tetradecenyl and dodecyl acetates, whereas the input from (E)-11-tridecenyl acetate appears to substitute for that of (E)-11-tetradecenyl acetate. Surprisingly, addition of dodecyl acetate to the tridecenyl acetates mixture decreases trap catches.

Key Words—Redbanded leafroller, *Argyrotaenia velutinana* (Walker), (Z)-11-tridecenyl acetate, (E)-11-tridecenyl acetate, (Z)-11-tetradecenyl acetate, (E)-11-tetradecenyl acetate, dodecyl acetate, attractant, pheromone.

INTRODUCTION

Three sex pheromone components found in the female redbanded leafroller moth, *Argyrotaenia velutinana* (Walker), are (Z)-11-tetradecenyl (Z11–14:Ac), (E)-11-tetradecenyl (E11–14:Ac) and dodecyl (12:Ac) acetates (Roelofs and Arn, 1968; Roelofs and Comeau, 1968; Klun et al., 1973; Roelofs et al., 1975). A 92:8:150 blend of these compounds is a potent attractant

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for males of this species. Roelofs and Comeau (1971a) found that (Z)-11-tridecenyl acetate (Z11–13:Ac) also attracts *A. velutinana* males, although Klun and Robinson (1972) reported that it did not. Comeau (1971) had preliminary evidence that the addition of 12:Ac to Z11–13:Ac results in a lower trap catch, but the effects of the presence of the geometrical isomer, (E)-11-tridecenyl acetate (E11–13:Ac), were not defined at that time. This paper reports an investigation on the attractiveness of Z11–13:Ac alone and in various combinations with E11–13:Ac, E11–14:Ac and 12:Ac.

METHODS AND MATERIALS

The Z11–14:Ac and E11–14:Ac were obtained from Farchan Chemical and the 12:Ac from Eastman Chemical. The Z11–13:Ac and E11–13:Ac were synthesized in our laboratory by the usual alkyne reductions of 11-tridecynyl acetate (Roelofs and Arn, 1968). The Z11–13:Ac was purified by $AgNO_3$ high-pressure liquid chromatography with benzene. Compound purity and treatment compositions were determined by GLC on 3% PDEAS (phenyldiethanolamine succinate on 100–120 mesh Chromosorb W-AW-DMCS) on a 1.8×4 mm glass column. All compounds were >99% pure.

Field tests were conducted in abandoned apple orchards in Sodus and Dresden, New York. Test chemicals were placed in natural polyethylene closures (OS-6, Scientific Products) and these dispensers were positioned in the bottom center of Pherocon® 1C traps (Zoecon Corp.). Traps were hung in trees at a height of 1.5 m and separated by 10 m in a randomized complete block design. When checked, traps were cleared of moths and rerandomized within blocks. Data were transformed to $\sqrt{(x+0.5)}$ and submitted to analyses of variance. Differences among means were determined by Duncan's new multiple range test.

RESULTS AND DISCUSSION

Previous field tests with *A. velutinana* pheromone have shown that a low percentage (ca. 8%) of E11–14:Ac is obligatory for trap catch (Klun et al., 1973), with the optimum trapping ratio of these two isomers approximating the naturally occurring blend (Roelofs et al., 1975). Behavioral studies showed that these two components together elicit long-distance upwind anemotaxis (Baker et al., 1976). A field test was conducted, therefore, with Z11–13:Ac in combination with various amounts of its geometrical isomer, E11–13:Ac, to determine if there is an optimum combination of these isomers

TABLE 1. CAPTURES OF MALE *Argyrotaenia velutinana* BY VARIOUS MIXTURES OF 11-TRIDECENYL AND 11-TETRADECENYL ACETATES (TEST CONDUCTED IN DRESDEN, NEW YORK, JULY 12–15, 1974)

Treatment						
Z11–13:Ac	:	E11–13:Ac	:	E11–14:Ac	$\bar{x}$/trap[a]
100	:	0	:	0	0.1 (g)
97	:	3	:	0	4.5 (de)
94	:	6	:	0	7.8 (cd)
91	:	9	:	0	9.1 (c)
88	:	12	:	0	18.2 (a)
85	:	15	:	0	12.7 (ab)
80	:	20	:	0	9.8 (bc)
97	:	0	:	3	7.2 (cd)
94	:	0	:	6	1.5 (fg)
91	:	0	:	9	1.0 (fg)
88	:	0	:	12	0.2 (g)
85	:	0	:	15	0.6 (g)
80	:	0	:	20	0.0 (g)
Pheromone					
10 mg 11–14:Ac's (92:8 of Z:E)+15 mg 12:Ac					14.7 (ab)
10 mg 11–14:Ac's (92:8 of Z:E)					2.7 (ef)
Unbaited trap					0.0 (g)

[a] Treatments contained 3.5 mg sample, unless otherwise noted, and were replicated three times and rerandomized twice. Means followed by the same letter are not significantly different at the 5% level.

for attraction of *A. velutinana* males. The results (Table 1) show that Z11–13:Ac alone did not lure males, but mixtures containing 3–20% E11–13:Ac did. Surprisingly, the 88:12 and 85:15 mixes of Z11–13:Ac to E11–13:Ac were as potent as the three-component pheromone system. These observations were consistent in a number of field tests over two years. In a typical test (also see Table 2), 10 replicate traps containing 10 mg Z/E11–14:Ac (93:7) captured 18 males; treatments combining the three pheromone components [10 mg Z/E11–14:Ac (93:7) and 15 mg 12:Ac] lured 278 males, whereas traps containing Z/E11–13:Ac (91:9) caught 598 males.

The discrepancy between previous reports on the attractiveness of Z11–13:Ac may be due in part to the use of samples of differing purities.[3]

[3] Another difference was in the dispenser load. Klun and Robinson (1972) used 14 μg Z11–13:Ac in 0.7 ml olive oil.

TABLE 2. CAPTURES OF MALE *Argyrotaenia velutinana* BY MIXTURES OF 11-TRIDECENYL, 11-TETRADECENYL, DODECYL, AND UNDECYL ACETATES (TEST CONDUCTED IN SODUS, NEW YORK, FROM JULY 19 TO AUGUST 2, 1973)

Treatment (5 mg)			
Z11–13:Ac		E11–13:Ac	$\bar{x}$/trap[a]
99.4	:	0.6	5.0 (d)
95.1	:	4.9	28.5 (b)
93.1	:	6.9	28.5 (b)
90.7	:	9.3	36.5 (a)
84.5	:	15.5	38.0 (a)
83.9	:	16.1	28.0 (b)
90.7	:	9.3+12:Ac (15 mg)	12.0 (cd)
90.7	:	9.3+11:Ac (15 mg)	6.0 (cde)
90.7	:	9.3+11:Ac (5 mg)	12.5 (c)
Pheromone			
10 mg 11–14:Ac's (91:9 Z:E)+ 15 mg 12:Ac			26.0 (b)
10 mg 11–14:Ac's (91:9 Z:E)			12.5 (c)

[a] Treatments were replicated five times and sampled five times. Means followed by the same letter are not significantly different at the 5% level.

The Z11–13:Ac sample used by Klun and Robinson (1972) contained 5% E isomer (J.A. Klun, personal communication), whereas the sample used by Roelofs and Comeau (1971a) contained ca. 10% of the E isomer.

Behavioral observations of wild males established that 12:Ac acts in conjunction with the 92:8 ratio of Z11- and E11–14:Ac, mediating an increase in the incidence of landing close to the chemical source (Baker et al., 1976). Laboratory behavioral studies (Baker et al., 1976) showed that the 12:Ac enhanced the wing-fanning response of males to Z11–14:Ac, but not to E11–14:Ac, indicating that 12:Ac might be acting in conjunction with the Z receptor sites. EAG (Baker and Roelofs, 1976) and single-cell electrophysiology (O'Connell, 1975) also suggest that 12:Ac interacts synergistically with the Z11–14:Ac antennal acceptors, as hypothesized by Roelofs and Comeau (1971b) in their model of acceptor-site specificity. It is tempting to suggest from the above data that Z11–13:Ac provides the synergized sensory input of Z11–14:Ac+12:Ac, whereas E11–13:Ac replaces E11–14:Ac. The unique chemical structure of Z11–13:Ac makes

it structurally similar to Z11–14:Ac and also very similar in overall length to 12:Ac.

Compared to the natural blend of E11– to Z11–14:Ac (ca. 8:92), a high ratio of E11–13:Ac to Z11–13:Ac (ca. 15:85) was required, possibly because of a lower intrinsic activity of the unnatural (E11–13:Ac) compound. When E11–14:Ac, instead of E11–13:Ac, was combined with Z11–13:Ac (Table 1), it was effective at low ratios but not at higher amounts of 6 and 9%. A possible explanation for these data is that the Z11–13:Ac compound replaced both the Z11–14:Ac and the 12:Ac of the natural 92:8:150 blend of Z11–14:Ac, E11–14:Ac and 12:Ac and gave a calculated attractant ratio of 3:97 for E11–14:Ac to Z11–13:Ac. Further research with lower ratios is necessary, however, to determine the optimum attractant blend for these two compounds.

Another test (Table 3) was conducted to substantiate further the roles of E11– and Z11–13:Ac. It was shown that Z11–13:Ac did not effect trap catch when added to the two pheromone components, Z11–14:Ac and 12:Ac, at the 5% level, but addition of E11–13:Ac to these components at the same level did produce an attractive blend. The 8% mix of E11–13:Ac with the components did not attract as many males as the natural blend using 8% E11–14:Ac, but this may be due to a lower intrinsic activity of the 13-carbon compound. These data indicate that E11–13:Ac can partially substitute for E11–14:Ac, but Z11–13:Ac cannot. However, when a high quantity (15 mg) of Z11–13:Ac was substituted for 12:Ac in the blend, trap catches were equivalent to the natural blend. A lower quantity (3 mg)

TABLE 3. CAPTURES OF MALE *Argyrotaenia velutinana* BY VARIOUS MIXTURES OF 11-TRIDECENYL, 11-TETRADECENYL, AND DODECYL ACETATES (TEST CONDUCTED IN DRESDEN, NEW YORK, JULY 9–13, 1974)

	Treatment (mg)					
	Z11–14:Ac	E11–14:Ac	Z11–13:Ac	E11–13:Ac	12:Ac	$\bar{x}$/trap[a]
	10.0	—	—	—	15	0.7 (c)
	9.2	—	0.8	—	15	1.0 (c)
	9.2	—	—	0.8	15	3.5 (b)
Pheromone	9.2	0.8	—	—	15	13.5 (a)
	9.2	0.8	15	—	—	11.4 (a)
	9.2	0.8	3	—	—	5.6 (b)
Unbaited trap						0.0 (c)

[a] Treatments replicated and rerandomized three times. Means followed by the same letter are not significantly different at the 5% level.

of Z11–13:Ac was not as attractive; similar results were obtained with lowered quantities of 12:Ac (Roelofs et al., 1975).

Although Z11–13:Ac by itself appeared to substitute for the pheromone components Z11–14:Ac and 12:Ac, surprisingly its effectiveness in doing so was reduced when the natural component 12:Ac was added to the lure (Comeau, 1971). This phenomenon has been observed over several years. A typical test (Table 2) shows that (1) maximum trap catch is obtained with Z/E11–13:Ac ratios of 85:15 and 91:9; (2) these 13-carbon acetate combinations are more attractive than the natural pheromone components; and (3) the addition of 12:Ac or undecyl acetate (11:Ac) to the 11–13:Ac's greatly reduces trap catch. Both 12:Ac and 11:Ac were effective in increasing trap catches when combined with the 11–14:Ac pheromone components (Roelofs and Comeau, 1971), but have the opposite effect when combined with the 11–13:Ac's. Possibly the hypothesized role of Z11–13:Ac in substituting for the sensory input of Z11–14:Ac+12:Ac could be diminished with the addition of 12:Ac. In the latter case, the role of Z11–13:Ac could be reduced to substituting only for Z11–14:Ac, in which case it may not be competitive with the natural component, a case similar to the substitution of E11–13:Ac for E11–14:Ac.

In a number of other Lepidoptera, pheromone analogs have been demonstrated to evoke the same behavioral reactions as the pheromones, but only when the analog stimulus was present in a dosage increased over that of the pheromone. For example, Shorey et al. (1976) found in *Pectinophora gossypiella* (Saunders) that at the same dispenser charges, the analog (Z)-7-hexadecenyl acetate was ca. 100-fold less attractive to males than the pheromone, (Z,Z)-7,11- and (Z,E)-7,11-hexadecadienyl acetates in a 1:1 ratio. Voerman et al. (1975) found Z11–13:Ac could be substituted for Z11–14:Ac in thetwo -component Z11–14:Ac and (Z)-9-tetradecenyl acetate pheromones of *Adoxophyes orana* (Fischer von Röslerstamm) and *Clepsis spectrana* (Treitschke), but with reductions in the levels of attractancy.

In contrast, our tests of *A. velutinana* field attraction showed that Z11– and E11–13:Ac in ca. an 85:15 ratio was at least as efficacious as the natural three–component mixture. The criterion of trap catch, however, is only an approximate measure of the complex orientation and precopulatory behaviors mediated by the pheromone. It is possible that at the natural rate of female pheromone emission an equivalent amount of 11–13:Ac's would be less attractive than the pheromone. Additionally, the threshold of response for activation, upwind orientation, and wing fanning (Baker et al., 1976) may be elevated with 11–13:Ac's compared to the pheromone.

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OVIPOSITIONAL BEHAVIOR OF *Bracon mellitor* SAY,[1] A PARASITOID OF THE BOLL WEEVIL (*Anthonomus grandis* BOHEMAN) III. ISOLATION AND IDENTIFICATION OF NATURAL RELEASERS OF OVIPOSITOR PROBING[2]

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Abstract—The chemical cues in the frass of larvae of the boll weevil, *Anthonomus grandis* Boheman, that elicit an ovipositional response in females of *Bracon mellitor* Say were isolated by a combination of column, thin-layer, and gas–liquid chromatography. Derivatization, analytical tests, and mass spectrometry were used to identify the biologically active materials as long-chain fatty acid esters of cholesterol. Bioassays with natural and synthetic cholesteryl esters were used to confirm activity. The activity of the cholesteryl esters was also confirmed by using females that had not been previously exposed to hosts.

Key Words—*Bracon mellitor*, *Anthonomus grandis*, host-finding, biological control, attractant, oviposition behavior.

[1] Hymenoptera: Braconidae.
[2] Mention of a proprietary or commercial product in this paper does not constitute an endorsement of this product by the U.S. Department of Agriculture.
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INTRODUCTION

Bracon mellitor Say has been reported as the most important parasite of boll weevil, *Anthonomus grandis* Boheman, in the southeastern United States (Cross, 1973). Adams et al. (1969) discussed the general biology of *B. mellitor*, and McGovern and Cross (1974) emphasized the physical aspects of its oviposition. However, very little research has been directed toward the isolation and identification of possible chemical cues used by *B. mellitor* in locating host larvae. Vinson et al. (1976a) reported that methyl *p*-hydroxybenzoate isolated from the frass of diet-reared boll weevil larvae elicited ovipositor probing and, because it was an artificial additive to the diet, might be "associatively learned" by *B. mellitor* females.

In nature, female *B. mellitor* must locate boll weevil larvae inside infested flower buds (squares) and bolls of cotton. Realistically, location of larvae inside these plant structures must be considered in light of cues produced by the cotton plant. Pierce (1908) was among the first to note the consistently higher rates of parasitization in boll weevil-infested squares failing to completely form the abscission layer and "hanging" on the cotton plant. Bottrell and Walker (unpublished data) further implicated the cotton plant's importance by recording significant responses of *B. mellitor* females to freshly abscissed squares independent of boll weevil infestation.

In the current study, we did not set out to mimic the natural ecological conditions present in *B. mellitor's* life system. Instead, we were interested in disclosing chemicals present in cotton bolls and squares which might be utilized by *B. mellitor* females to initiate ovipositional behavior once potential hosts were located.

METHODS AND MATERIALS

Insects

The weevils used in the study were either reared on artificial diet by the method of Vanderzant and Davich (1958) or were collected from field infested cotton squares. The *B. mellitor* were reared from field infested squares. After emergence, adults were held for 2 weeks as described (Vinson et al., 1976a).

Bioassay

The initial bioassay was designed to compare the response of *B. mellitor* to small (1 cm) uninfested squares picked from field cotton and to similar squares showing signs of weevil infestation on the plant before abscission

(infested squares). Thus, 10 uninfested or infested squares were exposed in a petri dish to 20 female parasitoids for 30 min at five time periods after collection. The numbers of females that probed the squares during each 5-min interval for the 30 min were recorded and averaged.

A second bioassay was designed to determine whether the response of the parasitoid demonstrated in the first test was caused by weevil larvae per se or by some modification of the plant produced by the larvae. In one test, parts of infested and uninfested squares were exposed to the female parasitoids as before. In a second test, parts of third-instar boll weevil larvae, fecal material collected from the anus by gently squeezing the larvae, or hemolymph collected with a capillary tube from larvae punctured by a needle were placed separately on a small area of a 9-cm filter-paper disk. If tissue was placed on the disk, it was macerated and the excess was removed. The filter paper was allowed to dry, then exposed to females as before. The numbers of females probing were again recorded at 5-min intervals but for only 25 min.

The third bioassay was used to monitor the active components during isolation. Ten μl of the candidate chemicals was applied to the center of one-half of a 9-cm filter paper disk, and 10 μl of solvent were applied to the other half as a control. Both spots (0.5-cm diam) were lightly outlined in pencil, the solvent was allowed to evaporate, and the paper disk was placed in a cage with 20 female and 20 male *B. mellitor*. The numbers of females probing the treated spot with their ovipositors were recorded at 5-min intervals for 25 min. The tests were replicated twice, and the results were expressed as the percentage of contacts by females that resulted in ovipositor probing.

Chemical Isolation and Identification

Frass from diet-reared and field-collected third-instar boll weevil larvae was extracted three times with chloroform–methanol (2:1v/v), and the extracts were washed according to Folch et al. (1957). The chloroform layer was dried over sodium sulfate, filtered, and concentrated on a rotary evaporator. The activity of the extract was confirmed by bioassay, and the active residue in pentane was place on a 2×20-cm Florisil® column (6% water deactivated). The column was washed with pentane (200 ml) and then with 200-ml volumes of increasing concentrations of methylene chloride in pentane. When bioassay of the column fractions revealed that the activity was concentrated in the 5–10% methylene chloride fractions, the active fractions were pooled, concentrated under a stream of nitrogen, and applied as a band to silica gel thin-layer plates. The plates were developed in pentane-benzene (1:1 v/v), air dried, and bands 2-cm wide were scraped into containers of methylene chloride. The silica gel was mixed thoroughly with the

eluting solvent and filtered. The procedure was repeated twice, and the pooled filtrates were concentrated and bioassayed. The two bands just below the solvent front elicited ovipositional probing activity in *B. mellitor* females and formed a bright pink–red color on TLC plates when sprayed with phosphoric acid: water (1 : 1), indicating a steroidal molecule. Treatment of the material with 0.5 N methanolic potassium hydroxide partially removed the original compound, which suggested an ester function. These preliminary tests and the relative nonpolar nature of the material suggested that an esterified sterol might be present.

The material was treated with 0.5 N sodium methoxide (Luddy et al., 1968), and the ether layer was analyzed for fatty acid methyl esters. The instrument used was a Varian® 2740 gas chromatograph equipped with a flame-ionization detector. The separations were made on a 2-m stainless-steel column packed with 12% diethylene glycol adipate on Gas Chrom Q®. Operating column temperature was 185°C. The fatty acid methyl esters were identified by cochromatography with authentic standards. A Varian® 485 digital integrator was used for peak quantification.

The solvent was removed from the ether layer under a stream of nitrogen and trimethylsilyl ether derivatives of the sterol moiety were formed by using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) as the derivatizing agent. The derivatives were analyzed on a 2-mm (ID) × 2-m glass column packed with 3% OV-17® on Chromosorb W® maintained at 240°C.

RESULTS AND DISCUSSION

When female *B. mellitor* reached a weevil-infested square, they moved over it and palpatated the substrate alternately with the antennae. If frass or damaged areas containing frass were encountered, the female stopped and examined the area with both antennal tips. The antennal examination sometimes took a minute or so or the female might move forward a short distance, raise her abdomen, and probe with her terebra. During ovipositor drilling, the female often continued to examine the area within reach with both antennae (Figure 1). The remainder of the process involved in oviposition was described by McGovern and Cross (1974). Fresh squares elicited less ovipositor probing than infested squares, even after 56 hr (Table 1).

The response of females to various parts of an infested and uninfested cotton square (Table 2) showed that the uninfested squares, and even the uninfested parts of an infested square, elicited no probing behavior. Probing behavior was always associated with frass and feeding activity. The bioassay with various parts of a boll weevil larva revealed that the frass and gut

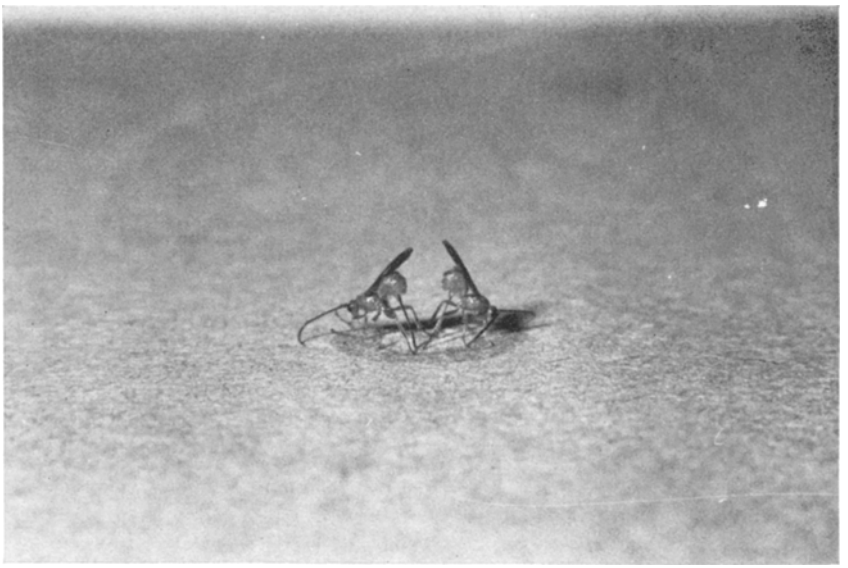

FIG. 1. Ovipositor drilling by *Bracon mellitor* females.

were most active in eliciting probing behavior (Table 3). However, some activity was present in other body regions.

The chemical analysis revealed that the active fraction of boll weevil larval frass contained sterol esters. The fatty acid composition of the sterol esters is shown in Table 4. The five fatty acids are common ones that are known to occur in most insects (Fast, 1966). The high linoleic acid content (45.9%) is consistent with the data reported by Svoboda et al. (1966) for several species of grasshoppers. The selective mechanism in the enzyme

TABLE 1. AVERAGE OVIPOSITIONAL RESPONSE OF *B. mellitor* TO FRESH AND INFESTED SQUARES OVER TIME

	Average number of females probing at indicated hour after collection (3 replicates)				
Material	1/4	4	28	32	56
Fresh squares	0	0.7	0.3	1.7	1.7
Infested squares	10	5	4	3	3.3

TABLE 2. OVIPOSITIONAL RESPONSE OF *B. mellitor* TO PARTS OF INFESTED AND UNINFESTED FIELD-COLLECTED COTTON SQUARES

Cotton part	No. females observed[a]	Average no. responding
Fresh boll	10	0.2
Fresh debracted square	10	0.9
Bracts of fresh square	10	0.1
Infested debracted square	10	5.8
Bracts of infested square	10	0
Damaged fresh square	10	1.6
Uncontaminated part of infested square	20	0
Weevil-contaminated part of infested square	20	6.8

[a] Replicated a minimum of two times with five observations of the number of females responding in 25 min.

TABLE 3. OVIPOSITIONAL PROBING RESPONSE OF *B. mellitor* TO PARTS OF SQUARE-REARED 3RD INSTAR BOLL WEEVIL LARVAE PLACED ON FILTER PAPER DISK

Weevil parts	No. females observed[a]	Average no. responding
Gut	20	2.8
Foregut	10	1.4
Midgut	10	2.2
Hindgut	10	3.2
Fecal material	20	5.0
Malpighian tubule	10	0
Head	10	0.6
Salivary gland	10	1.6
Hemolymph	10	1.2
Cuticle	10	0.4
Fat body	10	0

[a] Replicated a minimum of two times with five observations of the number of responding females in 25 min.

TABLE 4. FATTY ACID COMPOSITION OF CHOLESTERYL ESTERS OF BOLL WEEVIL LARVAL FRASS

Fatty acid	Percent
16:0	24.3
18:0	2.9
18:1	18.0
18:2	45.9
18:3	8.8

system responsible for sterol ester production apparently favors linoleate in these insects.

When the BSTFA derivative mixture was subjected to GLC, a single peak was detected. This peak was identified as cholesterol by comparing GLC retention time to that of an authentic standard, and also by GLC–mass spectrometry. From these identifications and the bioassay data, we can say that cholesterol esterified with five fatty acids acts as an ovipositional probing cue for *B. mellitor* parasitizing boll weevil larvae. Additionally, we found that pure cholesteryl linoleate elicited 22 ovipositional probes by female *B. mellitor* that had not been exposed to boll weevil larvae as opposed to three probes for the solvent control.

As noted, Vinson et al. (1976a) showed that a synthetic ester, methyl *p*-hydroxybenzoate, elicited ovipositional probing by *B. mellitor*. However, the analysis of cotton buds did not reveal any of this ester. Since methyl *p*-hydroxybenzoate is used in artificial diets for boll weevil larvae, which in turn are used for rearing *B. mellitor*, and since inexperienced (not exposed to hosts) *B. mellitor* did not respond to methyl *p*-hydroxybenzoate, the response of the parasitoid to methyl *p*-hydroxybenzoate is probably a learned one (Vinson et al., 1976b). The identification of a common metabolic product as an ovipositional probing stimulant for *B. mellitor* is not surprising since this parasitoid attacks 19 species of Coleoptera and 20 species of Lepidoptera (Cross and Chestnut, 1971), all of which probably produce cholesteryl esters as products of steroid metabolism (Thompson et al., 1973).

The ubiquitous nature of these esters might suggest *B. mellitor* would accept virtually all other insect larvae as hosts. Clearly, this is not the case. Reasons for this probably lie in the host habitat-finding and host-finding cues utilized by *B. mellitor* females and further implicate various host plants housing hosts known to be acceptable to *B. mellitor* as being essential ingredients in the host-finding process of this parasitoid.

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ALARM RESPONSE OF THE MARINE MUD SNAIL, *Nassarius obsoletus*: SPECIFICITY AND BEHAVIORAL PRIORITY[1]

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Abstract—*Nassarius obsoletus*, *N. vibex*, and *N. trivittatus* were exposed to juices of conspecifics and taxonomically and ecologically related gastropod species. Conspecific juices caused the strongest alarm responses, while those from taxonomically related species (congeners) induced less pronounced responses. Juices from nonrelated species induced no alarm response with one notable exception, the strong alarm response of *N. trivittatus* to *Urosalpinx cinerea* juices. Nonrelated gastropod species induced stronger feeding responses than more closely related species. The strongest feeding responses, however, were induced by juices from nongastropod mollusks. The apparent balance between feeding attraction to less related species and alarm response to more related species was further examined by manipulating the snails' diet. Starvation resulted in a great reduction of conspecific alarm responsiveness. Following the resumption of feeding, the original responsiveness was again observed. Although the alarm response is likely an antipredator device, conclusive evidence for its effectiveness in reducing predation is lacking. One of the predators of *Nassarius obsoletus* which may be responsible for maintenance of an alarm response is the green crab, *Carcinus maenas*.

Key Words—*Nassarius obseletus*, *N. vibex*, *N. trivittatus*, *Urosalpinx cinerea*, marine mud snail, alarm response.

INTRODUCTION

The burial alarm reaction of the mud snail, *Nassarius obsoletus*, in response to conspecific body extracts was first reported by Snyder (1967). Atema and

[1] This paper is based in part upon a thesis submitted by D.S. to Boston University for the degree of Master of Arts.

Burd (1975) studied the mud snail alarm response in the field and found that crushed conspecifics elicited burial and escape behavior within a 50-cm-radius area. Atema and Stenzler (1977) examined some of the biological and chemical properties of the alarm substance of *N. obsoletus*. While the function of the alarm response was never demonstrated by direct observation, circumstantial evidence supports the hypothesis that it serves as an anti-predation mechanism. This assumes that predation is greater upon snails that do not burrow into the substrate compared to those that do (Snyder, 1967).

In the laboratory, predation on *N. obsoletus* by the green crab, *Carcinus maenas*, and the moon snail, *Lunatia heros*, was observed regularly. Of these, *L. heros* is the only predator that was actually observed feeding on *N. obsoletus* in the marsh habitat, although the green crab is regularly found among the mud snails on the marsh. Both were tested for their ability to release alarm substance by predation on *N. obsoletus*.

Snyder (1967) pointed out that it would be advantageous for prey species living in the same habitat to respond to each other's alarm substances regardless of their taxonomic relationship, if their predators feed nonselectively. These substances would not have to be similar chemicals, although one might expect to find chemically similar alarm substances in closely related species, regardless of their habitat. Our study examines the question of response specificity of gastropod species related to *Nassarius obsoletus* found in similar and different habitats, and of nonrelated gastropod species found in the same habitat as *N. obsoletus*.

Field observations of the responses of *N. obsoletus* to crushed conspecifics showed that mud snails occasionally fed on conspecifics (Atema and Burd, 1975). Since both burial and feeding responses are initiated by juices released from crushed conspecifics, one might expect that starvation of *N. obsoletus* could cause a decrease in alarm reactivity and an increase in the food-searching behavior of the snail (Snyder and Snyder, 1971). This was tested in the laboratory.

METHODS AND MATERIALS

A. Intra- and Interspecific Responses to Alarm Substances

Nassarius obsoletus (Say) and fresh mud were collected periodically from the flats in Little Sippewissett Marsh, West Falmouth, Massachusetts. The snails, whose average wet weight without the shell was 0.9 g, were kept on mud in a shallow tank with running seawater and were well fed. The water temperatures in the tank ranged from 4 to 23°C depending on the seasons.

Nassarius vibex (Say) was collected with *N. obsoletus* in Little Sippewissett Marsh; additional specimens were obtained from Beaufort, North

Carolina. Periwinkles, *Littorina littorea* (L.), also occur among the *N. obsoletus* and were collected near the periphery of the area occupied by the mud snails. *Nassarius trivittatus* (Say) was collected on subtidal sandy areas off Martha's Vineyard, Massachusetts. The pulmonate snail, *Melampus bidentatus* (Say), was collected around the high-water mark on Little Sippewissett Marsh. Oyster drills, *Urosalpinx cinerea* (Say), were collected at Nobska Point, Woods Hole, Massachusetts, a rocky intertidal area.

All tests were conducted in 40 × 40-cm trays. To prevent chemical contamination from one test to the next, the trays were lined each time with a new sheet of plastic (Kordite Plastic, Mobil Chem. Co.), which did not affect the behavior of the snails. About 1 cm of mud from Little Sippewissett Marsh was placed in each tray and covered with 4.5 liters of fresh seawater, to a depth of 3 cm. The seawater was aerated through glass tubes which were cleaned between tests. The stream of bubbles mixed the water in each tray. In dye tests, gradual but thorough mixing of the water was observed during the time period of our tests. Snails were tested only once, and each experiment was replicated four times, unless otherwise stated.

The standard bioassay to test the reactivity of *N. obsoletus* to alarm substances was conducted as follows:

Ten *N. obsoletus* were arbitrarily taken from the holding tank, placed in a freshly prepared test tray and were allowed to acclimate for approximately 20 hr in the standing water. After 20 hr the temperature in the test tray was between 15 and 23°C, depending on the season. Since *N. obsoletus* shows a considerable amount of spontaneous burial behavior, we placed a half mussel, *Mytilus edulis*, in the center of the test tray to attract buried snails to the surface. Observation started 15 min following the introduction of the mussel, by recording each snail's position, direction of movement, and activity (eating, burrowing, stationary, crawling). A stimulus snail (about 0.9 g wet weight of meat) was then crushed and introduced next to the half mussel (time 0 min). Recordings of all ten mud snails were made at 2, 5 and 10 min after the introduction of the stimulus. As a control, clean chips of oyster shell were introduced to simulate the mechanical but not the chemical part of the stimulus introduction.

The following gastropod species were used as stimuli in the specificity tests: *Nassarius obsoletus* (Say), *Nassarius vibex* (Say), *Nassarius trivittatus* (Say) (all Prosobranchia, Neogastropoda, Nassariidae), *Urosalpinx cinerea* (Say) (Prosobranchia, Neogastropoda, Muricidae), *Littorina littorea* (L.) (Prosobranchia, Mesogastropoda, Littorinidae), and *Melampus bidentatus* (Say) (Pulmonata, Basommatophora) (Hyman, 1967). Depending on the size of the species, 1–11 crushed snails were used to make each stimulus 0.9 g wet snail weight.

The specificity of the alarm response was further tested by measuring the

responses of the other two *Nassarius* species, *N. vibex* and *N. trivittatus*, to the same six stimuli. The standard bioassay had to be modified when it was found that *N. vibex* did not respond to alarm substance while in the presence of a half mussel. It could be shown, however, that it does exhibit a burial response when exposed to alarm substance alone. Single *N. vibex* were, therefore, placed near the center of a test tray directly after a stimulus was placed in the center. The burial response to the six gastropod species and oyster chips was recorded after 5 min. The bioassay for *N. trivittatus* was similar to the standard assay, except sand was used as their natural substrate. Difficulties in obtaining this species made it necessary to expose each individual to more than one test. They were maintained in running seawater for one week between trials.

In the standard bioassay not all 10 snails placed in each test tray came to the surface during the attraction period. At time 0 min, the number of visible snails was always between 6 and 10 (mean number visible = 8.2 ± 0.1 SE) and recorded as 100% visible. For analysis, the number of snails observed at later recording times was expressed as percent of the visible snails at time 0. Thus, a score of over 100% was possible, when new snails emerged during a test. Visible snails are defined as those which were neither totally nor partly buried. Only the burial alarm response was used in the assay recordings; the less common response of crawling away from the source was disregarded. This results in a conservative measure of the total response, especially at 2 min. The Kramer Duncan Multiple Range Test has been used for all the tests of significance (unless otherwise indicated) at the $\alpha = 0.05$ level of significance.

B. Effect of Starvation on the Alarm Response of N. obsoletus

Marsh mud and about 700 snails were freshly collected (day 0) to test the effect of starvation on the alarm response. Mud snails are considered deposit feeders on diatoms and other unicellular organisms in the mud (Brown, 1969). However, like the other carnivorous species of *Nassarius*, *N. obsoletus* is also attracted to dead animal material, on which it can be maintained in the laboratory (Scheltema, 1964). All snails were kept on the original mud in running seawater. Additionally, one group (about 450 snails) was fed a limited supply of 4 mussels daily ("limited mussel diet"), and the other group (about 250 snails) was fed so that excess mussel was always present ("abundant mussel diet").

For the starvation experiment, naive snails were arbitrarily taken from the holding tanks and tested in a modified bioassay, which began by placing a half mussel in the center of the test tray. Ten snails were then placed just around the mussel. After 5 min, the recording was started when a crushed *N.*

obsoletus was placed next to the half mussel (time 0 min). Further recordings were made at 2, 5, and 10 min. In the control trays, only chips of oyster shells were placed next to the mussel. One experimental and one control test were performed once every few days for each feeding group.

On day 6, about half of the limited-mussel-diet snails were placed in a bare, seawater holding tank with running seawater to form the "starved" group. Snails remaining on the mud substrate were still given a limited supply of crushed mussel. On day 25 of the experiment, 70 snails from the limited-mussel-diet group were separated to form the mud-diet group, which received fresh, diatom-rich marsh mud every other day, as well as a limited mussel supply daily.

RESULTS

A. *Intra- and Interspecific Responses to Alarm Substances*

When a crushed mud snail was added to the center of a test tray with feeding *N. obsoletus*, the snails usually responded with proboscis withdrawal from the food within a few seconds. Upon leaving the center of the tray, a snail would occasionally make jerky motions, bringing its shell aperture

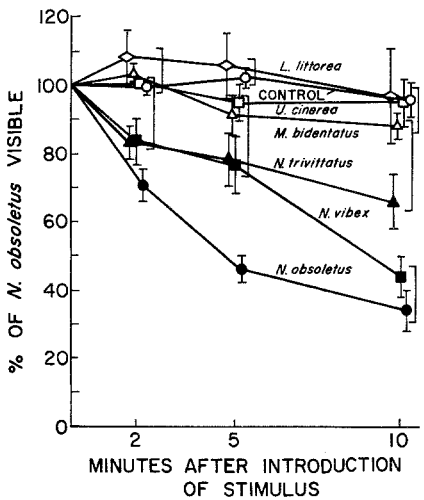

FIG. 1. The mean percent ±SE of *Nassarius obsoletus* visible after the introduction of oyster chips only: Control (○) (14 replicates), crushed *N. obsoletus* (●) (10 replicates), *N. vibex* (■) (8 replicates), *N. trivittatus* (▲) (8 replicates), *M. bidentatus* (△), *U. cinerea* (□), and *L. littorea* (◇) (4 replicates each). Values which are not significantly different are bracketed.

repeatedly down onto the mud surface. Burial occurred within the 10-min period, especially when a snail reached a wall of the test tray, although not all of the mud snails responded. The responses of mud snails after the introduction of a crushed conspecific versus introduction of oyster chips (control) show significant differences ($\alpha = 0.05$) at all time intervals (Figure 1). The responses of *N. obsoletus* to the other five stimuli are shown in the same figure. *N. obsoletus* responds somewhat less to crushed *N. vibex* and *N. trivattatus* than to crushed conspecifics, and it does not respond to crushed *M. bidentatus*, *L. littorea*, and *U. cinerea*.

The burial responses of *Nassarius vibex* to the same 6 species show that *N. vibex* responds less to crushed *N. obsoletus* and *N. trivittatus* than to crushed conspecifics; it does not respond to unrelated species nor to the oyster chip control (Table 1).

Figure 2 shows that *N. trivittatus* responds equally well to *U. cinerea* as to conspecifics. It responds less to *N. obsoletus*, even less to *N. vibex*, and not at all to *L. littorea* and *M. bidentatus*.

B. Effect of Starvation on the Alarm Response of N. obsoletus

As shown in Figure 3, by day 9, snails from the starved and limited-mussel-diet group showed a decrease in alarm reactivity, which persisted throughout the experiment. These snails continued to feed on the half mussel and sometimes on the conspecific. Snails placed in the mud-diet group,

TABLE 1. BURROWING RESPONSE OF *N. vibex* TO CRUSHED STIMULUS SNAILS[a]

Stimulus	Buried	Significance level
Control (oyster chips)	3	0.05*
Nassarius vibex (conspecific)	8	
Nassarius obsoletus	5	0.20
Nassarius trivittatus	5	0.20
Urosalpinx cinerea	1	0.005*
Littorina littorea	1	0.005*
Melampus bidentatus	1	0.005*

[a] Number of *Nassarius vibex* buried 5 min after the introduction of crushed stimulus snails (10 replicates each). Naive *N. vibex* were individually tested with one stimulus only. The number of buried snails was in each case compared to the *N. vibex* conspecific response. Significantly different responses are starred (Fisher Exact Test; Zar, 1974).

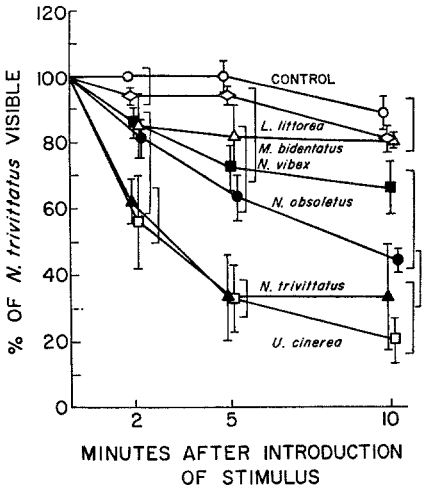

FIG. 2. The mean percent ±SE of *Nassarius trivittatus* visible after the introduction of oyster chips only: Control (○), crushed *N. obsoletus* (●), *N. vibex* (■), *N. trivittatus* (▲), *M. bidentatus* (△), *U. cinerea* (□), and *L. littorea* (◇) (4 replicates each). Values which are not significantly different are bracketed.

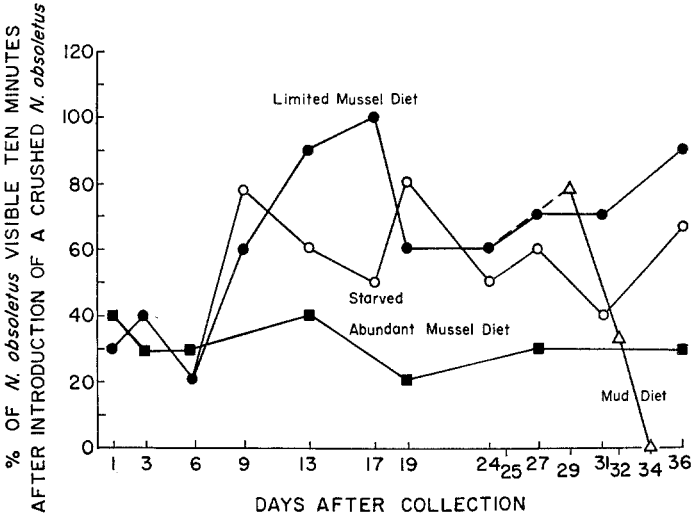

FIG. 3. The percent of *Nassarius obsoletus* visible 10 min after the introduction of crushed *N. obsoletus*: limited mussel diet (●), abundant mussel diet (■), mud diet (△), and starved (○).

however, demonstrated an increased reactivity to the alarm substance. Those snails which received abundant mussel supply throughout never showed a decrease in alarm reactivity. Very few burials were observed in the controls regardless of treatment (not shown).

C. Description of Predation on Mud Snails by the Moon Snail and Green Crab

The interaction between the mud snail and the moon snail, *Lunatia heros*, was studied by placing the two in a 20-cm-diameter finger bowl with mud substrate and 1 liter of seawater which was aerated. The following description has been pieced together from several observations, since the entire predatory act was not usually observed in the laboratory, and only seen once in the field.

Upon encountering the mud snail, the moon snail passes its massive foot over the *N. obsoletus*. As the mud snail is passed posteriorly under the moon snail, it is covered with mucus. *L. heros* carries the mud snail a short distance, tucked under the posterior portion of its foot, causing a visible lump behind its shell. The mud snail is then moved anteriorly and totally surrounded by the moon snail's foot. In this position, the moon snail begins to bore through the mud snail's hard shell with its radula. *L. heros* uses its proboscis to feed

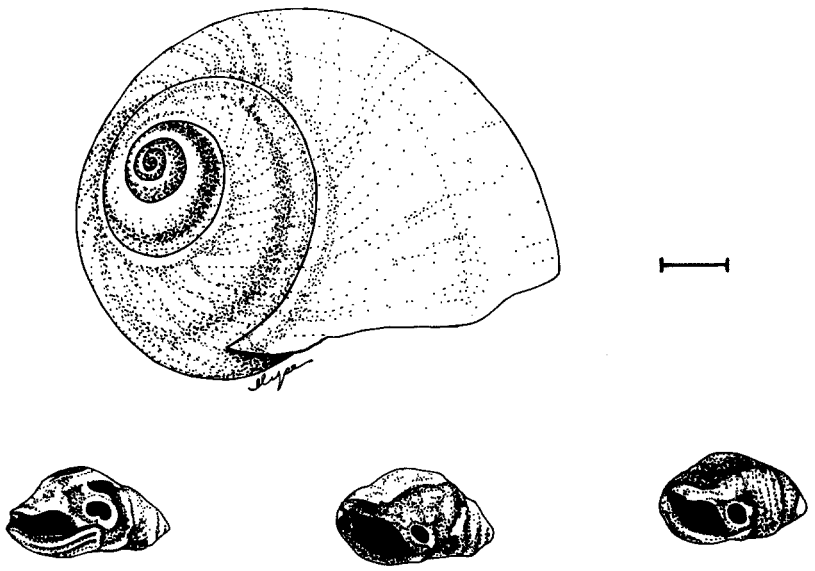

FIG. 4. Three *Nassarius obsoletus* which have been bored and eaten by *Lunatia heros* (larger snail) in laboratory. Scale: 1 cm.

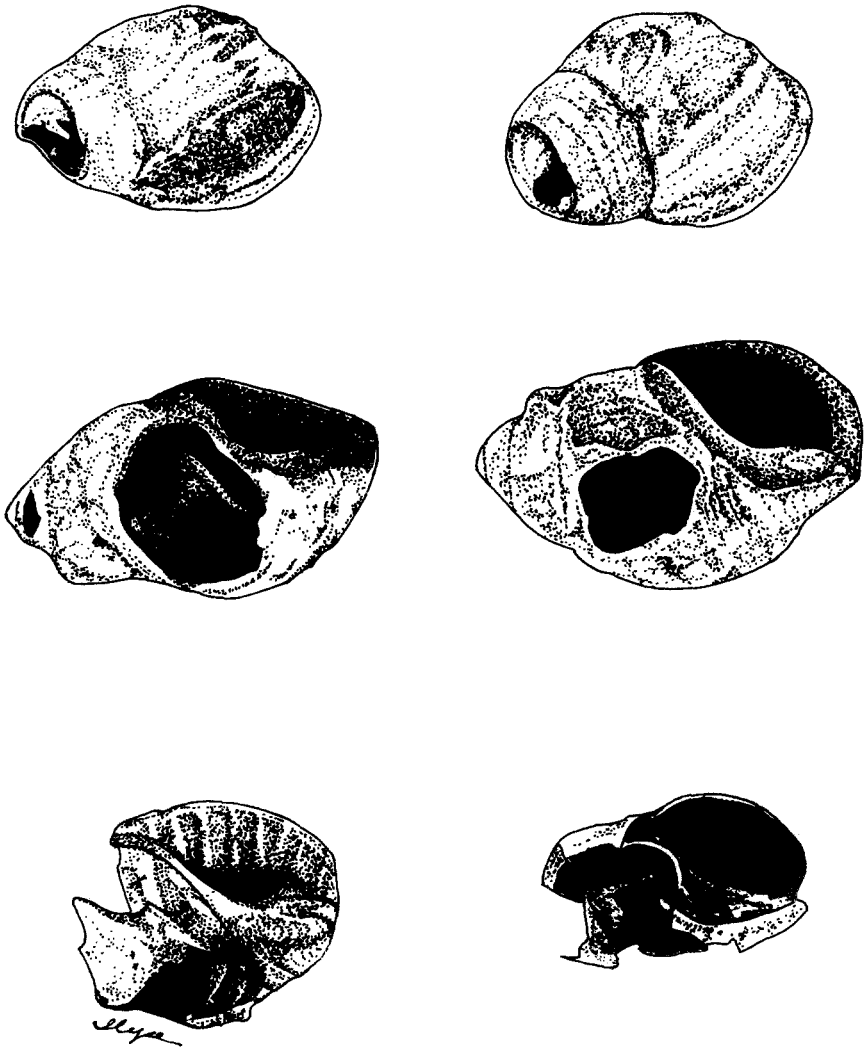

FIG. 5. *Nassarius obsoletus* cracked open and eaten by *Carcinus maenas* in the laboratory (right column); and empty shells found on the marsh among the mud snails (left column), apparently eaten by *C. maenas*.

on the mud snail through the bore hole. The whole process takes several days for completion. The hole is always bored in the same location (Figure 4). Bored shells were rarely found on the marsh.

Predation on mud snails by the green crab, *Carcinus maenas*, was also studied in large finger bowls. Typically, the green crab lifts the snail, apex up,

with one chela. It usually attempts to crush the weak, eroded apex with the other chela. When the apex is finally cracked, it is raised to the mandibles, where bits of shell and viscera are torn off. This is followed by further shell crushing at the same end by the chela, interspersed with feeding from and prying in the cracked end with the dactyl of the first walking leg. The whole process takes about 30 min. Usually, most of the shell is destroyed. However, some snails are discarded after about 10 min, if only the apex could be broken and eaten. In the latter case, the crab usually attempts to feed on another snail almost immediately. Although predation by green crabs was not observed in the field, cracked mud snail shells found there suggest its occurrence (Figure 5).

DISCUSSION

The experiments described in this paper have shown that not only *Nassarius obsoletus*, but also *N. vibex* and *N. trivittatus*, respond to crushed conspecifics with self-burial, which could be a defense mechanism against predation. Interspecific tests with taxonomically and ecologically related species showed that a clear but less pronounced response is caused by the crushed congeners, while the taxonomically unrelated gastropod species (*Melampus bidentatus*, *Littorina littorea*, and *Urosalpinx cinerea*) caused no alarm response in any of the *Nassarius* species except in one case. Snyder (1967) showed that *N. obsoletus* did not give burial responses to juices of 5 species of freshwater gastropods, but did show signs of food-seeking behavior when so stimulated. These 5 freshwater snails conversely showed no alarm responses to juices of *N. obsoletus*, although each possesses an alarm response to intraspecific juice. Thus, it appears that taxonomic relationships similar to those found in fishes (von Frisch, 1941; Pfeiffer, 1963) may exist in the occurrence of alarm substances and responses, while ecological relationships are of less importance. However, since *N. obsoletus* and *N. vibex* have been observed living together on marshes, they may have common predators, and sensitivity to each other's alarm substances may be advantageous. Therefore, an ecological basis for their interspecific alarm reactivity may exist here as well. The exceptional and strong response of *N. trivittatus* to crushed *Urosalpinx cinerea* could possibly be interpreted on an ecological basis, since both species inhabit the inter- and subtidal zones (Smith, 1964). This interaction is of special interest because crushed *U. cinerea* elicited no burial response in the other *Nassarius* species tested. It is thus unlikely that *U. cinerea* contains an alarm substance which is chemically similar to a *Nassarius* alarm substance. This idea is further supported by the taxonomic distance between these species. Apparently, *N. trivittatus* has developed a

special sensitivity to the *Urosalpinx* substance, which could be explained if one assumes that the two species share a predator. In general, interspecific alarm reactivity can provide strong evidence for chemical dissimilarity of alarm substances, but not for their similarity, since any species could evolve a response to more than one substance when such behavior is advantageous for its survival (Snyder, 1967). Among the three *Nassarius* species conspecific responses were stronger than the congeneric responses. Therefore, either their alarm substances are not identical, or possibly a species specific odor accompanies one generic alarm substance.

An interesting contrast appears between alarm burial and feeding attraction. While alarm reactivity increased with taxonomic relatedness, feeding attraction decreased. During tests of alarm responses, we observed that some individuals came to feed on the stimuli. In *N. obsoletus* tests, 1% fed on crushed conspecifics, 2% on *N. vibex*, and 3% on *N. trivittatus* in the presence of the half mussel. In contrast, 11%, 21%, and 21% of the *N. obsoletus* fed on crushed *L. littorea*, *M. bidentatus*, and *U. cinerea*, respectively. An 80% feeding response was shown to the bivalve mollusk, *Mytilus edulis*, alone. Similar results were obtained with *N. vibex*. These results concur with field observations of Atema and Burd (1975), who found strong attraction of *N. obsoletus* to *Modiolus demissus* (bivalve), weaker attraction to *L. littorea*, and strong alarm responses to conspecifics, accompanied by isolated cases of feeding. In the field, some burials were also observed in response to crushed *L. littorea*. What then represents food and what alarm for these snails, and what determines the dominance between the two chemical inputs? Why do some individuals and not others feed on conspecifics? By comparing the chemistry of alarm substances and feeding attractants and their effects on the snail's chemoreceptors and behavioral responses, these questions may be answered. Such work is now in progress. However, by manipulating the snail's hunger state, we already approached the question of balance between feeding and alarm responses.

As cited in Results, snails from the starved and limited-mussel-diet groups continued feeding and responded less to alarm substance than snails in the abundant-mussel-diet group. Furthermore, the mud-diet group returned to normal alarm response levels only after a week on the mud diet. We can conclude that, at least for the purpose of alarm responsiveness, snails from the limited-mussel-diet group were in fact starved. The limited-mussel-diet group probably had exhausted the original mud microflora in a few days, while a few mussels apparently did not appreciably add to their nutritional state. It took a few days of starvation to reach the poor level of alarm responsiveness by day 9. This parallels the return of alarm responsiveness found in mud-diet snails. Here it took seven days of fresh mud supply before alarm responsiveness returned to the levels measured in the apparently

well-fed abundant-mussel-diet group. Thus, quantity of food supply appears to have a profound influence on the response to alarm substance. Diet did not change the production of alarm substance measured in tests with crushed starved snails. Snyder (1967) reported a similar decrease in alarm reactivity after starvation in *Helisoma duryi* (Wetherby). Only after 16 days of starvation did he find a significant decrease in the intraspecific alarm response. Similar results were found for *Pomacea paludosa* (Say) (Snyder and Snyder, 1971).

Thus, responding to alarm substance appears not to be the highest priority behavior for snails under starvation stress. This may also explain the lack of alarm reactivity we observed in *N. vibex* at first. Since nutritional needs of this species were unknown, we may have been starving them. We also observed that copulating mud snails showed no alarm response to crushed conspecifics, thus demonstrating yet another behavioral priority. These priorities are probably determined by the snail's central nervous system. As Gelperin (personal communication) has shown in isolated ganglia-lip preparations, the ganglia from starved garden slugs (*Limax maximus*, Pulmonata) show greater responsiveness to food extracts touched to the slug's lips than the ganglia from well-fed animals.

While we have been able to implicate the effects of nutritional levels and taxonomic (and possibly ecological) relationships on the expression of the alarm response, we have not been able to demonstrate conclusively a biological function of the alarm response. We concur with Kempendorff (1942), Snyder (1967), Snyder and Snyder (1971), and several other authors, based on circumstantial evidence, that alarm burial serves an antipredation function. The evidence, however, deserves some closer scrutiny.

In order for the release of alarm substance to be an effective survival mechanism for a species, certain criteria should be met. First, of course, alarm substance must be liberated in superthreshold quantities during predation. Further, as already suggested by Snyder (1967), predators must be capable of feeding on additional individuals within their effective response time (i.e., the length of time the snails remain burrowed). Finally, the predator must search for prey within the "zone of danger," the area in which the concentration of the alarm substance is high enough to elicit the behavior. In the laboratory, the green crab, *Carcinus maenas*, is capable of feeding on additional snails within a short time period. *Limulus polyphemus* and certain fishes may be capable of doing this also. It is the only apparent predator able to do this. It is likely that our laboratory observation on crab predation will also hold true in field tests. But only field studies can determine if the crab searches in the immediate area for additional prey. It would seem, though, that this predator is fast enough to cover the 50-cm-radius "zone of danger" (Atema and Burd, 1975) in seconds to find undisturbed snails.

Since many burials are rather incomplete, it is also possible that *C. maenas* could easily find buried snails, in which case burial is not an effective survival response. Other animals, such as the horseshoe crab, *Limulus polyphemus*, and perhaps some fishes may have similar predator characteristics and could have reinforced the alarm response. Thus far, direct observations of predation on *N. obsoletus* are lacking, and only broken shells provide indirect field evidence for *C. maenas* predation. Direct field observations of predation would contribute evidence toward the hypothesis that alarm responses evolved to avoid predation. And it should be possible to test experimentally the survival value of the alarm response at the individual level simply by measuring survival from predation in reactive and nonreactive individuals. Even then, it will remain a difficult task to demonstrate at the population level that predation in *Nassarius obsoletus* is significantly reduced by responding to alarm substance.

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ALARM SUBSTANCE OF THE MARINE MUD SNAIL, *Nassarius obsoletus*: BIOLOGICAL CHARACTERIZATION AND POSSIBLE EVOLUTION[1]

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Abstract—The gastropod snail *Nassarius obsoletus* shows a dramatic self-burial response to the presence of crushed conspecifics. After it was shown that this burial alarm response could be reliably replicated in laboratory tests, a further characterization of the alarm substance was undertaken. Dilution experiments showed a very high response threshold resulting in a short effective radius of the substance in agreement with earlier field reports. Longevity experiments showed that the substance had lost some activity after 16 hr standing over marsh mud in sea water at room temperature; it became inactive after 24 hr. Superthreshold concentration in sea water was not necessary to keep the snails buried: Mud apparently provides an adsorption surface which can remain a stimulus source for previously unalarmed snails, and snails tend to remain buried after a short exposure to alarm substance, even when given a fresh environment. The substance is present in the snail's blood and tissues and is passively released. A potential natural predator capable of such release is *Carcinus maenas*, the green crab. Predator odor alone did not cause burial alarm responses. Preliminary chemical analysis indicates that the substance is water soluble, heat stable, and of high apparent molecular weight (over 100,000). A comparison with fish alarm substance and response is made in a discussion of the possible evolution of chemically triggered alarm responses. It is argued that *N. obsoletus* may have developed an alarm response to an existing nonspecific substance rather than a true alarm pheromone.

Key Words—*Nassarius obsoletus*, snails, alarm substance.

[1] This paper is based in part upon a thesis submitted by D.S. to Boston University for the degree of Master of Arts.

INTRODUCTION

Alarm responses elicited by chemical substances passively released from wounded conspecifics have been described for aquatic animals of widely different taxa, such as fish (von Frisch, 1938, 1941a, b; Pfeiffer, 1963, review), amphibian tadpoles (Eibl-Eibesfeldt, 1949; Hrbácek, 1950; Kulzer, 1954), gastropod snails (Kempendorff, 1942; Snyder, 1967; Snyder and Snyder, 1971; Atema and Burd, 1975; Stenzler and Atema, 1977), sea urchins (Snyder and Snyder, 1970), sea anemones (Howe and Sheikh, 1975), and turbellarians (Heinz, 1954). Their independent evolution in so many different groups is sufficient evidence for the biological significance of these substances. It is generally assumed that alarm substances serve as a defense system against predators (Snyder, 1967), although direct evidence for their effectiveness to reduce predator efficiency has not been demonstrated experimentally. Supporting evidence for the predator defense hypothesis can be found in the specificity with which prey species respond to alarm substances. For example, the fresh water minnow *Phoxinus laevis* quickly flees from areas where a wounded conspecific has left alarm substance; the intensity and pattern of the alarm response depends on the quantity of substance released as well as on a number of other factors (von Frisch, 1941a,b). The same minnow "freezes" and sinks slowly to the bottom when faced with the odor of its natural predator, the pike (Göz, 1941). There are two important aspects to this: (1) Responses to alarm substance are different from responses to predator odor, each having its own adaptive significance, and (2) the responses appear modifiable under varying environmental and physiological conditions.

Another example of predator-specific responses can be observed in the gastropod snail, *Nassarius vibex*. This snail buries itself in response to conspecific body juices (Snyder, 1967), but emerges from the sand and moves away from the odor of the predatory snails, *Fasciolaria tulipa* and *F. hunteria*, and the sea star, *Luidia alternata* (Gore, 1966). When actually touched by these predators or excised parts of them, *N. vibex* shows a violent flipping response. Other sea stars, *Astropecten duplicatus* and *Echinaster sentus*, do not elicit these responses in *N. vibex* (Gore, 1966). The responses are adaptive because all three predators could probably uncover a buried *N. vibex* and establish a good hold on it. Moving away is thus the safest avoidance. However, if contact does take place, violent flipping can prevent the predator from attaching to the snail. The burial response to conspecific body juice was apparently enforced by other predators, whose identities are thus far unknown. The other two sea stars are probably not predatory on *N. vibex*: *E. sentus* is not sympatric with *N. vibex*, and *A. duplicatus* may not pose such a serious danger to the snail population as to warrant the evolution of a

behavioral defense stimulated by odor. This leads to two related questions, which will be discussed at the end of this paper: (1) Why would predator odor detection alone not be sufficient since it could trigger a "selfish" escape response, and (2) how could potentially altruistic behavior of maintaining alarm substance have evolved?

The typical response of the mud snail, *Nassarius obsoletus*, to the presence of conspecific body juices is burial (Snyder, 1967; Atema and Burd 1975; Stenzler and Atema, 1977). The juices of other molluscan species from its salt marsh habitat elicit attraction (Atema and Burd, 1975). Snyder (1967) found that *N. obsoletus* had a long-lasting alarm response and a high threshold compared to those of the many gastropod snails he tested. Atema and Burd (1975) also noted that the alarm response in the field lasted perhaps as long as 24 hr, thus surviving two tidal flushings. This parallels the observation that minnows sometimes avoided their feeding area in the lake for days after alarm substance was released there (von Frisch, 1938, 1941a,b).

Attempts to characterize and identify an aquatic alarm substance have been made for fish. It was concluded that club cells in the skin of ostariophysine fishes are the source of their alarm substance, which is passively released by predator damage (Reutter and Pfeiffer, 1973). The substance was identified as a pterin with a molecular weight below 500, perhaps isoxanthopterin (Pfeiffer and Lemke, 1973). An alarm substance of the sea anemone, *Anthopleura elegantissima*, was described and identified with a molecular weight of 213.5 (Howe and Sheikh, 1975). The present paper describes biological and some chemical properties of the alarm substance of *N. obsoletus*, followed by a discussion of their evolution and biological significance.

METHODS AND MATERIALS

Characterization of Alarm Substance: Threshold, Longevity under Natural Conditions, Mud Adsorption, Lasting Response to Short Exposure

For laboratory testing, *Nassarius obsoletus* (Say) and mud were collected periodically from the flats in Little Sippewissett Marsh, West Falmouth, Massachusetts. The snails, whose average wet weight without the shell was 0.9 g, were kept on mud in a shallow tank with running ambient seawater. The water temperatures in the holding tank ranged from 4 and 23°C with the seasons. Tests were conducted with freshly collected snails and marsh mud in flat trays (40 × 40 cm) with new plastic liners for each test. A layer of 1 cm of mud was placed in the trays and covered with 4.5 liters of seawater, to a depth of 3 cm. Clean glass tubes introduced air into the water and

caused thorough mixing as indicated by dye tests. Each experiment was replicated four times unless otherwise indicated.

For data analysis and representation, the number of snails visible at any recording time in the tests was expressed in percentage. At time 0 min, the number of snails visible was always recorded as 100% visible. Visible snails are defined as those which were neither totally nor partly buried. Only the burial alarm response was used in these tests, disregarding the less common response of crawling away from the source. This results in a conservative measure of the total response, especially at 2 min. The Kramer Duncan Multiple Range Test has been used for all the tests of significance (unless otherwise indicated) at the $\alpha = 0.05$ level of significance.

To calculate the threshold concentration of alarm substance required to elicit the alarm response in *Nassarius obsoletus*, an extract was prepared by homogenizing 1.1 g wet weight of snail meat (without shell) in 5 ml distilled water. After filtration, 4 ml were introduced into prepared test trays without snails. After 10 min, when the extract was distributed throughout the tray, a half mussel was placed in the center as a focus for feeding attraction and 10 *N. obsoletus* were placed directly around it (time 0 min). The snails' activities were recorded at 2, 5, and 10 min after their introduction. The final concentration in the tray was calculated as 200 mg of snail meat/liter. Further dilutions of the original extract permitted the testing of concentrations of 75 mg/liter and 20 mg/liter. As a control identical tests were run, but with 4 ml distilled water added to the test trays instead of crushed snail extract.

The results show that only concentrations of 200 mg of snail tissue/liter elicited responses which were similar to the field alarm response (Atema and Burd, 1975) and the standard crushed snail bioassay (Stenzler and Atema, 1977) at 2, 5 and 10 min. Lower concentrations did not elicit the burial response, never differing significantly from controls at any time interval (Figure 1).

To test the longevity of alarm substance in seawater standing over marsh mud, its natural environment, the threshold test procedure was used with a snail extract concentration of 200 mg/liter. But here, the time interval between introduction of the snail extract and half mussel plus 10 mud snails was varied from 10 min to 1 hr, 11 hr, 16 hr, and 24 hr. The control was the same as in the threshold test. In longer tests the temperature in the test trays varied from 11 to 18°C due to daily temperature changes in the laboratory.

The test results showed a significant difference between control and test responses at all recording times for 10-min-, 1-hr-, 11-hr-, and 16-hr-old snail extract. There was no significant difference between the 10-min, 1-hr, and 11-hr responses at the 5-min and 10-min recording. Thus, the alarm substance lost some activity between 11 and 16 hr, and became inactive between 16 and 24 hr (Figure 2).

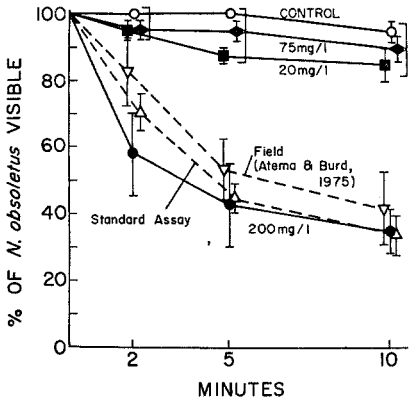

FIG. 1. The mean percent ±SE of *Nassarius obsoletus* visible after their introduction into water with a conspecific extract concentration of 200 mg snail meat/liter (●), 75 mg/liter (◆), and 20 mg/liter (■). The control snails were placed in trays free of snail extract (○). Values which are not significantly different are bracketed. For reference, the percent of *N. obsoletus* ±SE visible after introduction of a crushed conspecific in the laboratory standard bioassay (Stenzler and Atema, 1977) (△), and in the field (Atema and Burd, 1975) (▽).

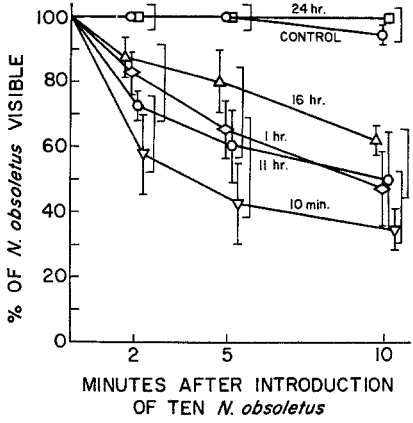

FIG. 2. The mean percent ±SE of *Nassarius obsoletus* visible after their introduction into water with a conspecific extract concentration of 200 mg snail meat/liter. The time intervals between the extract and *N. obsoletus* introductions were 10 min (▽), 1 hr (◇), 11 hr (○), 16 hr (△), and 24 hr (□). The control snails were placed in trays free of snail extract (○). Values which are not significantly different are bracketed.

To determine whether the long-lasting burial alarm reaction is in response to a short exposure to alarm substance, or is maintained by the constant presence of alarm substance in the water column, or by adsorption onto the mud, the following tests were done.

1. Superthreshold Concentration of Alarm Substance in the Water Column. Ten *Nassarius obsoletus* were placed in a freshly prepared test tray and given 20 hr acclimation. After a 15-min mussel-attraction period, a crushed conspecific was introduced and the snails' behavior was recorded 2, 5, and 10 min after that introduction. The water was then siphoned from the tray and the half mussel attractant and crushed *N. obsoletus* stimulus were removed. Then 4.5 liters of clean seawater were siphoned into the tray (water change took about 10 min) and a new half mussel was placed in the center of the tray. The number of mud snails visible was again recorded at 0, 2, 5, and 10 min after the introduction of the second mussel. In the control tests an identical procedure was followed, except that the same water was siphoned directly back into the tray from which it came, thus maintaining the same concentration of alarm substance. A second control for the possible effect of water changing itself was done by adding oyster chips to a test tray instead of crushed *N. obsoletus*. A clean water change was performed for this second control group.

These experiments showed the not surprising result that before water changes the responses of the two groups of *N. obsoletus* exposed to crushed conspecifics did not differ significantly (30% and 26% visible after 10 min) and that both were significantly different from controls (90% visible after 10 min). However, after the two different water change procedures, there was still no significant difference between the numbers of snails visible in the clean water tests and in control (same water) tests at all recording times (about 30%), while both groups remained significantly different from the oyster chip controls (about 90%).

2. Mud-Adsorbed Alarm Substance. To test this hypothesis, the same experiment was performed as above but without adding the 10 test snails for the first part. After the mussel and crushed snail were removed, the water was replaced with clean water. Ten mud snails which happened to be buried in the holding tank were manually unburied, rinsed 5 times in clean seawater, and placed in the tray without mussel present (time 0 min). Recordings were made at 2, 5, and 10 min after their introduction. As a control, 10 buried snails were manually unburied from the holding tank, rinsed, and placed in a fresh tray without mussel or crushed snail.

The tests for mud adsorption showed that when fresh snails were placed on possibly contaminated mud from the clean-water-change experiment, the number of *N. obsoletus* visible at 5 and 10 min differed significantly from controls (Figure 3).

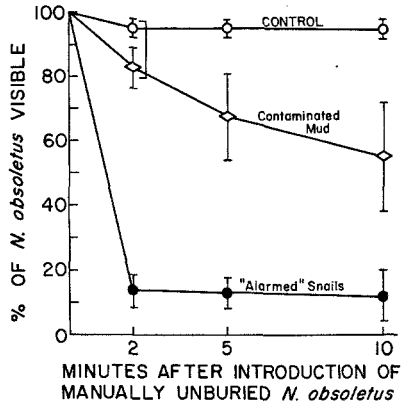

FIG. 3. The mean percent ±SE of *Nassarius obsoletus* visible in fresh trays after they were manually unburied from the holding tank (CONTROL) (○), and from test trays which were treated with crushed *N. obsoletus* and a clean water change ("Alarmed" Snails) (●). *N. obsoletus* were also manually unburied from the holding tank and placed in trays which were treated with crushed *N. obsoletus* and a clean water change (Contaminated Mud) (◇). Values which are not significantly different are bracketed.

3. Lasting Response to Short Exposure. Snails which had remained buried at the end of the first experiment after the clean water change were manually unburied, rinsed 5 times in clean seawater, and placed in a freshly prepared tray without mussel present. Recordings were made as before. Control as in the second experiment.

The results of the single dose response tests showed that most of the previously "alarmed" snails that were manually unburied and rinsed, buried again immediately in the fresh trays. The number of these snails at 2, 5, and 10 min differed significantly from control, which had not been exposed to alarm substance (Figure 3).

These last three tests show that a superthreshold concentration of alarm substance in the water column is not necessary to maintain the buried state. Apparently, the marsh mud provides an adsorption surface for the alarm substance, which remains a stimulus source for unalarmed snails, and previously alarmed snails stay in an alarmed state in the absence of the alarm stimulus.

Source and Mode of Release

To determine the source of the alarm substance in *Nassarius obsoletus*

we used the standard bioassay (Stenzler and Atema, 1977) with the following modifications: at time 0 min, 4 ml of filtered extract were pipetted over the feeding snails while 4 ml of filtered seawater served as the control. Two major portions of the snail were examined: the foot, which is protrusible through the aperture of the shell, and the visceral mass, which is permanently contained within the shell. Extracts were made by homogenizing these tissues in a tissue grinder with 5 ml of seawater. In tests with blood as a stimulus, a total of 0.5 ml of snail blood was taken from the feet of about 10 mud snails by making a few fine pin holes in their extended feet. This method was adopted after it was shown to yield similar results to blood drawn directly from the heart. The 0.5-ml sample was introduced at time 0 min in the test trays.

To test whether the alarm substance is actively released, a mud snail was hit between two large rocks so as to put a visible crack in its hard shell. This unusual treatment was considered alarming, while no damage to the inner tissues was observed. The crack-shell snail was then used as a stimulus source and placed in the bioassay test tray at time 0 min. In the control, chips of oyster shell were introduced to simulate the mechanical part of placing the mud snail into the test tray.

A more natural way to test for active release was to place a natural predator, the moon snail, *Lunatia heros*, with a mud snail in a bowl with mud substrate and aerated seawater. They contacted each other within 5 min. Afterwards, the mud snail was placed in a test tray to serve as a stimulus for 10 conspecifics to test if the alarm substance was being released actively as a result of this encounter. The oyster chip control was the same as in the standard bioassay.

Since active release of alarm substance may cease immediately after breaking contact with the predator, a longer test was done by again placing the two in the bowl. Within 18 hr, the *L. heros* was in a boring–feeding position and the mud snail concealed by the moon snail's massive foot (Stenzler and Atema, 1977). A 4-ml water sample was taken from the bowl at that time and tested for alarm substance release using the liquid extract bioassay. Water samples were tested 3 times per day for the following 3 days while the moon snail was still in the feeding position. Introductions of 4 ml of seawater served as controls.

To test if moon snail odor alone causes mud snails to actively release the alarm substance or to exhibit the alarm reaction, a *L. heros* was kept for 20 hr in a bowl with 1 liter of seawater. Four ml of this *L. heros* odor water were pipetted into the test tray, the normal test procedure was used, and 4 ml of seawater served as controls.

When another marsh predator was discovered, the green crab, *Carcinus maenas* (Stenzler and Atema, 1977), similar tests were conducted with crab

odor and mud snail predation water using a slightly different procedure, in which response snails were placed in stimulus water. This modification did not introduce significant changes in the snails' responses. One *C. maenas* and 5 *N. obsoletus* were placed in a 20-cm-diameter finger bowl with mud substrate and 1 liter of seawater. During the first 30 min, the crab had eaten one snail. The crab and the remaining snails were then removed, and 10 naive mud snails were placed in the bowl. Their behavior was recorded 2, 5, and 10 min after their introduction. The snails were then removed, mud stirred, and slime trails cleaned from the sides of the bowl. Five trials with new snails were recorded in that bowl. As a control, a crab and 5 snails were kept mechanically isolated from each other by a perforated plastic screen. Water from this bowl was tested as above after 30 min. Water with 30-min snail odor alone and crab odor alone were also tested. Since unusual responses were observed after 20 hr of exposure to crab plus snail odor, the responses of snails to 20-hr snail odor alone and crab odor alone were also examined.

RESULTS

The *Nassarius obsoletus* response to both conspecific foot and visceral mass tissue extracts and to blood differed significantly from the control at all time intervals. There was no significant difference between the responses to foot extract, visceral mass extract, and blood (Figure 4). The alarm substance is apparently present throughout the snail's tissues.

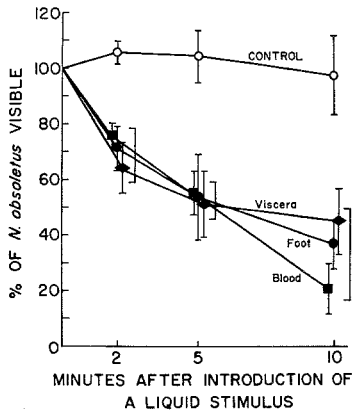

FIG. 4. The mean percent ±SE of *Nassarius obsoletus* visible after the introduction of 4 ml of seawater control (○) (6 replicates), *N. obsoletus* viscera extract (◆), foot extract (●), and 0. 5ml of blood (■). Values which are not significantly different are bracketed.

Active release of alarm substance does not appear to take place, since burial behavior was not significantly different from controls, whether the stimulus consisted of cracked-shell snails, or snails which had been in contact with *Lunatia heros*, or water from a bowl with a feeding *L. heros*. Similarly, 30-min crab plus snail odor (screen-separated animals) and 30-min crab or snail odor, or 20-hr snail odor alone had no effect. Only when *Carcinus maenas* crushed and began feeding on *N. obsoletus* did the surrounding water induce the alarm response in other snails. This indicates that physical damage to the snail's body is necessary to release the alarm substance. In fact, even when the shell of a mud snail was carefully peeled away completely except for a small attachment to the columellar muscle, the naked snail joined its feeding conspecifics without causing them to alarm. However, as soon as the last piece of shell was cut from the muscle, the alarm response was induced. This example dramatically illustrates the passive nature of alarm substance release.

When moon snail odor alone was introduced into the test trays, an unusual number of burials *and* unburials were observed (unlike the response to the alarm substance). Since only the number of snails visible at any time interval was used in the analysis, the overall response to this odor is not borne out in this representation. When 20-hr green crab odor was presented to the snails, they began to crawl out of the test bowls, but showed no signs of burying. Thus, distinctly different responses to predator odor and to alarm substance were observed in *Nassarius obsoletus*.

Preliminary Chemical Analysis

To determine some general chemical characteristics of the alarm substance, filtered snail homogenate was boiled for 5 min over a flame. The resulting precipitate was filtered out. The filtrate was tested and found as active as regular alarm substance.

In a second series of tests the filtered homogenate was extracted with hexane. The activity was recovered from the water contained in the resultant emulsion which formed above the water fraction. Neither the water fraction nor the hexane fraction was active. When the active fraction was boiled for 5 min in a water bath, the activity remained.

An attempt was made to determine molecular weight by running the hexane-purified extract through various grades of Sephadex columns. The activity remained at the eluant front even when G-100 was used, indicating an apparent molecular weight of about 100,000 or greater. Minor activity was recovered in a fraction of lower molecular weight. Further analysis is in progress.

DISCUSSION

This study has shown that the alarm response of *Nassarius obsoletus* occurs equally well and with a similar time course in the laboratory and in the field. Based on such tests further characterization of the alarm response and substance could be undertaken. Our threshold results agree with Snyder's (1967) in that a stimulus dilution of 75 mg snail meat/liter or less did not elicit the alarm response. In other words, the substance should cause a short-range effect. This is confirmed by the field observation that an area of only 50-cm radius is affected (Atema and Burd, 1975). Snyder found that the *N. obsoletus* response had the highest threshold of all those tested in his snail alarm survey study.

When Atema and Burd (1975) noted that few snails were visible the following day in previously tested areas on the marsh, they suggested that the alarm effect survived two tidal flushings. In the laboratory the alarm substance did not lose its activity until left standing 16–24 hr in seawater over marsh mud at room temperature. Further tests showed that the substance also adsorbed onto the mud and still retained its activity. Finally, "memory" may also be involved, since the snails remained buried after a brief exposure to the alarm substance, which was subsequently removed. Together, these results could easily explain the long-lasting response in the field. Similar observations on fish were made by von Frisch (1938), who noted that minnows (*Phoxinus laevis*) avoided a feeding area in the lake for several days after being exposed to alarm substance there. These fish appear to have a space memory connected with the alarm response.

The alarm responses of the mud snail and of the ostariophysine fishes are similar in that the alarm substances are likely only passively released. However, while in fish the substance is released from specialized cells in the skin of the animal (Reutter and Pfeiffer, 1973), in *Nassarius obsoletus* the substance appears to be present in the blood and all its tissues. Perhaps it should be mentioned that von Frisch (1941b) found low levels of alarm substance present in *Phoxinus* ovaries (100 times less than in skin), muscle tissue (20 times less) and gills (5–10 times less), which could lead us to believe that in fish the substance is not present *only* in the specialized club cells of the skin.

The release of the alarm substance was caused by *Carcinus maenas* (feeding on mud snails) but not by *Lunatia heros*. An explanation may be that the massive foot of *L. heros* (or mucus coating) sealed off the whole feeding area, or that its proboscis sealed off the bored hole through which it feeds (Stenzler and Atema, 1977), thus causing insufficient release of alarm substance in the surrounding water. *C. maenas*, on the contrary, is a sloppy feeder which scatters food particles while ripping and "chewing" its food

(Stenzler and Atema, 1977). It can be assumed that a predator like *C. maenas*, rather than like *L. heros*, selected for the evolution of the *N. obsoletus* alarm substance. In the field, the responses to predator odor alone are probably not meaningful, since mud snails are not likely to come into contact with anything like 1 liter of water that has surrounded a predator for 20 hr.

Another difference between *Phoxinus* and *Nassarius* alarm substances is their chemistry. As far as determined, the fish substance is a relatively small molecule with a molecular weight below 500 (Pfeiffer and Lemke, 1973), while the mud snail substance appears to be of high molecular weight, perhaps over 100,000. Snyder (1966) found the alarm substance of another gastropod *Helisoma duryi* to be of relatively high (5000–10,000) molecular weight. It has been shown that other high-molecular-weight compounds play an important role in eliciting proboscis extension in *Nassarius* (Carr, 1974). Therefore, both feeding behavior and alarm responses can be elicited by high-molecular-weight molecules, something not frequently considered in the past. The nutrition-dependent balance between feeding attraction and alarm repulsion (Stenzler and Atema, 1977) could further tempt us to speculate that perhaps the two responses are released by similar or even related molecules. This is not so strange as it may at first appear. *Nassarius obsoletus*, being a facultative scavenger, shows good feeding attraction to various snail body juices including in exceptional cases its own conspecific juices (see also Stenzler and Atema, 1977). It may be reasonable to assume that all this snail has done is evolve the behavioral alarm response to an already existing substance and its sensory processing. The following discussion of the possible evolution of alarm responses may provide further context for this speculation.

Evolution of Alarm Response and Substance

The simplest assumption about the evolution of alarm responses is that detection of conspecific body juices decreases the probability of conspecific individuals becoming prey. The response thus increases the fitness of the species, presumably because the presence of these juices would indicate that a predator was actually "at work." This would make a difference from detecting a predator by its odor, since the latter would cause snails to stop feeding and bury whenever the predator is near, even when he does not "hunt." Snyder (1967) and Snyder and Snyder (1971) have speculated along these same lines, arguing that the balance between time and energy spent on feeding versus alarming must be subjected to predator pressure. The snail's choice at the individual level may be starvation versus being eaten. When this pressure is not too great and when feeding is time consuming, the snails benefit from not responding to predator presence per se. Perhaps in terms of the whole population the resulting loss of few individuals is outweighed by the avail-

ability of a greater number of well-fed individuals. In other words, whenever we find alarm responses but not predator odor detection, as in *Nassarius obsoletus*, we can assume relatively light predator pressure on a population that must graze extensively to feed itself.

For a species to arrive at this state of conspecific body juice detection, the individuals must develop (1) a chemoreceptor site capable of recognizing a conspecific metabolic product which is liberated in the environment during predation, and (2) the appropriate escape behavior. It was speculated above that *N. obsoletus* may already have had the proper receptor sites, and only needed to modify the behavioral response to its input.

This may be the approximate state in which we find contemporary *Nassarius* species. It was shown that three species of the *Nassarius* genus respond to each other's alarm substance, that they respond strongest to the conspecific substance and not to juices from other gastropod genera (Stenzler and Atema, 1977). The one exception to this rule was the strong response of *N. trivittatus* to the juices of the sympatric, but taxonomically not closely related, *Urosalpinx cinerea* (Stenzler and Atema, 1977). This exception supports the assumption of the evolutionary state of *Nassarius*; we do not have to invoke complex molecular relationships between *Nassarius* and *Urosalpinx* juices if we assume that it became advantageous for *N. trivittatus* to respond to *U. cinerea* juices. Sharing a common predator would be sufficient "reason" for this development. *Nassarius* species seem to be predisposed to develop this type of a response.

If indeed the receptor sites and the appropriate response are the only evolutionary adaptations, then the alarm substance does not serve a specific signal function. In fact, detection of alarm substance appears very similar to prey or predator detection. In the latter case it seems very clear that no "intentional" (chemical) signal has evolved to establish "communication" between prey and predator (Burghardt, 1970). Thus, the alarm substance should not be considered part of a communication system and should not be classified as a pheromone.

The situation changes when we consider fish alarm substances. Although these substances may be present in small amounts in various tissues and organs (von Frisch, 1941), there are good arguments that the main source of it is in the club cells of the skin. These cells are thus far not known to have any function (Reutter and Pfeiffer, 1973). If such a function cannot be demonstrated, we can argue that in the course of evolution the substance has taken on a special signal function, carried largely if not entirely for the purpose of warning conspecifics. The fish alarm substance would thus be a true pheromone. Since a metabolic price must be paid for the production and maintenance of club cells and pheromone, we now deal with a case of altruism: It is likely that the victim does not itself benefit from the metabolic

expense incurred. The altruistic aspects of this evolution are even more striking when we see that a varying threshold has evolved in the responding fish population: Some low-threshold individuals, usually the perhaps more expendable males, start darting away long before their schoolmates detect the alarm substance. The behavior of these "alarmists" acts as an amplifier and visually causes alarm in the rest of the school. It also draws the attention of the predator, which will inevitably attack the "odd one" (Gandolfi, Mainardi and Rossi, 1968a,b).

Finally it should be mentioned that alarm responses seem to be rather common among gastropod snails (Snyder, 1967), and it is reasonable to assume that this group is predisposed to develop the appropriate receptor sites, neurobiology, and behavior. The same could be said for the ostariophysine group of fishes (Pfeiffer, 1963). Perhaps the most intriguing questions we are left with are the mechanisms by which these and other chemoreceptor sites evolve and the neurobiological processes that make such dramatic shifts in behavior possible.

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CHEMICALLY MEDIATED HOST FINDING BY *Biosteres (Opius) longicaudatus*, A PARASITOID OF TEPHRITID FRUIT FLY LARVAE

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Abstract—Host finding by the fruit fly parasitoid *Biosteres (Opius) longicaudatus* Ashmead was found to involve attraction to fermentation products emanating from rotting fruit, a probable site for location of host larvae. Bioassays conducted in the greenhouse with all saturated 1-, 2-, and 3-carbon primary alcohols, aldehydes, and organic acids indicated that acetaldehyde was the most active agent followed by ethanol and acetic acid. Rotting fruit was attractive irrespective of the presence of host larvae, and attraction was attributed to fungal fermentation products rather than to kairomones from host larvae.

Key Words—Host finding, *Biosteres (Opius) longicaudatus* Ashmead, ethanol, acetaldehyde, fungi, fermentation, Tephritidae, parasitoid.

INTRODUCTION

Appreciable attention has been directed recently to elucidation of the physical and chemical stimuli used in host finding by parasitic wasps, and a number of kairomones have been chemically identified (Vinson, 1975, 1976). In addition to cases involving kairomones, wherein the parasitoids respond to compounds produced by the host insects themselves, there are other instances in which parasitoids are attracted to compounds associated with their hosts but not produced by them. For example, Spradbery (1970) found that certain

parasitoids of siricid woodwasps are attracted to odors produced by fungal symbionts of their hosts, and Vinson (1975) reported that some parasitoids are attracted to odors liberated from wounded plant tissues. Although these types of host indicators cannot be regarded as true kairomones, they function similarly. There appears to be no recognized term available to describe such compounds as they do not comply with any of the definitions given by Nordlund and Lewis (1976) for various types of allelochemics.

The object of the present study was to investigate the mechanisms of host finding used by the braconid *Biosteres* (*Opius*) *longicaudatus* Ashmead. This solitary endoparasitoid attacks larvae of a variety of Tephritidae (Greany et al., 1976), normally encountering them in decomposing fruit. We obtained evidence indicating that host finding involves attraction to specific fermentation products emanating from rotting fruit. These compounds were produced not by host larvae, but by fungi growing in the fruit.

METHODS

Bioassay Techniques

Cultures of *B. longicaudatus* were maintained using larvae of the Caribbean fruit fly, *Anastrepha suspensa* (Loew), as the laboratory host (Greany et al., 1976). Bioassays were conducted in screen cages in a greenhouse maintained at $28 \pm 4°C$ and ca. 50% relative humidity. The cages were $36 \times 36 \times 46$ cm and were constructed of aluminum frame with fiberglass window screen covering all surfaces other than the end that contained a sleeve. Candidate attractants and solvent controls (100 μl ea.) were applied to 4.3 cm-diam pieces of filter paper enclosed in evaporators. The evaporators were 20-dr snap-cap plastic vials modified by replacing the bottom with nylon netting (ca. 1-mm^2 openings) and with a 7-mm-diam orifice in the cap. Two evaporators (treatment and control) were suspended in each cage 10 cm from the top and 15 cm from either end (16 cm apart), with the netted end abutted against the upwind side of the cage. Air was drawn through the cages at a rate of 1.5 m/sec by the greenhouse exhaust fan.

Bioassays were performed with 100 5–6-day-old *B. longicaudatus* females, and at least 1 hr was allowed for acclimation prior to bioassay. The parasitoids were used not more than twice each day, with at least 4 hr between bioassays. All tests were conducted between 9:00 AM and 4:00 PM; no time-of-day effects were noted during this period. The peak number of parasitoids observed inside and on the surface of the vials within 5 min after application of material to the evaporators was recorded for both the test and control units.

Due to the fact that a simple cage was used as an olfactometer, not

all test insects were exposed to the odorants; i.e., some were located on the top and sides of the cage out of the air stream of either the treatment or control evaporator. We therefore tried to increase the proportion of the test population that was attracted by using other types of olfactometers, including Y tubes. None of these other olfactometers allowed the parasitoids to fly freely, and they proved less satisfactory than the screen cages because orientation to the odor source apparently occurred only after initiation of flight. Thus, a fairly large number of parasitoids (100) was exposed so as to obtain observable differences in capture rates between treatment and control units. Tests conducted in the laboratory failed to elicit desired responses, whereas these activities were readily displayed in the greenhouse, perhaps due to the high intensity light that prevailed there.

Microbiological

Cultures of microorganisms were obtained from rotting peaches and were isolated on acidified potato-dextrose-agar. Colonies that developed after 7 days at 22–23°C were identified by the methods of Alexopoulas (1962) and Barnett and Hunter (1972).

Chemical

Crude extracts were prepared from rotting peaches using methanol, which preliminary studies showed to be superior to less polar solvents for extraction of the attractant. The peach pits were removed and the tissue was homogenized in a blender. The homogenate was centrifuged and the supernatant held aside. The precipitate was rehomogenized in reagent-grade methanol with 25 ml/10 g tissue, and this homogenate was also centrifuged. The supernatants from both steps were combined and distilled at atmospheric pressure using an 8-cm Vigreux column. Three fractions were collected (64–66°C, 67–90°C, plus pot residue), and the activity of each was monitored by bioassay.

Gas–liquid chromatographic (GLC) analyses of the test materials were conducted using a Packard Model 804® equipped with a flame ionization detector and 2 m × 2.3 mm (ID) stainless-steel columns operated with an He carrier gas flow rate of 20 cc/min. All active distillation fractions were analyzed by GLC on Porapak Q, 80/100 mesh (Waters Associates). This column was operated either isothermally at 170°C or was programmed from 140 to 220°C at 8°/min.

Identification of the major peak in active distillation fractions was facilitated by comparing the GLC retention times of known standards with

that of the major peak, and by comparing the retention times of 3,5-dinitrobenzoate (3,5-DNB) derivatives of standard alcohols with the retention time of the derivatized unknown. Derivatives were prepared from the corresponding benzoyl chlorides according to the procedure of Shriner et al. (1956). Benzoate derivatives were analyzed by GLC on 5% OV-101 on 80/100 mesh Chromsorb G-HP; the column was operated isothermally at 200°C.

RESULTS AND DISCUSSION

In preliminary studies of host finding by *B. longicaudatus*, it was noted that the parasitoid females, but not males, were attracted to peaches that had begun to decompose either due to mechanical injury or because of infestation by Caribbean fruit fly larvae. Further studies showed that fresh, uninfested peaches were not attractive. As the presence of host larvae apparently was not necessary to produce activity, all subsequent extractions were made using rotting but uninfested fruit. These findings agree with those of Nishida and Napompeth (1974), who found that *B. longicaudatus*, *Opius oophilus* Fullaway, and *O. incisi* Silvestri were attracted to uninfested fruits as readily as to infested ones; however, they did not state whether the fruit was rotting. Nishida (1956) also demonstrated that *O. fietcheri* Silvestri was attracted to host plant tissue rather than to host larvae. Thorpe and Caudle (1938) described other instances in which parasitoids were attracted to the food plants of their hosts.

The fungus obtained from rotting fruit was identified as *Monolinia fructicola* (Wint.), known to cause brown rot in plums, apples, pears and peaches (Dunegan 1953). To establish the role of this fungus in attraction, fresh peaches were disinfected with 1% NaOCl, rinsed with sterile water, inoculated with pure colonies of *M. fructicola*, and allowed to incubate. After moderate decomposition, an extract was prepared and found to attract a mean ($\pm$SE) of $7.7 \pm 2.0\%$ of the females vs. $3.1 \pm 0.8\%$ for the control units (100 μl of each solution applied; 10 replicates; significantly different at the 1% level by χ^2 analysis). This extract was found to contain 0.13% ethanol by GLC analysis. Next, pure cultures of *M. fructicola* were incubated in potato-dextrose broth, which was then centrifuged and the supernatant bioassayed. This solution was active also, attracting a mean of $8.0 \pm 1.0\%$ of the females vs. $3.1 \pm 1.0\%$ for the controls (10 replicates, significantly different at the 1% level by χ^2 analysis). These tests suggested not only that it was the fungus that produced the attractant(s), but also that it could do so even without being incubated on fruit. A similar test was conducted using a pure culture of *Penicillium digitatum* Sacc. grown on disinfected grapefruit. This extract (0.37% ethanol by GLC) was active also ($8.2 \pm 1.0\%$ vs. $1.8 \pm$

0.5% attraction to treatment and control units, respectively), indicating that attraction was probably due to a common fermentation product(s).

Following demonstration of the role of fungi in attraction, chemical fractionation studies were performed to isolate and identify the attractant(s). Distillation fractions collected from 64 to 66°C and from 67 to 90°C were both active, but the higher boiling fraction was more active. Gas chromatography of both fractions showed a major peak with a retention time slightly greater than that of methanol, but identical to that of ethanol. Coinjection of ethanol with active fractions resulted in addition to the major peak rather than appearance of a new peak, suggesting that the peak corresponded to ethanol. Furthermore, derivatization of an active distillation fraction to the 3,5-DNB yielded a product with a retention time identical to that of a 3,5-DNB derivative of ethanol, corroborating identification of the peak as ethanol. Gas chromatography showed that the lower boiling fraction of most crude extracts contained about 0.2–0.4% ethanol, presumably carried over by codistillation with methanol, whereas the higher boiling fraction contained about 0.7–0.95% ethanol.

These studies suggested that ethanol might serve as an attractant for *B. longicaudatus* females; hence bioassays were performed using aqueous solutions of ethanol. Aliquots of 100 μl of a 50% solution attracted a mean of 8.5% of the parasitoids, whereas absolute ethanol attracted 14.5% (Table 1). When a 10% solution was tested, however, the level of response was less than expected (Table 1), and no significant difference was detected between treatment and control units. The ethanol concentration of crude extracts averaged less than 1% by GLC analysis, yet they were more attractive to the parasitoids than 10% ethanol, indicating involvement of additional components in the active fractions Tests were therefore conducted on 1-, 2-, and 3-carbon saturated alcohols, aldehydes, and organic acids. Of these, acetaldehyde was found to be ca. 50 times as attractive as ethanol, with 1% acetaldehyde capturing as many parasitoids as 50% ethanol (Table 1). Acetic acid also showed some activity (Table 1). Attraction was not observed in response to 10 or 50% methanol or propanol, 0.1 or 1.0% formaldehyde or propionaldehyde, or 0.1 or 1.0% formic or propionic acid. Although attempts were not made to isolate acetaldehyde or acetic acid from the crude extracts tested in the present study, oxidation of ethanol to acetaldehyde in fruit is a well-known phenomenon (Davis, 1970), and acetaldehyde and acetic acid have previously been reported from peaches (Power and Chestnut, 1921).

Ethanol has been reported as an attractant for a number of other insects, including the scolytid beetle *Trypodendron domesticum* (L.) (Kerck, 1972). Other instances of attraction to ethanol and acetic acid are cited by Dethier (1947). Acetaldehyde was reported to be slightly attractive to the codling

TABLE 1. RESPONSE OF *Biosteres longicaudatus* FEMALES TO CERTAIN FERMENTATION PRODUCTS[a]

Concentration (%)	No. trials	No. parasitoids counted[b]				
		Total[c]			Mean[d]	
		Treatment		Control	Treatment	Control
Ethanol						
10	9	24	(NSD)	14	2.7±0.6 (a)	1.6±0.3 (a)
50	17	152	(**)	18	8.9±0.7 (b)	1.1±0.3 (a)
100	10	145	(**)	14	14.5±2.7 (c)	1.4±0.3 (a)
Acetaldehyde						
0.01	9	41	(*)	24	4.6±0.7 (a)	2.7±0.5 (a)
0.1	10	73	(**)	3	7.3±0.7 (b)	0.3±0.1 (b)
1.0	13	113	(**)	15	8.7±0.6 (b)	1.2±0.3 (b)
Acetic acid						
1.0	10	32	(**)	19	3.2±1.0 (a)	1.9±0.7 (a)
10	10	38	(**)	14	3.8±0.6 (a)	1.4±0.4 (a)

[a] Each evaporator contained 100 μl of an aqueous dilution of the candidate attractant (treatment unit) or distilled water (control unit).
[b] Peak number of *B. longicaudatus* females present during 5-min bioassay period; each cage provisioned with 100 females.
[c] Treatment and control totals significantly different at the 5% (*) or 1% (**) level by χ^2 analysis (NSD = not significantly different).
[d] Means followed by different letters in the same column for a given compound are significantly different at the 1% level by Duncan's new multiple range test.

moth, *Laspeyresia pomonella* (L.) (Eyer and Medler, 1940), but no other published accounts of attraction to acetaldehyde were discovered.

Field trials are needed to verify the attractiveness of ethanol, acetaldehyde, and acetic acid alone and in combination upon presentation to a wild population of *B. longicaudatus*. While additional compounds and physical cues may be used in host finding by *B. longicaudatus* females, the findings of the present study agree well with our field observations in which *B. longicaudatus* females were seen probing for hosts only on fruits which had begun to decompose. Upon inspection, these fruits generally contained nearly mature host larvae, the preferred stage. Considering the number of tephritid species attacked by *B. longicaudatus* (Greany et al. 1976), and the even wider host plant ranges of these fruit flies, it is significant that the parasitoids apparently use fermentation products as host indicators. These compounds would normally be associated with infested fruit, irrespective of the species of host or host plant.

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SYNERGISTIC INHIBITORY EFFECTS OF *p*-COUMARIC AND FERULIC ACIDS ON GERMINATION AND GROWTH OF GRAIN SORGHUM

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Abstract—The data support the hypothesis that there is a synergistic phytotoxic effect when *p*-coumaric and ferulic acids are found together. Equimolar mixtures of both acids showed greater reduction in sorghum seed germination, shoot elongation, and total seedling growth than either phytotoxin caused when alone. Repeated experiments showed mixtures containing 5×10^{-3} M *p*-coumaric and 5×10^{-3} M ferulic acids reduced germination to 34% of controls after 24 hr and 59% by 48 hr. The same concentration of either phenol-treated seeds alone showed 69 and 92% germination at comparable times. The phytotoxic action of the combination approximated the inhibitory effect on germination of 10^{-2} M ferulic acid and was a greater reduction than caused by 10^{-2} M *p*-coumaric treatments. Sorghum seedling growth was more sensitive than germination, with an equimolar mixture of 2.5×10^{-4} M *p*-coumaric and 2.5×10^{-4} M ferulic acids reducing seedling dry weight significantly below weights of seedlings treated separately with 2.5×10^{-4} M *p*-coumaric or ferulic acids. Further dilutions showed a 1.25×10^{-4} M concentration of either phenol was stimulatory to seedling growth, whereas a mixture of these two produced inhibition.

Key Words—synergism, ferulic acid, *p*-coumaric acid, allelopathy, phytotoxicity, inhibition, sorghum.

INTRODUCTION

Paracoumaric acid and ferulic acid, both phenolic compounds, have been found together in many plants and soils and have been shown repeatedly

to inhibit both seed germination and overall growth of several plants. Whitehead (1964) identified *p*-coumaric and ferulic acids together in four different types of soil. Guenzi and McCalla (1966a,b) found several phenols, including *p*-coumaric and ferulic acids, in residues from oats, wheat, sorghum, and corn and in soils under these crops. Wang et al. (1967) found both *p*-coumaric and ferulic acids to be significantly inhibitory to the growth of young sugar cane at a concentration of 50 ppm, and they reported inhibition from a mixture of five phenolic acids which included these two. They also extracted both of these phenols from soils in Taiwan, with *p*-coumaric acid usually being present in larger quantities. They found phenols present in the soil at concentrations that would inhibit the growth of corn, wheat, and soya beans growing in nutrient culture solutions. Hennequin and Juste (1967) found both *p*-coumaric and ferulic acids to be phytotoxic to germination and seedling growth of maize, and they reported finding both phenols in many cultivated soils. More recently, Chou and Muller's (1972) work on the allelopathic nature of *Arctostaphylos glandulosa* var. *zacaensis* showed these two phenols in soils under this shrub, along with several other phytotoxins.

Abdul-Wahab and Rice (1967) found *p*-coumaric acid to be one of the primary inhibitors in *Sorghum halepense*. Del Moral and Muller (1970) reported *p*-coumaric and ferulic acids and eight other phenols in the leachate from the litter of *Eucalyptus camaldulensis*. The 10 isolated phenolic toxins inhibited herb growth in laboratory, greenhouse, and field tests. Rasmussen and Rice (1971) extracted large quantities of both *p*-coumaric and ferulic acids from shoot residue of *Sporobolus pyramidatus*, and both were found to be very inhibitory to germination of *Amaranthus palmeri* seed. These are also two of the toxins found in *Celtis laevigata* leaves (Lodhi and Rice, 1971), and other examples could be noted. Demos et al. (1975) reported that both phenols inhibited hypocotyl growth of mung bean.

Thus *p*-coumaric and ferulic acids are often found together in both plant and soil, and they also occur in combinations with other inhibitory phenolic compounds. Although both have been shown to have phytotoxic effects on germination and/or plant growth and development, evidence is minimal on the effects that these phenols may have when they are in combination with one another as they are often found. Several investigators have mentioned the possibility of additive and/or synergistic effects (Wilson and Rice, 1969; Einhellig, et al., 1970; McCalla, 1971), but few experiments have demonstrated this conjecture. Lodhi (1975) reported apparent cumulative effects of *p*-coumaric, ferulic, and caffeic acids isolated from soils.

Because of the small quantities of phenolic inhibitors often noted in allelopathic plants or the soil, it is important to establish whether their inhibitory effects on plant growth act in supportive ways, or whether their effects are completely independent of each other, perhaps even cancelling

each other. This project was carried out to determine the possibility of additive or synergistic inhibitory effects of *p*-coumaric and ferulic acids on seed germination and plant growth.

METHODS AND MATERIALS

Grain sorghum (Hybrid 701, Gurney's Nursery, Yankton, South Dakota) was chosen as the test species for germination and growth bioassays because of its sensitivity to phenols and because it is an agronomic crop often infested by allelopathic weeds.

Seed germination bioassays were accomplished in petri dishes (15 cm) containing a disk of Whatman No. 1 paper. In each experimental group 200 seeds were used for a test group, with 50 seeds per petri dish. After initial screening experiments established the threshold level of inhibition of sorghum germination by these two phenols, the experimental design involved determining seed germination rate in separate *p*-coumaric and ferulic acid-treated groups and in combinations. Aqueous solution of 10^{-2} M or 5×10^{-3} M solutions of *p*-coumaric and ferulic acids, 10 ml/petri dish, were added to make the appropriate tests. Combination dishes contained 5 ml of 10^{-2} M *p*-coumaric and 5 ml of 10^{-2} M ferulic acids, making an effective concentration of 5×10^{-3} M *p*-coumaric and 5×10^{-3} M ferulic acids in these chambers (total phenolic molar concentration equals 10^{-2} M). Since no difference in germination was found in procedures using a buffered medium compared to deionized water, control seed dishes contained 10 ml distilled water with the pH adjusted to that of the test solutions (3.9). Seeds were allowed to germinate in darkness at 25°C with germination checked periodically from 12 to 72 hr using a 2-mm extrusion of the radical as the germination criterion. Germination experiments were replicated five times.

In two replicas of similar design to that used in seed germination, shoot and root lengths were measured after 7 days for all those seeds that showed germination.

Experiments designed to test the effects of *p*-coumaric and ferulic acids on total plant growth utilized the test phenols dissolved in nutrient culture (Floyd and Rice, 1967; Rasmussen and Einhellig, 1975). Sorghum seedlings were germinated in vermiculite for 8 days, individually transplanted to 35-ml light-free vials containing a 2:5 aqueous dilution of Hoagland's (Hoagland and Arnon, 1950) nutrient solution, and then allowed several days acclimatization before treatment. Seedlings were treated by replacing the diluted nutrient solution with solutions containing appropriate phenols dissolved in 2:5 Hoagland's; thus all groups had equal nutrient solution. Plants were grown 10 days in a Percival growth chamber illuminated with 20,000 lux

over a 16-hr photoperiod and day/night temperatures of 30/18°C. Guided by initial empirical tests of the effects of various concentrations of phenols, test solutions for these growth experiments were: control, 5×10^{-4} M *p*-coumaric, 5×10^{-4} M ferulic, 2.5×10^{-4} M *p*-coumaric, 2.5×10^{-4} M ferulic, and a mixture of equal volumes of 5×10^{-4} M ferulic with 5×10^{-4} M *p*-coumaric. This latter test mixture gave a solution with both 2.5×10^{-4} M *p*-coumaric and 2.5×10^{-4} M ferulic acids for a combined vial molarity of 5×10^{-4} M phenolic acids. When sorghum growth was terminated, dry weights were taken after 48 hr at 105°C and comparisons made using a *t*-test.

Seedling growth was also tested using more dilute solutions, 1.25×10^{-4} M *p*-coumaric and 1.25×10^{-4} M ferulic, as well as a mixture with the equivalent of these two phenolic concentrations. This latter experiment followed the previous procedures except that plants were grown under more variable, winter greenhouse conditions with supplemental light providing a minimum of 10,000 lux for 12 hr.

RESULTS

Since all five replicas of the seed germination experiments showed similar trends, data of Table 1 show only two of these replicas and the combined totals. The data demonstrate that both 10^{-2} M *p*-coumaric and 10^{-2} M ferulic acid treatments severely reduced seed germination and, although continuous germination counts are not shown for 60- and 72-hr intervals, some reduction carried through the duration of the experiment. Initially 5×10^{-3} M concentrations of these phenols depressed germination, but within 48 hr germination of seeds under these conditions was approaching that of controls (Table 1). In the combination solution of *p*-coumaric plus ferulic acid the germination percentages demonstrate that the mutually cooperative action of these substances produced an effect greater than any component taken alone (synergism). It can be noted that the inhibitory effect of the mixture was greater than the inhibitory effect of 5×10^{-3} M *p*-coumaric plus 5×10^{-3} M ferulic treatments (Table 1). While the inhibition shown in the *p*-coumaric–ferulic acid mixture is approximately an additive effect of the components at 24 hr, by 36 hr inhibition shown in the mixture is considerably greater than the sum of the separate components.

Effects of 10^{-2} M concentrations of both *p*-coumaric and ferulic acids are also shown for comparison (Table 1) since the phenolic mixtures could be construed to have an effective concentration of total phenols of 10^{-2} M. Germination data from the phenolic combination are considerably below that of 10^{-2} M *p*-coumaric, and they approximate that of the 10^{-2} M ferulic-acid condition (Table 1).

TABLE 1. EFFECTS OF *p*-COUMARIC AND FERULIC ACIDS ON SORGHUM SEED GERMINATION

Treatment	Exp.	Seed germination								
		24 hr			36 hr			48 hr		
		No. germ	Dif. from control	% Of control	No. germ.	Dif. from control	% Of control	No. germ.	Dif. from control	% Of control
Control	1	105			157			166		
	2[a]	117			161			171		
	Total[b]	484			754			827		
10^{-2} M ferulic acid	1	33	(−72)	31.4	85	(−72)	54.1	101	(−65)	60.8
	2[a]	49	(−68)	41.9	87	(−74)	54.0	109	(−62)	63.7
	Total[b]	155	(−329)	32.0	364	(−390)	48.3	501	(−326)	60.6
10^{-2} M *p*-coumaric acid	1	49	(−56)	46.7	110	(−47)	70.1	131	(−35)	78.9
	2[a]	57	(−60)	48.7	109	(−52)	67.7	130	(−41)	76.0
	Total[b]	213	(−271)	44.0	463	(−291)	61.4	571	(−256)	69.0
5×10^{-3} M ferulic acid	1	90	(−15)	85.7	148	(−9)	94.3	170	(+4)	102.4
	2[a]	109	(−8)	93.2	153	(−8)	95.0	168	(−3)	98.2
	Total[b]	334	(−150)	69.0	647	(−107)	85.8	766	(−61)	92.6
5×10^{-3} M *p*-coumaric acid	1	95	(−10)	90.5	151	(−6)	96.2	163	(−3)	98.2
	2[a]	83	(−34)	70.9	145	(−16)	90.1	158	(−13)	92.4
	Total[b]	332	(−152)	68.6	651	(−103)	86.3	763	(−64)	92.3
Combination:	1	40	(−65)[c]	38.1	85	(−72)[c]	54.1	98	(−68)[c]	59.0
5×10^{-3} M ferulic with	2[a]	58	(−59)[c]	49.6	94	(−67)[c]	58.4	119	(−52)[c]	69.6
5×10^{-3} M *p*-coumaric acid	Total[b]	*165*	(−319)[c]	*34.1*	374	(−380)[c]	*49.6*	486	(−341)[c]	*58.8*
Summation of data:	1		(−25)			(−15)			(+1)	
5×10^{-3} M ferulic acid	2[a]		(−42)			(−24)			(−16)	
and 5×10^{-3} M	Total[b]		(−302)			(−210)			(−125)	
p-coumaric acid (added—not in combination)										

[a] Duplicate experiment.
[b] Total data of 5 duplicate experiments.
[c] Synergistic effects shown.

TABLE 2. EFFECTS OF *p*-COUMARIC AND FERULIC ACIDS ON SEEDLING GROWTH OF SORGHUM

Treatment	Experiment	Mean shoot length[a] (mm ± SE)
Control	1	42 ± 1.2
	2[b]	25 ± 0.8
10^{-2} M ferulic acid	1	16 ± 0.6[c]
	2[b]	11 ± 0.4[c]
10^{-2} M *p*-coumaric acid	1	21 ± 0.6[c]
	2[b]	9 ± 0.3[c]
5×10^{-3} M ferulic acid	1	25 ± 0.6[c,d]
	2[b]	16 ± 0.5[c,d]
5×10^{-3} M *p*-coumaric acid	1	26 ± 0.5[c,d]
	2[b]	13 ± 0.4[c,d]
Combination:		
5×10^{-3} M *p*-coumaric with	1	17 ± 0.6[c]
5×10^{-3} M ferulic acid	2[b]	8 ± 0.4[c]

[a] Based on all seeds germinated after 7 days.
[b] Duplicate experiment.
[c] Difference from control mean significant at 5% level or better.
[d] Difference from combination mean significant at 5% level or better.

TABLE 3. EFFECTS OF *p*-COUMARIC AND FERULIC ACIDS ON SORGHUM GROWTH

Treatment	Experiment	Mean[a] oven-dry weight (mg ± SE)
Control	1	141.6 ± 13.9
	2[b]	177.8 ± 15.2
5×10^{-4} M ferulic acid	1	95.2 ± 12.6[c]
	2[b]	83.3 ± 9.3[c]
5×10^{-4} M *p*-coumaric acid	1	101.4 ± 16.1
	2[b]	62.3 ± 4.4[c]
2.5×10^{-4} M ferulic acid	1	129.4 ± 12.6[d]
	2[b]	142 ± 8.1[c,d]
2.5×10^{-4} M *p*-coumaric acid	1	128.7 ± 8.9[d]
	2[b]	134.3 ± 12.1[c,d]
Combination:		
2.5×10^{-4} M ferulic with	1	93.6 ± 11.1[c]
2.5×10^{-4} M *p*-coumaric acid	2[b]	75.9 ± 6.4[c]

[a] Each mean represents 13 plants.
[b] Duplicate experiment.
[c] Difference from control mean significant at 5% level or better.
[d] Difference from combination significant at 5% level or better.

TABLE 4. EFFECTS OF *p*-COUMARIC AND FERULIC ACID ON SORGHUM GROWTH

Treatment	Mean[a] oven-dry weight (mg ± SE)
Control	94 ± 6.2
2.5×10^{-4} M ferulic acid	71 ± 3.5[b]
2.5×10^{-4} M *p*-coumaric acid	68 ± 4.4[b]
1.25×10^{-4} M ferulic acid	101 ± 7.4[c]
1.25×10^{-4} M *p*-coumaric acid	102 ± 7.7[c]
Combination:	
1.25×10^{-4} M ferulic acid with 1.25×10^{-4} M *p*-coumaric acid	83 ± 5.1

[a] Each mean represents 12 plants.
[b] Difference from control mean significant at 5% level or better.
[c] Difference from combination significant at 5% level or better.

Ferulic and *p*-coumaric acids were both significantly inhibitory to the growth of sorghum shoots and roots. While control seed germination produced a long primary root and usually adventitous roots, treated seedlings were very short or immeasurable and roots were often dark-tipped. After 7 days germination, treated seedlings showed an average shoot length reduction below that of the control shoots (Table 2). However, the *p*-coumaric–ferulic acid mixture inhibited shoot elongation more than either 5×10^{-3} M *p*-coumaric or 5×10^{-3} M ferulic acids alone, an effect that can be considered synergistic.

Experiments with sorghum seedlings demonstrate that seedling growth was even more sensitive to phenolic treatment than germination since 5×10^{-4} M *p*-coumaric or 5×10^{-4} M ferulic significantly inhibited growth in all cases, and 2.5×10^{-4} M concentrations of both compounds showed significant growth reduction in one of the duplicate experiments (Table 3). The mixture of 2.5×10^{-4} M *p*-coumaric with 2.5×10^{-4} M ferulic produced significantly more inhibition than either of these phenolic acids alone, demonstrating their cooperative influences on growth.

The growth experiment utilizing 1.25×10^{-4} M concentrations of both phenols and a combination of these two showed that the action of the mixture of 1.25×10^{-4} M *p*-coumaric with 1.25×10^{-4} M ferulic acid was inhibitory whereas the separate phenolic dilutions slightly stimulated sorghum seedling growth (Table 4).

DISCUSSION

When the data of Tables 1–4 are evaluated by comparing the total effects of a mixture of *p*-coumaric and ferulic acids to the separate action of either phenol alone, it is clear that a cooperative inhibitory action occurred in each case. The total effect is greater than the sum of the effects of each phenolic acid taken independently. This synergistic effect is most evident in seed germination as time progresses (48 hr), but even at early germination counts there is at least an additive inhibition (Table 1). Sorghum seedling growth was more sensitive to these two phenolic acids, showing strong inhibition at concentrations of 2.5×10^{-4} M and 5×10^{-4} M (Table 3), whereas threshold inhibition of germination required a 10-fold increase in concentration (5×10^{-3} M) for either *p*-coumaric or ferulic acids (Table 1). When evaluating the synergistic effects of a *p*-coumaric–ferulic acid mixture on growth, the cooperative action is most obvious with the dilute mixture of 1.25×10^{-4} M *p*-coumaric and 1.25×10^{-4} M ferulic acids which showed inhibition of seedling growth. Separate treatments of these phenolic concentrations actually stimulated seedlings.

Several mechanisms for *p*-coumaric or ferulic phytotoxic action have been suggested. Gortner and Kent (1958) considered *p*-coumaric to be a coenzyme for activation of IAA oxidase of pineapple, and low concentrations of ferulic acid also activated this enzyme system. Zenk and Muller (1963) also found both *p*-coumaric and ferulic acids to be activators of the IAA oxidizing system and, additionally, *p*-coumaric appeared to reduce uptake of IAA. Kadlec (1973) reported *p*-coumaric acid to greatly inhibit photosynthetic rate in sorghum leaves, and Demos et al. (1975) reported *p*-coumaric inhibited respiration and prevented substrate-supported Ca^{2+} and PO_4^- transport in isolated hypocotyl mitochondria of mung bean. However, Demos et al. (1975) did not find ferulic acid to affect respiration.

While mechanisms for cooperative effects of *p*-coumaric and ferulic acid inhibition have not been studied, the synergistic action shown in germination and growth of sorghum supports previous contentions that small quantities of several individual phytotoxic phenols may act together in producing allelopathic effects noted in natural communities and agronomic fields.

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FEMALE PHEROMONE IN THE BLACK MOLLY FISH (*Mollinenesia latipinna*): A POSSIBLE METABOLIC CORRELATE[1]

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Abstract—The female black molly *Mollinenesia latipinna* emits a water-borne pheromone which increases general activity and social contacts among males. Two hormones, thyroxine and thiouracil, administered to the females increase these behaviors in males. Apparently the hormones do not functionally compete in this species, and both seem to elevate the emission of the male stimulant.

Key Words—Black molly fish, *Mollinenesia latipinna*, pheromone, thyroxine, thiouracil, general activity stimulus, social contact.

INTRODUCTION

There is growing dissatisfaction with the classic definition of a pheromone (Doty, 1976). Contrary to earlier expectations, pheromones appear to be modifiable at all taxonomic levels and rarely evoke stereotypical reactions (Thiessen, 1976; Thiessen and Rice, 1976). Instead, pheromones are dramatically influenced by metabolic and environmental perturbations and require continuous monitoring and interpretation by both the sender and the receiver (Thiessen, 1976). Although we do not believe that the concept of a pheromone need be abandoned, it is clear that it is becoming an anachronism within our current understanding and is in need of revision.

Of special interest are recent studies suggesting that insects and mammals acquire pheromones by pirating chemicals directly from their environment

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(Birch, 1974; Noirot et al., 1975; Gilbert and Raven, 1973; Skeen and Thiessen, 1976) and that ambient temperature and body thermoenergetics specify pheromone production and sensitivity (Thiessen, 1976; Thiessen et al., 1976). Such findings are in a minority but are beginning to shift attention from the assumed genetic invariance of pheromone systems to the environmental malleability of chemical information processes.

In a continuing effort to outline ways in which pheromone communication adjusts to intrinsic and extrinsic influences, we report on the black molly (*Mollinenesia latipinna*). This investigation demonstrates that females produce a pheromone which stimulates male behavior. The quantity and/or the intensity of the pheromone increases when the females are exposed to thyroxine. Surprisingly, thiouracil, when administered to females, has a similar effect on the male, suggesting that thyroxine and thiouracil are not counteractive at the tissue level.

METHODS AND MATERIALS

Animals and Apparatus

Adult *M. latipinna* (lyretail black or common black mollys) were maintained in aquaria as illustrated in Figure 1. Five 5-gal aquaria, four of which contained 7 females each and one which contained only water (blank), were shelved above three 30-gal aquaria. Ten males were maintained in each of the 30-gal aquaria. No visual contact was possible between any of the tanks. The aquaria were floored with sand and small pebbles and contained rocks and artificial plants. The physical environments were constructed as similar as possible. All tanks were constantly filtered and maintained at 27°C. Room lights were cycled every 12 hr and tests and observations were conducted midway through the light cycle. The fish were fed once a day with Geisler's Basic Flake, approximately 4 hr prior to any observation or manipulation. Food was also routinely placed in the blank aquarium. In order to facilitate the quantification of fish activity, the female tanks were lined on the front surface into 8 equal squares, and the male tanks were lined into 16 equal areas. Two 500-ml separation flasks with separate delivery tubes and funnels in each male aquarium allowed for a constant flow of pheromone ("female-primed" water) into the male tanks (Figure 1 shows only one flask and associated funnels).

The observational and testing sequence was divided into two phases, the "pheromone" and the "metabolic" phases. The first was intended to establish the presence of a female pheromone and the second was an attempt to alter the production of the female pheromone by subjecting females to thyroxine or thiouracil in their water. With no exceptions, daily behavioral

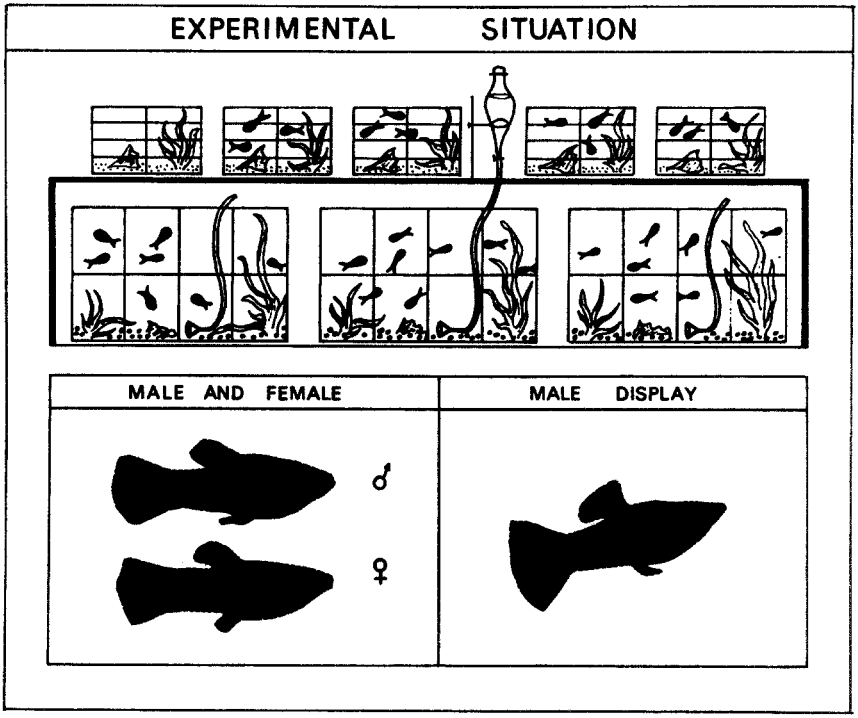

FIG. 1. Experimental arrangement showing the gravity flow system for introducing water from female or blank aquaria (above) into male aquaria (below). Male and female are depicted in lower part of figure, with the male on the right showing a typical fin display to a female pheromone.

observations were conducted for 10 min. Water from the female or blank aquaria was delivered with the separation flasks described above. The pheromone phase consisted of (1) 2 weeks of adaptation following the introduction of the fish, during which time no systematic observations were made; (2) 5 days of observation without water exchange between tanks (baseline measure); (3) 7 consecutive days in which water from a female (pheromone) or blank aquarium was drained from the delivery flasks into each of the male tanks (the order of presentation of the two types of water was alternated daily); and (4) 2 days of introducing water from the blank aquarium into the male aquaria, followed by one day in which water from the female aquaria and two days in which water from the blank aquarium were introduced into the male aquaria. Step 4 in the pheromone phase was conducted to control for the possibility that the systematic alternation of fish and plain

water in step 3 biased the results. The female aquarium used for donor water was randomly selected each day.

The metabolic phase consisted of the daily random presentation to each male aquarium of water coming from female aquaria. The females were subjected to the following manipulations: (1) no manipulation of females (control), (2) thyroxine, (3) thiouracil, or (4) thyroxine and thiouracil added to water in the blank aquarium. The hormone manipulation is described below. The introduction of water was by way of the separation flasks, as before. Prior to the metabolic phase 5 days of habituation were given to each aquarium of males, consisting of the daily presentation of blank water. No behavioral scores were recorded during this period.

During behavioral observations, the experimenters turned off the filter system of the relevant tank and sat quietly in front of the tank for 10 min, allowing the fish to adapt to the change in stimulation. For the next 10 min one experimenter recorded general activity, while the other experimenter recorded social contacts among fish. An activity count was registered whenever a fish crossed a line on the front of the aquarium. A "social contact" was registered whenever two fish approached each other or when one approached another or when two fish swam in parallel within 2.5 cm of each other. Since these measures did not distinguish between the tanks of females under any condition, only data for the males are presented. Random samples of behaviors were videotaped for closer scrutiny at a later time.

For delivery of water into the male tank, 1000 ml of water was removed from a female tank or the blank tank and allowed to equilibrate for 5 min in two 500-ml separation flasks. Each flask was attached to a Tygon tube leading to a small funnel resting in the bottom of the male tank. The two funnels in each tank opposed each other about 25 cm apart. At the beginning of each 10-min observation period the gravity flow was started from the two flasks, lasting approximately 3 min. Water was replaced in the female tank after each test.

For the second phase of the experiment (metabolic phase) thyroxine or thiouracil was introduced to the females in the following way. In two aquaria thyroxine (T_4) was given to the females along with the daily food ration. In one aquarium dose level began at 1 mg/day and reached 16 mg/day by test day. In another aquarium the dose began at 2 mg/day and ended with 32 mg/day. In a third tank thiouracil was added with the food beginning with 1 mg/day and ended with 16 mg/day. During the test periods dose was maintained at 8 and 16 mg/day for thyroxine for the respective tanks, and 8 mg/day for thiouracil. The total number of days the females were under hormone stimulation was 10. In order to get maximum dissolution of the hormone it was first mixed with water as a paste and added to the food immediately before introduction into the female aquaria.

The presentation of the female or blank water was conducted as before, except that the order of presentation of the water was randomized for each test. Each male group was presented with water from each of the conditions over a 4-day period. On the final day of testing the males were subjected to water from the blank aquarium containing a mixture of 0.3 mg thyroxine and 0.4 mg of thiouracil, which was our rough estimate of the average amount of free hormone circulating in female aquaria receiving hormones. Since dose–response reactions to thyroxine were not evident, these results were combined. Similarly, no differences appeared between conditions involving water from the blank aquarium and water from the blank aquarium containing thyroxine and thiouracil. These results were also combined.

In summary, baseline measures of male activity and social contacts were followed by identical measures during exposure to female pheromone water or water only. Two different sequences of presentation were used to establish the effect of male stimulation by female pheromone. Finally, males were stimulated by water from the blank tank, control water (female water), or water from females subjected to hormonal stimulation by thyroxine or thiouracil. The same behavioral measures of activity and social contacts were recorded. Friedman analyses of variance for related measures were applied to the basic outcomes of this experiment, and exact probabilities assessed for order effects in the three replications (three independent aquaria).

RESULTS

Daily swimming activity and the frequency of social contacts were closely related for each of the three groups of males (Fig. 2). Pearson coefficient of correlation (r) ranged from 0.68 to 0.90 ($P<0.001$). There were obvious differences between the two basic measures, as many of the social contacts involved fin displays as well as movement. Still, the high correlations suggest that activity and social contacts reflect a common process.

Figure 3 shows the results from the pheromone and metabolic phases for all three male aquaria combined. Assuming that the probability of an increase or decrease in activity and social contacts is equally likely when pheromone water is added to the male aquaria, the probability of finding 6 out of 6 possible changes in an upward direction is $n^{1/2}$ or 0.02. In other words, activity and social contacts were stimulated by the introduction of water from female fish aquaria, above that of water alone. The order of daily presentation of female water or water from the blank tank was immaterial for this effect. The apparent additional increases in male behaviors when females were stimulated by thyroxine or thioracil are referred to below.

Figures 4 and 5 show a breakdown of average swimming activity and

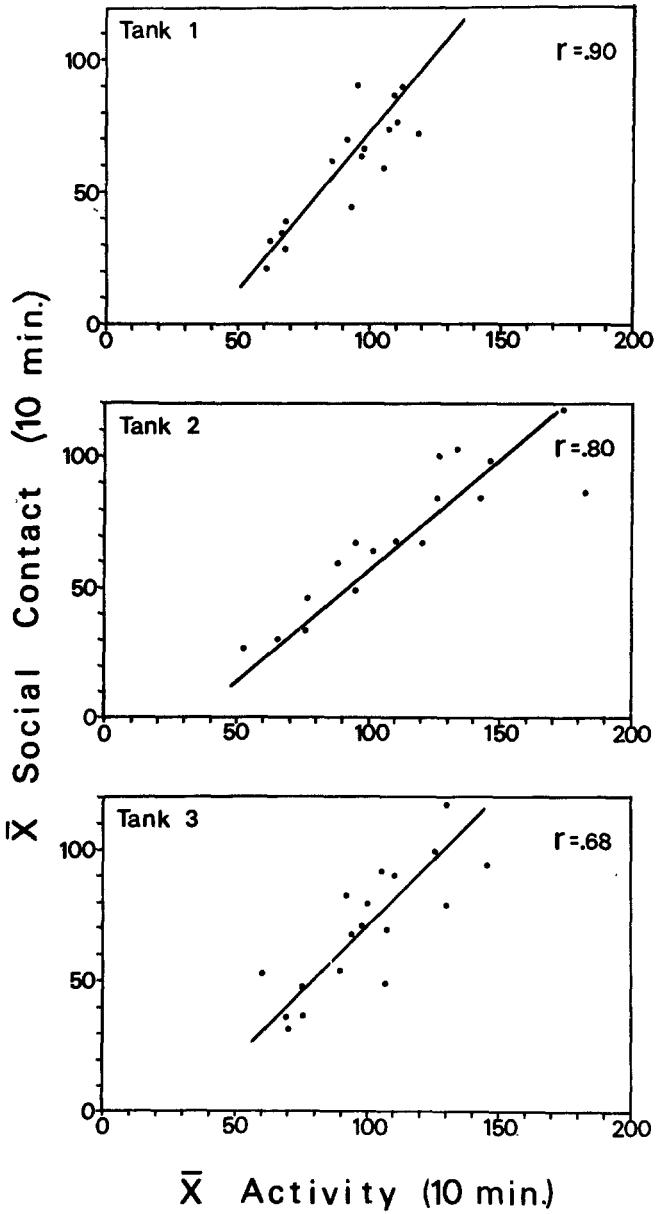

FIG. 2. Relationship between swimming activity (liue crossings) and social contacts (approach, close parallel swimming, or fin display) for the three male groups over the course of the experiment.

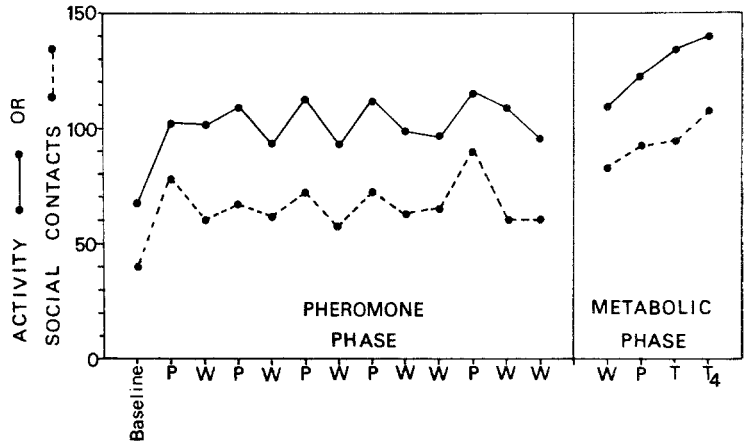

FIG. 3. Male activity and social contacts for the three aquaria combined during the pheromone and metabolic phases of the experiment. P = pheromone (female water), W = water from blank tank, T = thiouracil, T_4 = thyroxine.

social contacts for each of the three male tanks. In general each male group responded similarly and followed the same trends for the combined results noted in Figure 3. In the pheromone phase of testing the males of all three tanks showed increasing levels of activity and social contact in the order: baseline < blank water < fish water. Friedman analyses of variance were equally significant for all male groups ($\chi_r^2 = 6$; $df = 2$, $P = 0.03$). The probability of finding the same rank order of effects in all three male aquaria is $\frac{1}{3!3!3!}$ or 0.005. The pheromone effect was therefore evident for both swimming activity and social contacts.

Surprisingly, for the metabolic phase of testing both thyroxine and thiouracil administered to females increased both male behaviors. Witht he exception of activity for aquarium 2, which showed no obvious pattern, the order of stimulation was: blank water + hormone < control < thiouracil < thyroxine. Friedman analysis of variance for activity was nonsignificant ($\chi_r^2 = 3.4$; $df = 3$, $P = 0.47$) but was highly significant for social contacts ($\chi^2 = 9$; df = 3, $P = 0.002$). The probability of finding the consistent rank order for activity in two tanks is $\frac{1}{4!4!4!}$ or 0.001. Taken as a whole, then, it appears that thiouracil, and especially thyroxine, when administered to females increased the stimulating effect of female fish water. The hormones alone placed in the male aquaria had no stimulating effect.

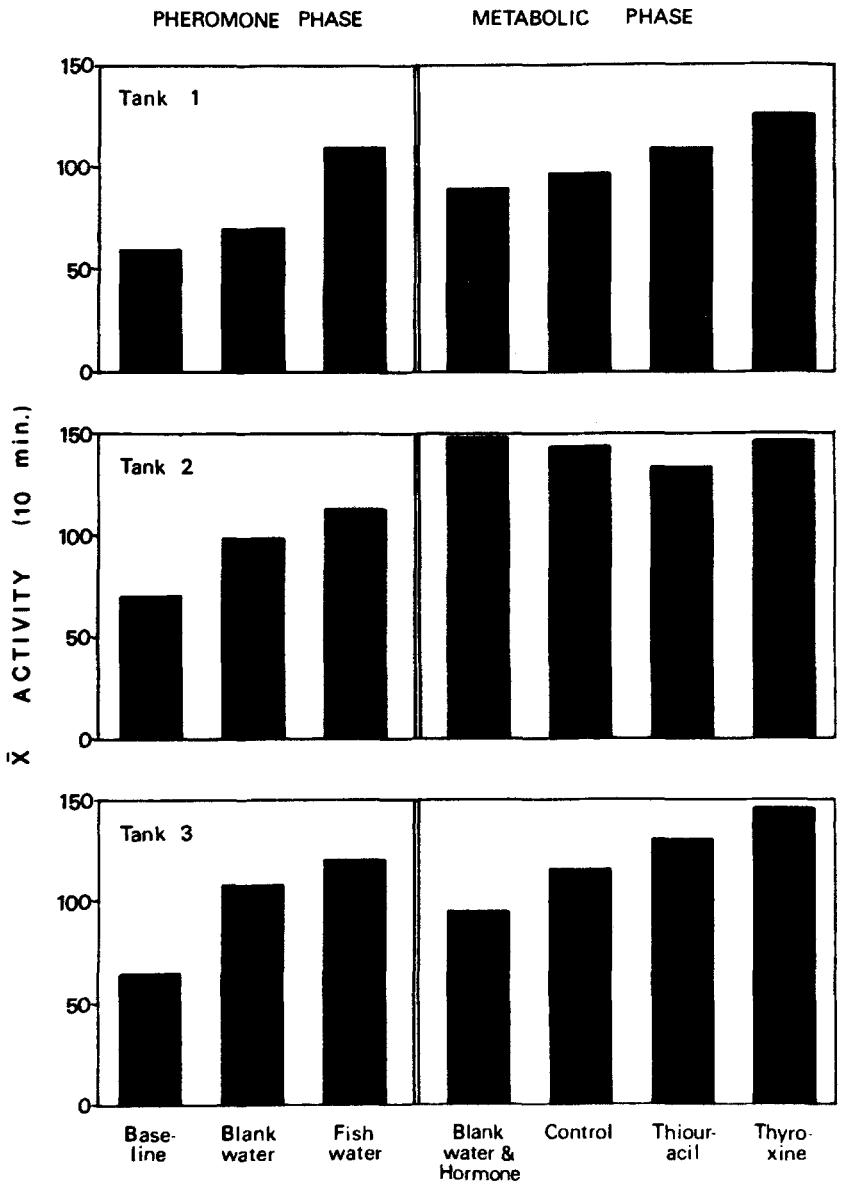

FIG. 4. Male activity during the pheromone and metabolic phases of the experiment. Control = water from non-hormonally-stimulated females.

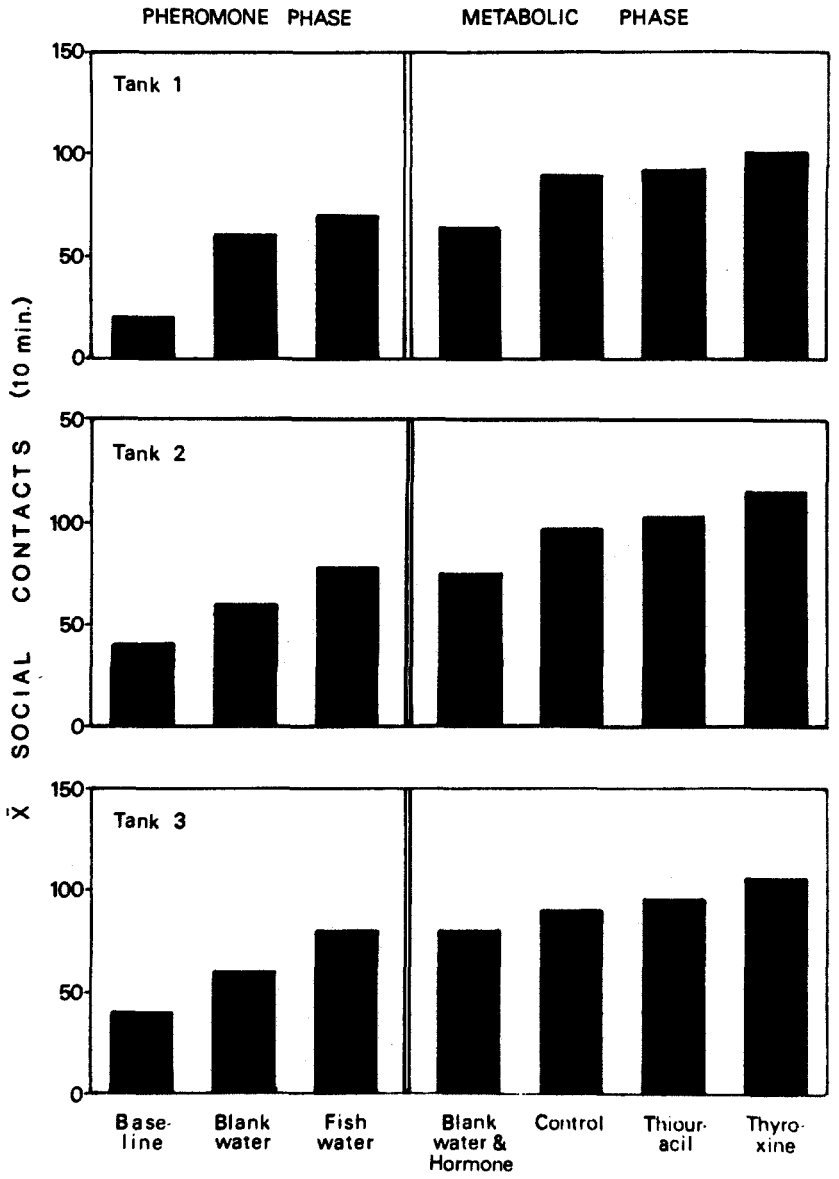

FIG. 5. Male social contacts during the pheromone and metabolic phases of the experiment. Control = water from non-hormonally-stimulated females.

DISCUSSION

The black molly may be an informative species for the study of metabolic aspects of pheromone regulation. The female produces a water-borne substance which stimulates swimming activity and social interactions among males. It may also evoke sexual responses, but we have no information on this.

Direct observation of the males and videotape inspection indicate that the chemical manipulation of the females resulted in a quantitative increase in pheromone production, rather than a qualitative shift in the type of pheromone. Females stimulated by thyroxine and thiouracil induced males to greater levels of activity and social contact but did not alter the mode of reaction.

The pheromone production by the female is apparently influenced by alteration in tissue metabolism. In all tests, and for all three male groups, water from females subjected to 10 days of thyroxine increased swimming activity and social contacts. We suspect that thyroxine increases the metabolic rate in the female, thus increasing the synthesis and release of pheromones. The notion is not entirely compelling, however, for at least two reasons. First, thiouracil, a putative antagonist to thyroid activity, did not decrease pheromone production, as expected, but instead apparently increased production. Second, periodic viewing of female activity revealed no obvious changes associated with either thyroxine or thiouracil. It is still possible, however, that both chemicals affect cellular metabolism in a similar way independently of the thyroid. The data in regard to metabolic and thermoenergetic parameters of fish are still incomplete and confusing (Fry and Hochachka, 1970). We could find no information on the energetics of *Mollinenesia latipinna.*

A metabolic hypothesis is still viable, however, as the males did react more to water from females who were exposed to thyroxine and thiouracil. These chemicals when presented alone were ineffective and did not interact with fish food or artificial plants to produce the active substance. A reasonable possibility is that the ingestion and absorption of thyroxine and thiouracil alters the metabolic character of pheromone producing cells to increase their secretion. It need not be assumed that a specialized pheromone gland is involved, as long as the excretory substance has signaling characteristics understood by the male. A relatively nonspecific cellular reaction to thyroxine and thiouracil would seem compatible with this notion.

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BEHAVIOR AND PHEROMONE STUDIES WITH *Attagenus elongatulus* CASEY (COLEOPTERA: DERMESTIDAE)

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Abstract—Males of *Attagenus elongatulus* Casey responded to a source of female odor with a characteristic sequence of actions which included: extension of the antennae, elevation of the body by leg extension, rapid zig-zag running, and intermittent bobbing up and down. The courtship sequence also consisted of several activities and was usually followed by a typical copulatory act of less than 1-minute duration. Virgin females exhibited calling behavior which was associated with pheromone release. Female calling activity, female pheromone content, and male responsiveness followed a diurnal cycle with peak values occurring during the earlier hours of the 16L:8D photoperiod. Quantitative bioassay indicated that a 50% male response level (RD_{50}) occurred at ca. 0.014 female equivalent (FE) exposure.

Key Words—*Attagenus elongatulus*, Dermestidae, pheromone, behavior, calling, bioassay.

INTRODUCTION

Pheromones must be considered a major mode of intraspecific communication in insects, and as such they offer several possibilities for the manipulation of populations and behavior of both useful and destructive insects. The often undisturbed and dark environment of a stored product could reduce or eliminate visual cues which might be utilized, thus increasing the importance of sex pheromones for mate-finding.

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The first evidence for a pheromone in a stored-product insect was reported by Valentine (1931) in work with *Tenebrio molitor* L., the yellow mealworm. Bar-Ilan (Finger) et al. (1965) and Burkholder and Dicke (1966) presented the first evidence for sex pheromones in the Dermestidae. One of the first identified coleopteran sex pheromones was megatomoic acid, (E,Z)-3,5-tetradecadienoic acid, of the black carpet beetle, *Attagenus megatoma* F. (Silverstein et al., 1967). Related genera have been demonstrated to possess non-species-specific pheromone components, e.g., the sex pheromones of *Trogoderma* spp. (Rodin et al., 1969; Vick et al., 1970; Levinson and Bar Ilan, 1970; Silverstein, 1971). In addition, the sex pheromone of *Anthrenus flavipes* LeConte has recently been identified (Burkholder et al., 1974; Fukui et al., 1974).

Woodroffe (1958) described a "calling" posture assumed by females of *Ptinus clavipes* Panzer f. *mobilis* Moore. The distinct postural behavior consisted of bending of the forelegs and straightening of the hindlegs until the insect was "standing on its head." The posture was commonly maintained for long periods of time and was more prevalent in culture jars which contained large numbers of virgin females. No conclusion was made relating the calling to release of sex pheromones. Similar calling behavior was reported by Burkholder et al. (1974) and Hammack et al. (1976b) for the first time in the family Dermestidae, with several species of *Trogoderma* and *Anthrenus* exhibiting calling behavior.

Wojcik (1969) described the mating behavior of eight species of stored-product insects including the dermestids *A. flavipes* and *Trogoderma glabrum* Herbst. The intent of this research was to study the pheromone related behavior of *Attagenus elongatulus* Casey. This paper is to our knowledge the first description of the courtship and mating behavior and diurnal activity in an *Attagenus* species.

METHODS AND MATERIALS

Insects were reared on *Trogoderma* medium previously described by Hammack et al. (1973) for the rearing of *T. glabrum*. Cultures were maintained in ca. 1-liter, wide-mouth canning jars held at $27.5 \pm 1°C$ and $60 \pm 10\%$ relative humidity under a 16:8 L:D light regime. At intervals of 5–7 days after the cultures had begun to provide pupae, larvae and pupae were separated from the medium by sifting through an 18-mesh U.S. Standard sieve. Pupae were removed by aspiration. The pupae were initially segregated by sex and held in petri dishes lined with filter paper. The male pupae were typically smaller than female pupae. Dishes of pupae were checked every 1–2 days for adults, at which time the adults were removed and the sex

confirmed. The terminal segment of the male antennal club was several times as long as the corresponding segment of the female antenna. Male pupae and adults were held in female-free incubators under rearing conditions until needed.

In studies on the male response to a source of female pheromone, individual males were placed in filter-paper-lined petri dish arenas to which a standard 12.5-mm-diam antibacterial assay disk treated with hexane extract of macerated females was added.

For the studies on the courtship and mating behavior of the insects, male on female adults were confined singly in 1.5-dr shell vials with an assay disk on the bottom. An insect of the opposite sex was then added.

To study the calling behavior of female insects, individual females were confined in numbered 1.5-dr shell vials, the bottoms of which were lined with an assay disk. Each "calling vial" had a 5×20-mm piece of filter paper, pleated at three points, inserted to the bottom. The technique is similar to that employed by Hammack and Burkholder (1976a) in experiments on *Trogoderma* calling. Experiments on diurnal female calling were conducted in the rearing room. To study the diurnal incidence of female calling, 50 virgin adult females, 6–8 days old, were observed at half-hour intervals over the 16-hr photophase. Observations for each hour were averaged.

Experiments on the incidence of calling in aged females were conducted in a white painted room held at conditions similar to the rearing room. To estimate calling activity among different age groups of virgin females, 25 adults in each of nine age groups from 1–2 to 9–10 days old were observed at 0.5 hr intervals during the photophase.

Tests were conducted to determine if the observed calling behavior could be associated with the release of the sex pheromone. Standard biological assay disks were suspended above a calling or noncalling female for 7 min each. Both disks were immediately assayed with a single replicate of ten 5–7-day-old males each. A second test was conducted in which assay disks were suspended above noncalling and calling females for periods of 1, 3, and 5 min. Light intensity in the rearing room at the location of the experiment was ca. 126 lx and in the white room intensity was ca. 760 lx at the center of the experiments. Light was provided by cool white fluorescent tubes in both rooms.

Pheromone extracts of females for male bioassays were prepared as follows. Females of the appropriate age and number were placed in 125 $\times$ 15-mm screw-top culture tubes which contained a known amount of hexane and ca. 4 ml of 4.0-mm-diam glass beads. The tubes containing the insects were vibrated on a vortex mixer until the insects were completely macerated. Serial dilutions of the extracts were made with hexane to provide the desired number of female equivalents (FE) per 10-μl aliquot. A FE was defined as

the number of females extracted per 10-μl aliquot of hexane. Extracts were stored in darkness at $-40°C$.

The olfactometer used in bioassays was similar to that described by Vick et al. (1970). One day before males were to be bioassayed they were placed individually in olfactometer vials and stored overnight in a female-free incubator under rearing conditions. All bioassays were conducted in a female-free bioassay room with temperature and humidity identical to the rearing room and incubators. Only quiet, resting males with retracted antennae and head were used in bioassays. Males were exposed to the pheromone source for 1 min. A positive response was recorded as the time in seconds up to 1 min at which the male became aroused and began locomotory movement. Ten males were tested per replicate unless otherwise indicated.

To determine the effect of male age on response to a female extract, male insects 1–2 to 1–10 days old were bioassayed against a single extract of 7–8-day-old virgin females. Ten replicates of each group were tested, except at 1–2 and 9–10 days for which 64 insects and 7 replicates, respectively, were tested.

To determine if a differential male response threshold existed over the photophase, at hourly intervals throughout the photophase 5 replicates of 4–6-day-old males were bioassayed against a single hexane extract of 25, 7–9-day-old virgin females.

The effect of female age on pheromone content was determined by extraction and bioassay of 25 virgin females of each age group from 0–1 to 7–8 days old. All extracts were made at hour 6 of the photophase. Five replicates of males were tested for each age group.

To determine female pheromone content as a function of time of day, extracts of 10, 5–7-day-old virgin females each were made at 1 hr intervals during the photophase. Each extract was bioassayed against five replicates of 10, 4–6-day-old males each.

A quantitative relationship between pheromone titre and male activity was estimated. A single female extract of 0.064 FE was prepared from 25, 3–8-day-old virgin insects. Serial dilutions of the extract were prepared to yield the following concentrations: 0.032, 0.016, 0.008, 0.004, 0.002, 0.001, and 0.0005 FE/10-μl aliquot. Five replicates of 15 males each were bioassayed at each concentration.

RESULTS

Observations on the Male Response to the Sex Pheromone

The response of the male to the female extract consisted of a sequence of increasing levels of excitation. A typical response sequence is depicted in

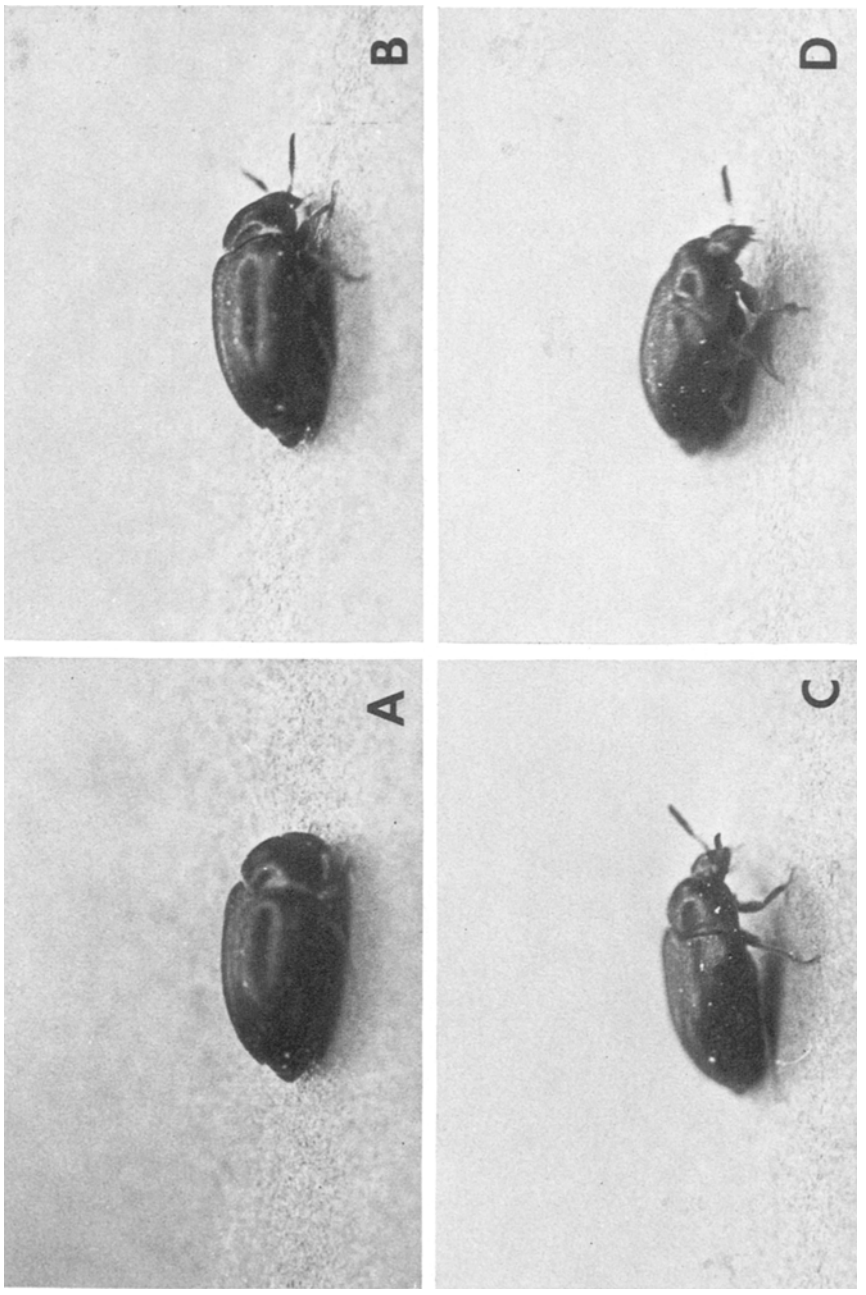
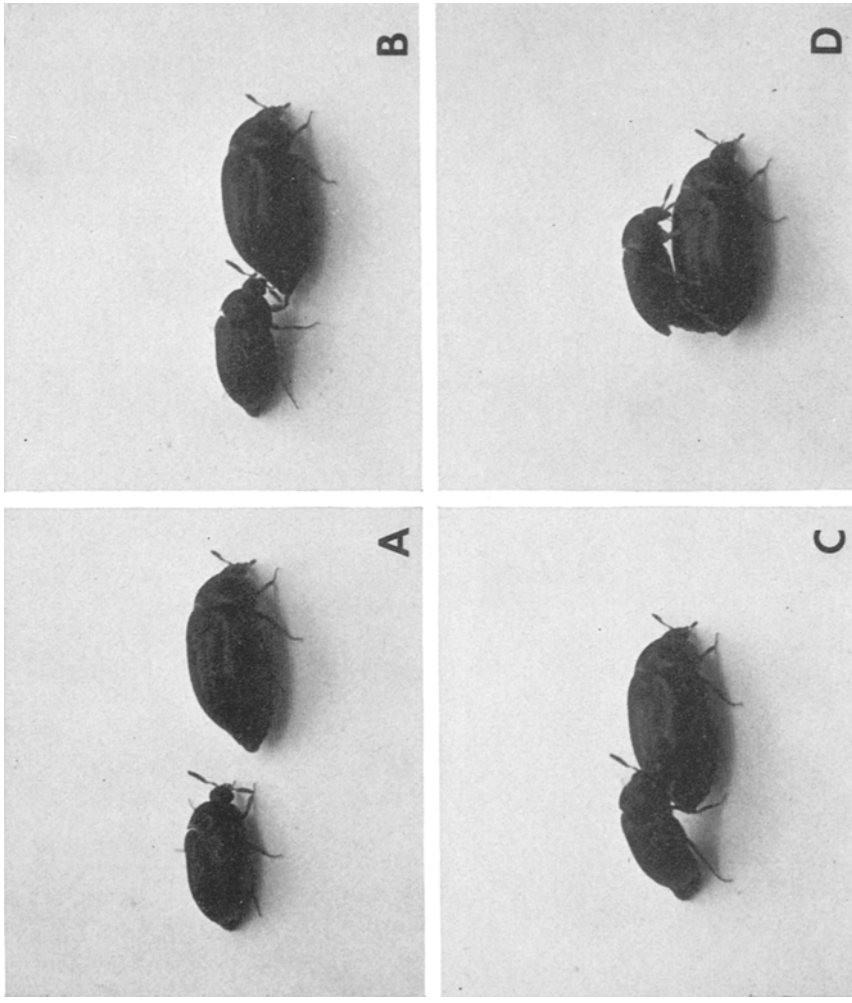


FIG. 1. A–D: Male *A. elongatulus* responding to the female sex pheromone.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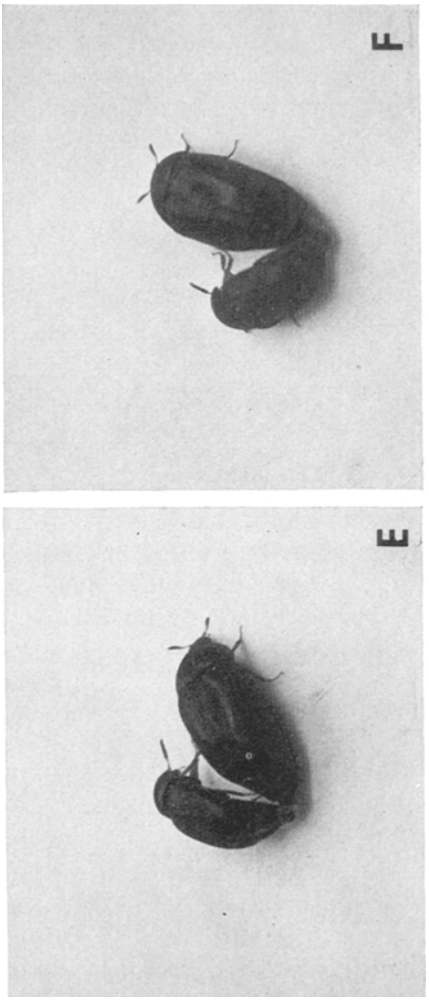

FIG. 2. A–F: Sequence of the courtship and mating behavior of a male–female pair of *A. elongatulus*.

Figure 1 A–D. In Figure 1A the male is in resting state with head and pronotum withdrawn and antennae retracted. The first level of response is shown in Figure 1B. The male has partially protracted the antennae, but the head remains withdrawn and the body is close to the substrate. Under sufficient stimulation the male will advance to the second stage, as in Figure 1C. Antennae are fully protracted, the head is extended forward and the body is elevated by extension of the first and second pair of legs. While in this level of excitation the male will often bob up and down and haltingly move its head. Additional stimulation results in an active searching state as in Figure 1D. When the response behavior was observed in a large arena, ca. 1×1 m, the male would occasionally run in a zig-zag manner, often returning to a lower level of excitation between periods of searching. During the active searching stage, males were observed to occasionally raise the elytra or to make short, hopping flights of a few centimeters.

The duration of any level of excitation was variable. The males occasionally progressed from the resting state to active searching in one fast, continuous motion, but most commonly paused for variable lengths of time at the lower levels of excitation.

Observations on the Behavior of Courtship and Mating

The typical courtship and mating behavior of *A. elongatulus* is depicted in the sequential photographs of a single male/female pair in Figure 2A–F. The following description was based on the close observation of 20 male/female pairs of 4–6-day-old virgin adults. All observations were made between hours 6 and 7 of the photophase.

The female is very receptive to mating when apparently resting, with abdominal segments partially protruding and antennae protracted, as in Figure 2A. The male approaches the female in an excited state after sex pheromone stimulation and promptly makes palp contact with the body of the female (Figure 2B). The male immediately begins to stroke the female's elytra with his forelegs while orientating in the same direction as the female (Figure 2C). Repeated palp contact and stroking continues as the male mounts the female from one side (Figure 2D). While in this position, the male extends his genitalia and begins to probe for the female's genitalia. When genital contact has been made and copulation has begun, the male stops stroking the female and palp contact is discontinued (Figure 2E). The male remains in this position for ca. 5–10 sec and then, while maintaining intromission, turns his body, facing a different direction than the female, commonly at a 45–90° angle away from the female (Figure 2F), but occasionally 180°. The male is very still while in this position except for occasional twitching of tarsi or legs. In some mating attempts the male will become dis-

oriented and mount the female while she is facing in the opposite direction. The male will then briefly probe the female's head with his genitalia before moving to the correct position. The female shows no visible activity during copulation, but when mating is completed she begins to move away from the male, often giving a slight tug to break the coupling. The female then often becomes briefly active, avoiding the male, and will often bump the male away from her.

The duration of copulation, timed from when a coupling was made to the time at which the connection was broken, averaged 56 sec (range 45–75 sec), SD = 7 for the 20 male/female pairs.

When 10–13 males each of 1, 24, 48, and 72 hr of age were confined individually with a 6–9-day-old virgin female, males of 48 hr or less did not successfully copulate, but of the males 72 hr old, 7 of 10 mated with females which subsequently laid eggs. When 10–13 virgin females each of 1, 24, 48, and 72 hr of age were confined with 6–9-day-old males no successful mating occurred. Among the 1-hr-old females, 3 of 13 copulated, but separation was difficult and none of the females laid eggs. The same females were mated when 5 days old and produced viable eggs.

Description of Calling Behavior

When virgin female adults were held in petri dishes provided with pleated strips of filter paper upon which to crawl, they often assumed various unusual postures similar to the calling posture described in other species of Dermestidae. Figure 3 pictures a female *A. elongatulus* in a typical calling posture. The female had crawled close to the top of the filter paper and thrust her body outward by extension of the legs. Calling females were easily disturbed and reacted quickly by retracting the legs, head, and antennae. Calling postures in *A. elongatulus* were more subtle than the head-stand posture of *A. flavipes* (Burkholder et al., 1974).

Female Calling at Various Times of the Photophase

Results are presented in Figure 4. Calling was observed first 1 hr after the onset of light and by the second hour a period of maximum calling had begun. Calling continued at a high level for 5 hr after which time the calling incidence rapidly decreased. Calling has not been observed during scotophase.

Calling among Females of Various Ages

No incidents of calling were observed among females 1–2 and 2–3-days-old. At 3–4 and 4–5 days calling had begun (28 and 76%, respectively)

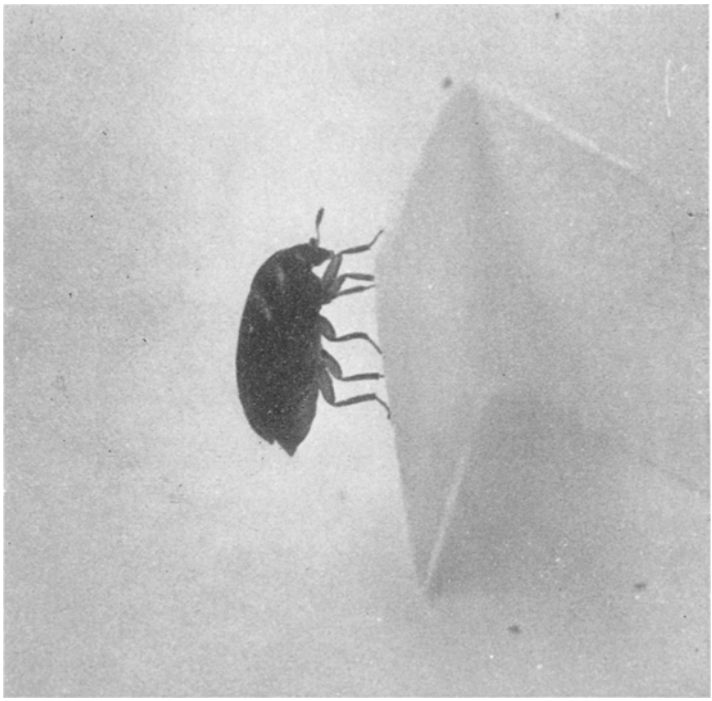

FIG. 3. A virgin female *A. elongatulus* in a typical calling posture, photographed on a vertical surface of a strip of pleated filter paper.

and among females 5–6 days or older, every female had been observed to call at some time during the day. As in the previous experiment the incidence of calling was greatest between hours 2 and 7 of the photophase.

Relation of Female Calling to Release of the Sex Pheromone

The disk exposed to a calling female elicited a response from 7 of 10 males, and the assay disk exposed to a noncalling female did not elicit a single male response. Disks exposed to calling females for 3–5 min each elicited 10 of 10 male responses as compared to the zero response to non-calling control disks. Assay disk exposed to calling females for as little as 1 min still elicited a positive response from 7 of 10 males tested. Apparently, the calling female had released a quantity of the sex pheromone and an undetermined amount of the compound had contaminated the assay disk.

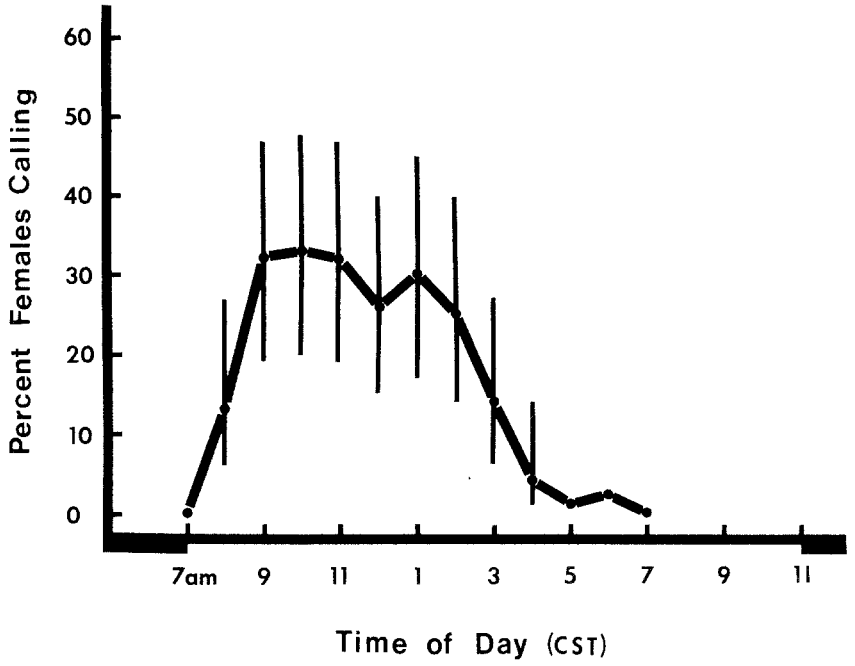

FIG. 4. Diurnal calling activity in 50 *A. elongatulus* virgin females 6–8 days-old. The vertical lines represent the 0.95 binomial confidence interval about each point.

Introductory Bioassays

Observations of *A. elongatulus* and the demonstration of a sex pheromone in *A. megatoma* by Burkholder and Dicke (1966) suggested the presence of a sex pheromone in *A. elongatulus*. Bioassays were therefore conducted to determine a suitable male age group and female extract concentration. On the basis of these tests, males 4–6 days old and concentration of 5×10^{-3} FE were chosen to begin the pheromone studies.

Response of Males of Various Ages to the Sex Pheromone

The results are presented in Figure 5. No male responses were observed among the 1–2-day-old insects, and only one 2–3-day-old male responded. Percent male response reached 40% at 4–5 days of age and continued at a high level through the oldest age tested. Males older than 10 days were not tested since they became too active to obtain a sufficient number of resting

FIG. 5. Percent response of *A. elongatulus* males of various ages to an extract of 7–8-day-old virgin females. The vertical lines represent the 0.95 binomial confidence interval about each point.

males. Based on these data, males 4–10 days old were considered usable for standardized bioassay procedures.

Male Response over the Photophase

The results are summarized in Figure 6. Male response at the beginning of the photophase was 8.0%. Response increased rapidly and a period of maximum responsiveness was observed between 10:00 AM and 2:00 PM. After this period, the response level rapidly dropped to zero by 7:00 PM. Based on these results, bioassays were conducted between 10:00 AM and 2:00 PM unless otherwise indicated.

Effect of Age on the Pheromone Content of Virgin Females

The results are presented in Figure 7. The data indicate that 0–1-day-old females contained a significantly lower pheromone titre than females 1–2

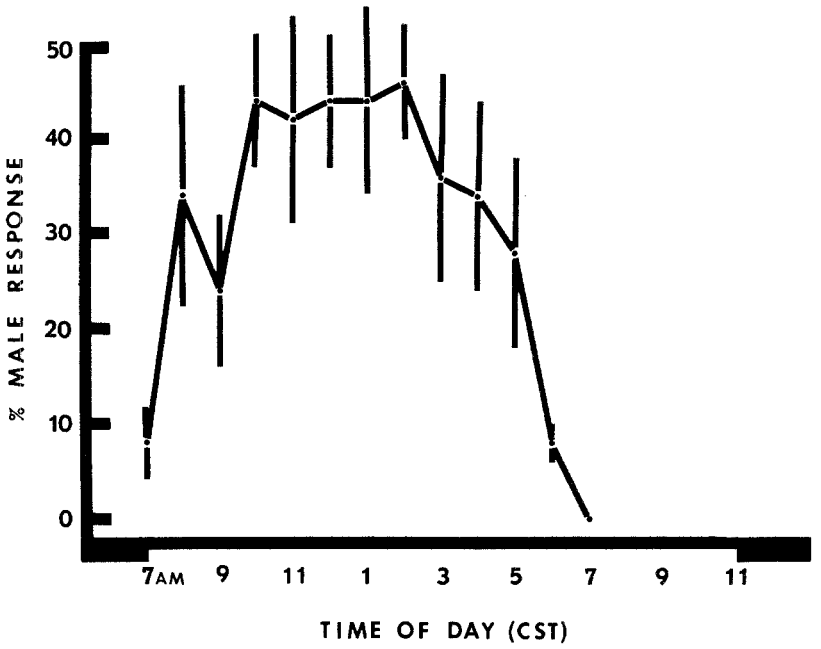

FIG. 6. Response of 4–6-day-old *A. elongatulus* males at 1-hr intervals over the photophase to an extract of 6–7-day-old virgin females. The vertical lines indicate the 0.95 confidence interval about each point.

days old or older. At 1–2 days of age the female pheromone titre did not differ significantly from the older age groups.

Effect of Photophase on Female Pheromone Content

It was clear that the female pheromone content, as estimated by the percent male response, rapidly increased from a low level at the onset of light to a period of significantly higher pheromone content. Following the period of increased titer, the male response gradually decreased to a level which was statistically equal to that at the beginning of the photophase. The data are presented in Figure 8.

Quantitative Bioassay of Female Pheromone Content

The probit response of the males is presented in Figure 9. The calculated 50% response threshold (RD_{50}) after a 1-min exposure to the extract was equal to 0.014 FE. Extrapolation of these data to Figure 6 indicates that

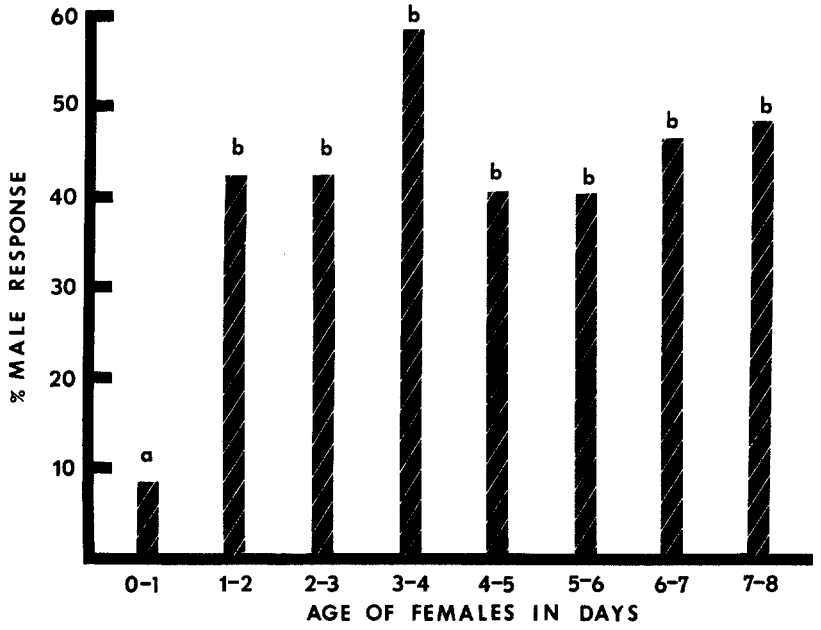

FIG. 7. Response of *A. elongatulus* males to extracts of 5–7-day-old virgin females of various ages. Bars indicated by the same letter were not different at a level of 0.95 by Duncan's Multiple Range Test.

male responsiveness is ca. 10 times greater during the active period than during the earliest and latest hours of the photophase. Likewise, the female pheromone content (Figure 8), based on whole insect macerations, is ca. 10 times greater during the hours corresponding to the calling peak than during the first and last hours of the photophase.

DISCUSSION

The response of *A. elongatulus* males to a source of sex pheromone was similar to that described by Burkholder and Dicke (1966) for the black carpet beetle, *A. megatoma*. It is thought that successive levels of excitement are reached as the response threshold for any level is exceeded. At very low pheromone concentrations males were observed to progress to the level of leg and antennal extension, but locomotory movements did not often occur

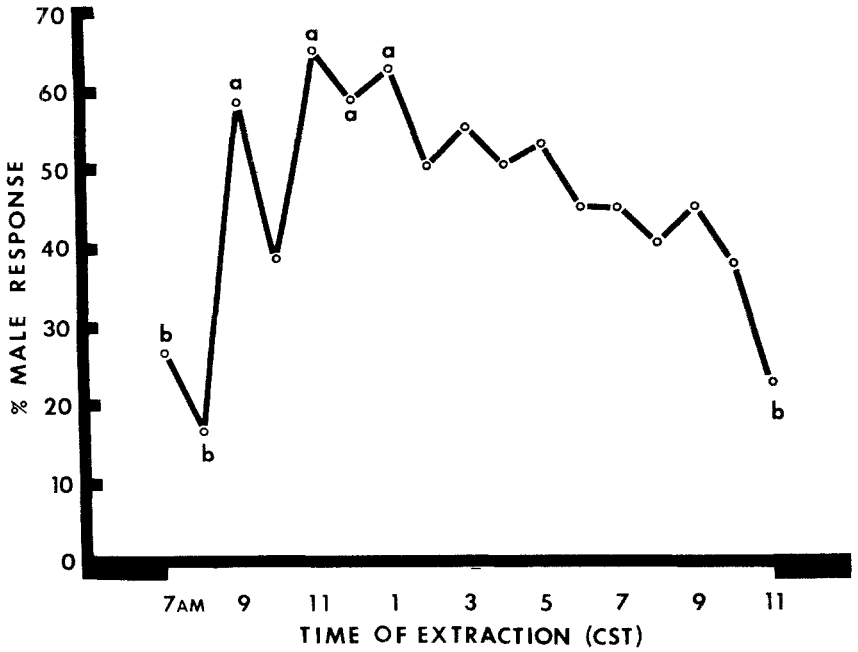

FIG. 8. Response of 4–6-day-old *A. elongatulus* males to extracts of 5–7-day-old virgin females made at 1-hr intervals over the photophase. Points indicated by the same letter were not different at a level of 0.95 by Duncan's Multiple Range Test.

within the 1 min time limit of each observation. Bartell and Shorey (1969), in work with the light-brown apple moth, *Ephiphyas postvittana* (Walker), demonstrated that the frequency of occurrence of successive response steps could be increased by increasing the quantity of pheromone tested, thus supporting the hypothesis that each step in the behavioral sequence had a higher threshold than the preceding one. Likewise, the orientation pattern of male Khapra beetles clearly depends on the pheromone concentration available (Levinson, 1975).

The time spent in copula by *A. elongatulus* pairs was much less than that reported by Wojcik (1969) for *A. flavipes* and *T. glabrum*. He reported mean times of 3.9 min and 2.2 min, respectively, as opposed to less than 1 min for *A. elongatulus*. He also reported very low (10%) rates of successful matings with the two species. With *A. elongatulus* pairs, data have shown mating success greater than 90% when pairs were confined for ca. 1 hr (Barak and Burkholder 1977). Wojcik (1969) did not mention the time of day of his studies or the light regime and temperature under which the insects were

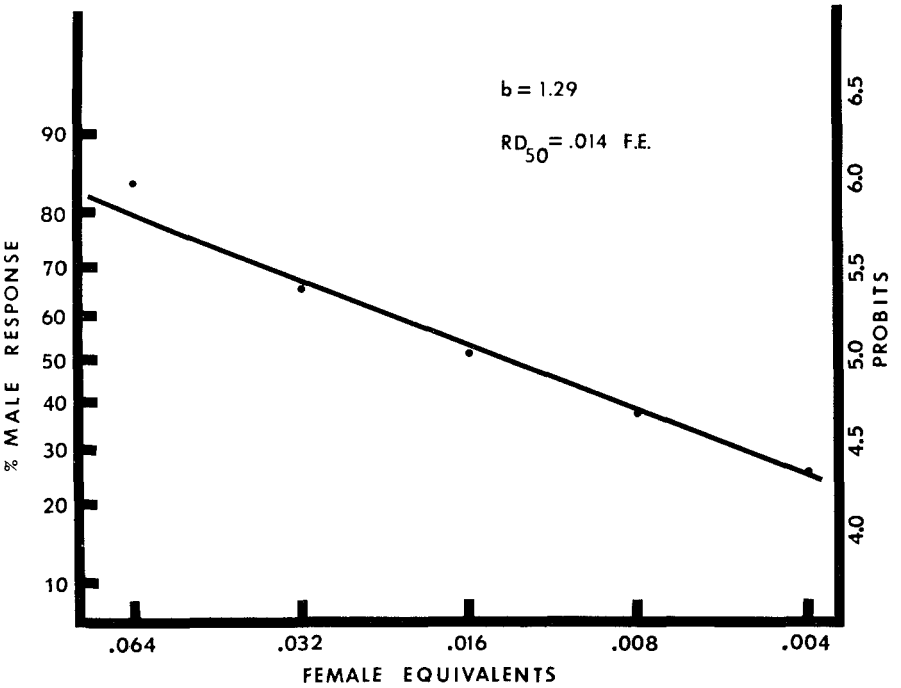

FIG. 9. Probit response of 4–9-day-old *A. elongatulus* males to various concentrations of an extract of 3–8-day-old virgin females. The calculated 50% response threshold (RD_{50}) was 0.014 FE.

held. The time of photophase can be critical in the elicitation of certain insect behaviors. The data of Figures 4, 6, and 8 have demonstrated that with *A. elongatulus* female calling, male response, and female pheromone content follow a diurnal rhythm. All experiments on the mating of *A. elongatulus* were done during the period of peak activity. Vick et al. (1973a) have demonstrated that with *T. glabrum* and *T. inclusum* the mating frequency correlated significantly with the male response cycle. They also reported that mating occurred without female calling and speculated that mating was not obviously linked to calling behavior. However, Hammack et al. (1967b) have shown that with *T. glabrum*, calling females were more receptive to mating than noncalling females. The data of Vick et al. (1973a) did not demonstrate a higher pheromone content in *Trogoderma* females over the photophase, but the insects were held in crowded conditions not favorable to calling. Hammack et al. (1976b) have demonstrated that filter papers exposed to calling *T. glabrum* females elicited higher male response than filter papers exposed to noncalling females. Our data also demonstrate this in *A. elongatulus*. Ap-

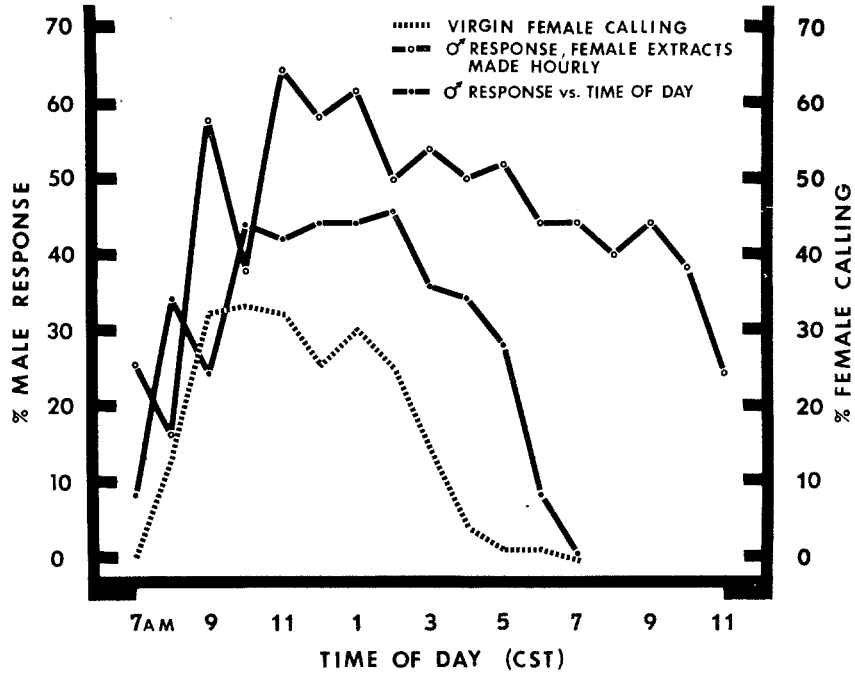

FIG. 10. A comparison of the diurnal activities of female calling, male response and female pheromone content of *A. elongatulus* adults.

parently the release of the pheromone is associated with calling behavior. The data of Figure 8 demonstrate a high female pheromone content in females held in dishes, which coincides with the peak female calling and male response.

Hammack and Burkholder (1976a) have given evidence that the calling activity of *T. glabrum* is circadian and is photoperiodically controlled. Sower et al. (1970) also demonstrated an endogenous rhythm in the calling behavior of *Trichoplusia ni* (Hübner). From our data and the results of other workers, it seems likely that the diurnal activities of *A. elongatulus* are circadian and are photoperiodically entrained. This would serve the useful function of coordinating male attraction with the period during which the females would be releasing the pheromone and be most receptive to mating (Figure 10). The existence of specific diurnal periods of activity must be considered in the planning of an insect control program which would utilize the behavioral effects of pheromones.

Hammack et al. (1973) have demonstrated the presence of glandular epithelia in the 7th abdominal sternites of 6 species of *Trogoderma*. It was postulated that the glandular tissues are the site of pheromone production.

Females of *Trogoderma* species expose the abdominal sternites while in calling postures, and it is possible that calling behavior facilitates the dispersion of pheromone into the environment. It is not unreasonable to postulate that the site of pheromone production in *A. elongatulus* is in the abdominal sternites and that the calling behavior is a means of facilitating pheromone dispersion. Previous data (Barak and Burkholder, 1977) have shown that females did not mate readily until they were 3–4 days old and that after this time fecundity and mating success rapidly declined, with the number of progeny produced closely approximating a calculated regression line. Females had passed the statistical age of maximum fecundity and mating success (3–4 days old) when 100% calling was first observed in individuals 5–6 days old. This might suggest that in large populations calling might be seen at much lower levels than observed in the laboratory since mating could occur as much as 2 days before maximum calling. Hammack et al. (1976b) have shown that the incidence of calling in mated females of *T. glabrum* was greatly reduced. In the related species, *A. megatoma*, Burkholder (1970) demonstrated that males did not respond at a high level to female extracts until the males were 4–5 days old and that females were not highly attractive until they had reached the same age. Burkholder (1970) also reported that *A. megatoma* males that had the distal segments of the antennae removed failed to respond to female odor, but that some insects copulated with the females after as much as 10 min of close contact. Obviously, excitation by perception of the female odor via the antennal club was important, but not necessary, for copulation. Perhaps perception of other compounds via the palps is necessary to facilitate the copulatory sequence.

The presence of another pheromone or the same compound perceived via the palps could "hold" the male and stimulate repeated copulating attempts. Males have been observed to attempt copulation with nonreceptive females for several minutes, a much longer period than that necessary for habituation in *T. inclusum* (Vick et al., 1973b).

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ANNOUNCEMENT

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The Eight International Congress of the International Society of Psychoneuroendocrinology will be held at the Atlanta Hilton Hotel, Atlanta, Georgia, USA., May 8–12, 1977. Registration will be on Sunday, May 8. For information please write to Dr. Richard P. Michael, Department of Psychiatry, Emory University School of Medicine, Atlanta, Georgia 30322, USA.

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ACTINIDINE FROM THE DEFENSIVE SECRETIONS OF DOLICHODERINE ANTS

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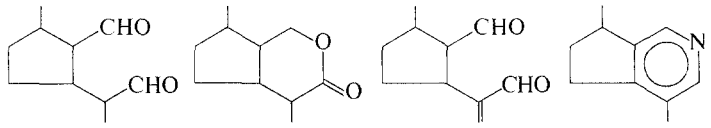
Abstract—The alkaloid actinidine has been identified as an anal gland product of two species of dolichoderine ants in the genus *Conomyrma*. The biosynthetic implications of this finding are discussed.

Key Words—actinidine, iridodial, *Conomyrma*, anal glands, biosynthesis, dolichoderine ants.

INTRODUCTION

Cyclopentanoid monoterpenes have been identified as characteristic anal gland products of dolichoderine ants in a wide variety of Old and New World genera. The widespread occurrence of iridodial (Structure I) (Cavill et al., 1956a; Trave and Pavan, 1956; McGurk et al., 1968), irido- and isoiridomyrmecin (Structure II) (Fusco et al., 1955; Cavill et al., 1956b), and dolichodial (Structure III) (Cavill and Hinterberger, 1960a, 1961) led Clark et al. (1959) to propose a synthetic scheme which included acyclic precursors and Cavill and Hinterberger (1960b) to propose later the conversion of iridodial to the alkaloid actinidine (Structure IV). Although neither the proposed precursor aldehydes, citral and/or citronellal, nor actinidine have been detected in dolichoderine ants, Bellas et al. (1974) have identified these compounds in the pygidial gland secretions of rove beetles.

We have analyzed the anal gland products of 11 ant species in the dolichoderine genus *Conomyrma* (nine of which appear to be undescribed species) and have identified the iridodials, dolichodial, iridomyrmecin, and either 2-heptanone and/or 6-methyl-5-hepten-2-one as major glandular



(I) (II) (III) (IV)

products.[3] In addition, two of these species produce actinidine as a major constituent of the capacious anal glands.

METHODS AND MATERIALS

Collection and Storage of Ants

Workers of *Conomyrma* species were collected and extracted with chromatoquality methylene chloride. After drying over anhydrous sodium sulfate these extracts were used directly for gas chromatographic–mass spectroscopic analyses.

To determine the source of the detected compounds, anal glands were dissected from the ants and extracted with methylene chloride. Analyses were performed as in the case of extracts of whole ants.

Gas Chromatography–Mass Spectroscopy

A Finnigan 3200E automated gas chromatograph–mass spectrometer was used in both electron impact (EI) and chemical ionization (CI, methane) modes. Columns of 5-ft length containing 1 or 3% OV-17 on Supelcoport 60–80 were programmed from 40 to 280°C at 8–10/min.

Synthesis

Actinidine was synthesized by the method of Cavill and Zeitlin (1967) from iridodial.

RESULTS AND DISCUSSION

Gas chromatographic–mass spectroscopic analyses using the CI mode demonstrated the presence of 2-heptanone ($M+1$, $m/e = 115$), 6-methyl-5-hepten-2-one ($M+1$, $m/e = 127$), at least two iridodials ($M+1$, $m/e = 169$),

[3] The taxonomy of these species is now being studied by Dr. W.F. Buren, Jr. The results of this investigation will be published separately.

iridomyrmecin (M+1, *m/e* = 169), and dolichodial (M+1, *m/e* = 167). The mass spectra of these compounds were congruent with those of standard compounds when each was analyzed using the EI mode.

An additional major peak (M+1, *m/e* = 148), which exhibited practically no fragmentation, was also observed in extracts of two *Conomyrma* species (*Conomyrma* sp. 1-Wellborn, Texas, and *Conomyrma* sp. 2-Athens, Georgia). Reexamination of these extracts in an EI mode yielded the same gas chromatographic peak which exhibited *m/e* 147(50), 146(26), 132(100), 131(20), 130(18), 117(42), 105(5), 103(17), 91(15), 89(6), 77(33), 65(18), 55(17), 53(22) and 51(22). Synthetic actinidine possessed the same retention time (eluting more slowly than the iridodials but before dolichodial and iridomyrmecin) and mass spectra in both CI and EI modes as the natural product. Both were soluble in dilute hydrochloric acid and were separated from neutral materials by this technique.

Actinidine joins the iridodials, dolichodial, and iridomyrmecin as part of the battery of defensive compounds synthesized in the anal glands of dolichoderine ants. Its occurrence as a concomitant of iridodial lends credence to the biosynthetic pathway in which it is proposed that actinidine is produced from the action of ammonia or its equivalent on the dial (Cavill and Hinterberger, 1960b).

Acknowledgments—We are grateful to A.C.F. Hung for collecting *Conomyrma* sp. 1. We thank W. F. Buren, Jr., for taxonomic information on *Conomyrma* species 1 and 2 which both appear to be undescribed species. We thank the MARC program of the National Institutes of Health for funds to purchase the Finnigan gas chromatograph–mass spectrometer.

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SEX PHEROMONE OF THE STABLE FLY[1]: EVALUATION OF METHYL- AND 1,5-DIMETHYLALKANES AS MATING STIMULANTS[2]

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Abstract—Each of 20 methyl-branched and 1,5-dimethyl-branched alkanes that comprise the active principle of saturated hydrocarbons of the female stable fly, *Stomoxys calcitrans* (L.), was synthesized and evaluated for mating stimulant activity. The compounds that showed the highest degree of activity in bioassays were 15-methyl- and 15,19-dimethyltritriacontanes.

Key Words—stable fly, *Stomoxys calcitrans* (L.), pheromone, sex pheromone, branched alkanes, mating stimulant.

INTRODUCTION

We recently reported the isolation and preliminary identification of compounds that induce mating strike behavior in male stable flies, *Stomoxys calcitrans* (L.) (Uebel et al., 1975a). Cuticular lipids of virgin female flies were fractionated by liquid column chromatography, and the activity of each fraction was determined by a mating strike bioassay that is now routinely

[1] Diptera: Muscidae.
[2] Mention of a proprietary or commercial product in this paper does not constitute an endorsement by the U.S. Department of Agriculture.
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employed in our laboratory (Uebel et al., 1975b). The greatest activity appeared to be associated with saturated hydrocarbons whose identities were determined by gas–liquid chromatography and mass spectrometry. The compounds were either methyl-branched C_{31}, C_{33}, C_{35}, and C_{37} alkanes or a homologous series of 1,5-dimethyl-branched compounds. Although activity was also associated with female-borne alkenes (Uebel et al., 1975a; Muhammed et al., 1975), our characterization of these materials is still incomplete. We describe here the synthesis and biological activity of 20 branched alkanes that, along with penta- and heptacosanes, constitute the major portion of the female saturated hydrocarbons.

METHODS AND MATERIALS

The monomethyl-branched alkanes were synthesized as shown in Figure 1. Ethylidenetriphenylphosphorane was generated from the phosphonium salt (Maercker, 1965) and allowed to react with an alkyl bromide. The resulting mixture was diluted with 1,2-dichloroethane, washed with water, and dried. Removal of the solvent provided the α-methylphosphonium salt, which was converted to a phosphorane and reacted with an aldehyde. Hydrogenation of the olefin from this sequence provided the methyl-branched alkane. Purity of the product (>97%) was assessed by gas–liquid chromatography (GLC) and thin-layer chromatography (TLC). Satisfactory elemental analysis was obtained for 11-methylhentriacontane (within 0.2% of theory for C, H).

The dimethylalkanes were synthesized by the method outlined in Figure 2. Details of this procedure have been published elsewhere (Sonnet, 1976). Intermediates in the synthesis were routinely purified by column chromatography; the final product was recrystallized at −20°C from acetone and its purity assessed by GLC (>96%) and TLC. Satisfactory elemental analyses were obtained for 11,15-dimethylhentriacontane (within 0.2% of theory for C, H).

$$CH_3CH_2\overset{\oplus}{P}Ph_3\,\overset{\ominus}{Br} \xrightarrow[(2)\ CH_3(CH_2)_8CH_2Br]{(1)\ n\text{-BuLi}} CH_3(CH_2)_9\overset{CH_3}{\overset{|}{C}}H\overset{\oplus}{P}Ph_3\,\overset{\ominus}{Br} \xrightarrow[(2)\ CH_3(CH_2)_{18}CHO]{(1)\ n\text{-BuLi}}$$

$$CH_3(CH_2)_9\overset{CH_3}{\overset{|}{C}}{=}CH(CH_2)_{18}CH_3 \xrightarrow{H_2/Pt} 11\text{-}C_{31}H_{63}(CH_3)$$

FIG. 1. Synthesis of methyl-branched alkanes.

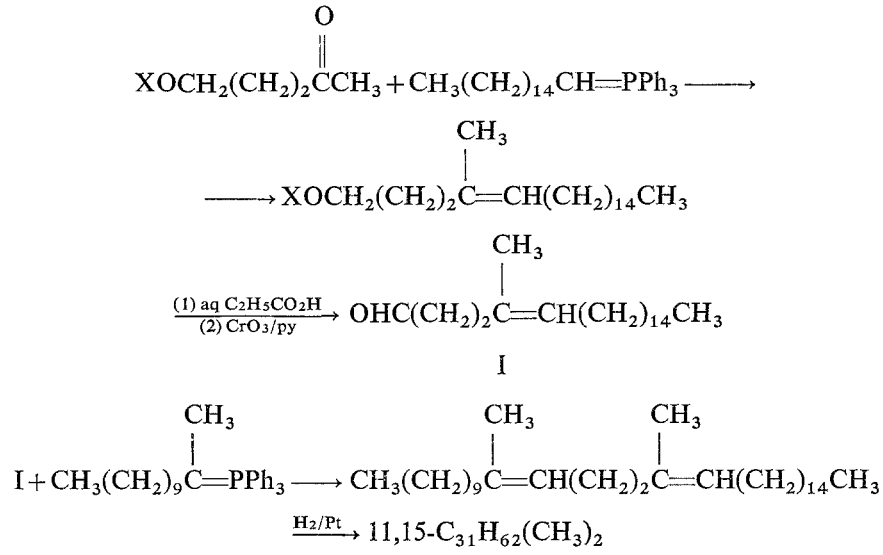

FIG. 2. Synthesis of 1,5-dimethylalkanes, X = tetrahydropyranyl group.

The bioassays used in these tests were: (1) the assay described in our initial report on the subject of fly pheromones (Uebel et al., 1975b), and (2) a similar assay wherein the strike activity was mechanically recorded (Harris et al., 1976). For the first assay, laboratory-reared male stable flies that were less than 24 hr old were placed in quart mason jars. When they were 3–5 days old, they were exposed to other male stable flies (3–5 days old) bearing the test material. Activity of the test compound was estimated by counting the mating strikes made by the males upon the treated males. Strikes made by flies on untreated virgin females of the same age were counted over the same time period (5 min), and the activity of the test material was then expressed as the quotient of the strikes made on the treated males divided by the strikes on the females (activity quotient). Untreated male flies were, of course, inactive in this assay.

For the second assay, two treated male house flies, *Musca domestica* L., were affixed to the pen holders of an electronic recorder (a GLC recorder), and the mechanical activity generated by a group of 50 male stable flies making mating strikes was recorded on a time baseline.

RESULTS AND DISCUSSION

The activities of the branched alkanes were originally estimated by

measuring the activity quotients of GLC trappings (Uebel et al., 1975a). We observed that activity was greatest in the C_{31} and C_{33} branched alkanes. This observation was borne out by testing the synthetic hydrocarbons. A comparison of data derived from both assay methods indicated that the most active compounds were 11,15-dimethylhentriacontane and 15-methyl- and 15,19-dimethyltritriacontane. Both assays indicated consistently high activity with the C_{33} chain length. While the second method of assay (Harris et al., 1976) tended to corroborate the first (Uebel et al., 1975a), there was a significant disparity in the evaluation of 11-methylhentriacontane. This

TABLE 1. MATING STRIKE ACTIVITY PRODUCED BY SYNTHETIC BRANCHED HYDROCARBONS

Compound	Assay 1[a]	Assay 2[b]
11-$C_{31}H_{63}(CH_3)$	+++	
13-$C_{31}H_{63}(CH_3)$	++	+
15-$C_{31}H_{63}(CH_3)$	++	+
11,15-$C_{31}H_{63}(CH_3)_2$	+++	++
11-$C_{33}H_{67}(CH_3)$		
15-$C_{33}H_{67}(CH_3)$	+++	+++
11,15-$C_{33}H_{66}(CH_3)_2$	++	+++
13,17-$C_{33}H_{66}(CH_3)_2$	++	+++
15,19-$C_{33}H_{66}(CH_3)_2$	+++	+++
13-$C_{35}H_{71}(CH_3)$	+	++
15-$C_{35}H_{71}(CH_3)$	+	+
17-$C_{35}H_{71}(CH_3)$	+	++
11,15-$C_{35}H_{70}(CH_3)_2$	+	+++
13,17-$C_{35}H_{70}(CH_3)_2$		+
15,19-$C_{35}H_{70}(CH_3)_2$	+	+
13-$C_{37}H_{75}(CH_3)$	+	+
15-$C_{37}H_{75}(CH_3)$	+	
11,15-$C_{37}H_{74}(CH_3)_2$		
13,17-$C_{37}H_{74}(CH_3)_2$	+	+++
15,19-$C_{37}H_{74}(CH_3)_2$		

[a] Assay 1 (Uebel et al., 1975a) gave activity quotients as described in the text. If the quotient was $\geq 0{\cdot}7$, the compound was rated (+++); $\geq 0{\cdot}4$ (++); $\geq 0{\cdot}1$ (+); 200 μg of compound employed.

[b] Assay 2 (Harris et al., 1976) tended to be more subjective because the activity was assigned on the basis of number of seconds of mechanical activity during a timed run; 200 μg of compound employed.

compound was consistently very active in the first assay but was inactive in the second, although the only material difference in the assays was the use of male house flies. Hence, 11-methylhentriacontane should remain in consideration as a potential stimulant. The activities reported in Table 1 for the synthetics represent the average evaluation of several trials and should be regarded as qualitative. Among the compounds synthesized was 11-methyltriacontane, a compound not found in our isolation study. The fact that this compound was inactive in both assays encourages us to believe in the pheromonal quality of the other isolated and synthesized materials.

CONCLUSIONS

The hydrocarbons constituting the major portion of the female-borne alkanes were synthesized and evaluated as mating stimulants for male stable flies. Activity was associated primarily with C_{31} and C_{33} methyl- and dimethyl-branched alkanes. Our previous investigation with face flies, *Musca autumnalis* De Geer (Uebel et al., 1975b) and our current results with house flies (Uebel et al., 1976) also show that pheromone activity is not associated with a single chemical species. Rather, several closely related materials act together to produce a modestly active chemical communication system. Although there is difficulty in assessing the degree to which tactile and/or visual processes are involved in the total mating communication, the repeated observation that several materials showed varying degrees of relatively independent activity suggests that the receptive processes of these species have developed in adaptation to the cuticular hydrocarbons.

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AN UNUSUAL POLYENE FROM MALE STABLE FLIES[1,2]

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Abstract—The major component of the cuticular lipids of male stable flies, *Stomoxys calcitrans* (L.), was identified as (Z,Z)-1,7,13-pentacosatriene. The identification was confirmed by synthesis. This material is of unknown biological function; it is apparently not produced by female stable flies.

Key Words—stable fly, *Stomoxys calcitrans* (L.), pheromone, sex pheromone, polyene, mating stimulant, (Z,Z)-1,7,-13-pentacosatriene.

INTRODUCTION

In an initial report of an investigation of the nature and biological activity of cuticular lipids derived from female stable flies, *Stomoxys calcitrans* (L.), we noted that test male flies bearing saturated hydrocarbon fractions from female flies stimulated caged male flies to make copulatory strikes (Uebel et al., 1975); we also defined the major chemical components of the alkanes. At about the same time, Muhammed et al. (1975) reported that pheromonal activity in stable flies was primarily associated with female-borne *trans*-alkenes that acted as mating stimuli and a female-borne polyolefin that acted as a sex attractant.

In fact, our published gas–liquid chromatograms (Uebel et al., 1975)

[1] Diptera: Muscidae.
[2] Mention of a proprietary or commercial product in this paper does not constitute an endorsement by the U.S. Department of Agriculture.
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indicated that the male stable fly may possess a polyolefin, and we found no such material present in the extracts of female stable flies that were carefully reared in isolation from male flies. Our observations have since been confirmed: The male stable flies do transfer this particular material to female flies when the sexes are not isolated (Harris et al., 1976). Since this material is a major component ($\sim$70%) of the male unsaturates and is sex-specific, our curiosity was aroused. Perhaps information about its structure and its availability through synthesis would help clarify its biological function.

METHODS AND MATERIALS

Batches of male and female stable flies that had been reared in isolation were washed with petroleum ether. The extracts were concentrated and eluted through a column of Florisil with hexane to provide the hydrocarbons (16.7 μg/fly). The hydrocarbon fraction was then further fractionated into alkanes and alkenes with varying degrees of unsaturation by using approx 1% load on silver nitrate-impregnated Florisil (100 g of 60–100 mesh Florisil coated with 25 g of silver nitrate). Separations were monitored with 20% silver nitrate-impregnated silica gel G (250 μm) thin-layer plates (Analtech, Inc.) and by gas–liquid chromatography (GLC) by using 5% SE-30 on Gas Chrom Q. Mass spectra were obtained with a Finnigan Corp. 1015 quadrupole mass spectrometer interfaced with a glass Gohlke separator to a gas chromatograph equipped with a glass column containing 3% OV-1 on Varaport. Infrared spectra were obtained as 3% CCl_4 solutions with a Perkin Elmer 457A Spectrophotometer. Some of the polyolefin material (11 μg/fly) was further purified by collections from the gas–liquid chromatograph (5% SE-30) and microozonized. The ozonides were reduced to aldehydes (Beroza and Bierl, 1967) by the addition of triphenylphosphine (Ph_3P), and the resulting aldehydes were identified by GLC with coinjections of authentic standards.

RESULTS AND DISCUSSION

Hydrogenation (H_2, PtO_2) of the unknown yielded the unbranched hydrocarbon, pentacosane; gas chromatography/mass spectrometry indicated that the substance was a triene ($M^+ = 346$). Major infrared absorption occurred at 3000, 3080, 1640 weak, 910 strong, and 990 strong, revealing that at least one double bond was terminal (i.e., a vinyl group existed) and that any internal double bonds were cis. Ozonolysis of the triene yielded two major aldehydes, one of which was identified as dodecanal

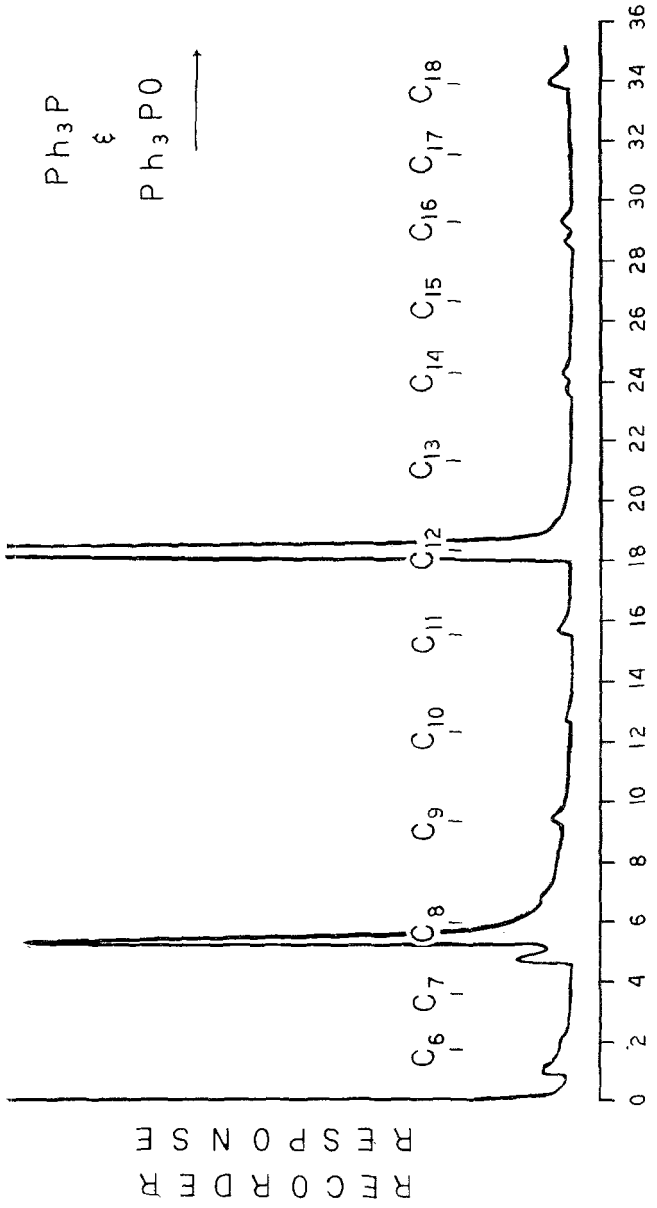

FIG. 1. Gas–liquid chromatogram (5% SE-30 programmed from 50°C at 4°/min) of the ozonolysis products from the triene of male stable flies. C with subscript indicates the retention time of aldehyde standards.

$$Cl(CH_2)_6OTHP \xrightarrow[(4)\ \text{dodecanal}]{\substack{(1)\ NaI \\ (2)\ PPh_3 \\ (3)\ n\text{-BuLi}}} CH_3(CH_2)_{10}CH{=}CH(CH_2)_5OTHP$$

$$\xrightarrow[(2)\ CrO_3/py]{(1)\ H_3O^+} CH_3(CH_2)_{10}CH{=}CH(CH_2)_4CHO$$

$$\xrightarrow{Ph_3P{=}CH(CH_2)_5OTHP} CH_3(CH_2)_{10}CH{=}CH(CH_2)_4CH{=}CH(CH_2)_4OTHP$$

$$\xrightarrow[(2)\ CrO_3/py]{(1)H_3O^+} CH_3(CH_2)_{10}CH{=}CH(CH_2)_4CH{=}CH(CH_2)_4CHO \xrightarrow{Ph_3P{=}CH_2} 1$$

FIG. 2. Synthesis of 1,7,13-pentacosatriene. OTHP = 2-tetrahydropyranyloxy.

(Figure 1). The other produced a GLC peak that fell between those of heptanal and octanal. This peak was ascribed to hexanedial by comparison with the ozonolysis product from cyclohexene (5% SE-30). The only structure fitting these data is (Z,Z)-1,7,13-pentacosatriene,[6] $CH_3(CH_2)_{10}CH{=}CH(CH_2)_4CH{=}CH(CH_2)_4CH{=}CH_2$.

Our initial efforts to identify this material were somewhat thwarted by difficulty in separating monoolefins from the triene, and our ozonolyses products contained spurious aldehydes. However, a very careful separation led to a triene, the ozonolysis of which yielded the two aldehydes shown in Figure 1, an indication that the triene was a nearly homogenous material. Minor peaks in Figure 1 may indicate small amounts of isomers or contamination of our triene with dienes.

The synthesis of this triene is depicted in Figure 2. Since the Wittig condensations were carried out in tetrahydrofuran, the internal olefinic linkages are ~15–20% trans. Hence, the infrared spectrum of the synthetic compound showed a weak 965 cm^{-1} band. However, the synthetic material was identical in all other respects (mass spec fragmentation pattern, GLC retention, and nature and ratio of ozonolysis products). Intermediates in the synthesis were purified by column chromatography; their identities and purities were assessed by infrared and thin-layer chromatographic techniques.

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[6] D.A. Carlson, ARS, Gainesville, Florida, independently reached a similar conclusion (private communication).

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INHIBITION OF THE ATTRACTANT PHEROMONE RESPONSE IN *Ips pini* AND *I. paraconfusus* (COLEOPTERA: SCOLYTIDAE): FIELD EVALUATION OF IPSENOL AND LINALOOL[1]

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Abstract—Linalool, a compound from male *I. pini*, previously suggested as an inhibitor for *I. paraconfusus*, has no obvious effect on the response of *I. paraconfusus* to ponderosa pine bolts containing male *I. paraconfusus*. *I. pini* from California and New York equally inhibit the response of *I. paraconfusus* to male *I. paraconfusus*. Ipsenol, one component of the attractant pheromone of *Ips paraconfusus*, inhibits attacks by *Ips pini* on ponderosa pine logs baited with male *I. pini*. The concentration of ipsenol used appears to be critical for effective suppression of attacks.

Key Words—*Ips*, *paraconfusus*, *I. pini*, pheromone, allomone, inhibition, ipsenol, linalool, Scolytidae.

INTRODUCTION

Ipsenol (2-methyl-6-methylene-7-octen-4-ol) was identified by Silverstein et al. (1966) as one of three synergistic alcohols forming the attractant pheromone of *Ips paraconfusus* Lanier. Linalool (3,7-dimethyl-1,6-octadien-3-ol), an isomer of ipsenol, is produced by male *Ips pini* (Say) and female *I. paraconfusus* (Young et al., 1973). Both compounds were implicated as allomones in the mutual inhibition of attractant pheromone response between *I. paraconfusus* and *I. pini* (Birch and Wood, 1975). Bolts of ponderosa pine (*Pinus ponderosa* Laws) containing males (the pheromone-producing sex) of both species side by side attracted significantly fewer beetles of each species

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than did bolts containing males of either species alone. Ipsenol duplicated the activity of male *I. paraconfusus* in decreasing trap catches of *I. pini* when eluted next to a bolt containing male *I. pini*. Linalool reduced trap catches of *I. paraconfusus*, but the results were not significant (Birch and Wood, 1975).

In this study we aimed to clarify the status of linalool as an inhibitor for *I. paraconfusus*. Since *I. pini* from California and New York show considerable differences in their pheromone systems (Lanier et al., 1972) we also investigated the ability of male *I. pini* from California and New York to inhibit the response of *I. paraconfusus* to bolts containing male *I. paraconfusus*. In addition, we tested the effectiveness of ipsenol in preventing attacks of *I. pini* on logs baited with male *I. pini*, in order to evaluate the potential of ipsenol as a practical deterrent to *I. pini* attacking fallen ponderosa pine.

METHODS AND MATERIALS

Beetles of both species were obtained from infested ponderosa pine collected from the McCloud Flats area of the Shasta-Trinity National Forest, Siskiyou County, California. Adult beetles were sexed after they emerged in the laboratory in emergence cages similar to those described by Browne (1972). They were stored under refrigeration for up to two weeks before being used in field tests. The New York *I. pini* were reared from white pine (*Pinus strobus* L.), and shipped on strips of white pine phloem to California, where they were removed from phloem and refrigerated.

Ipsenol was obtained from Chemical Samples Company, Cleveland, Ohio, at 95+% purity and was further purified by gas–liquid chromatography (GLC) until one GLC peak was detected (approximately 98+% pure). Linalool was obtained from Matheson, Coleman, and Bell at "research grade" purity. Only one GLC peak could be detected in the sample.

Inhibition of I. paraconfusus *by Linalool*

Field experiments on *I. paraconfusus* were conducted at McCloud Flats in 1974 and 1975 and at Miami Creek, Sierra National Forest (Madera County, California) in 1975. Field tests followed established procedures (Wood et al., 1968; Bedard and Browne, 1969; Birch and Wood, 1975). Bolts containing 25 or 50 male *I. paraconfusus* were placed inside wire-mesh cylinders coated with Stikem Special®, and set on pipe standards 1 m above ground level. The beetles were contained in holes in the bolts by fine screen, with which the entire bolt was also wrapped to prevent attacks by the wild population. Each test consisted of alternations of treatments at 50-m intervals in a line.

The three tests with linalool followed three different procedures. In the first (McCloud, July 14–18, 1974), linalool was evaporated from 5-μl capillary tubes open at both ends (Drummond Micro-Caps®). The tubes were held vertical by taping them to the inside of an inverted 35-mm-film canister with holes drilled in the screw cap (Birch and Wood, 1975). Two 5-μl Micro-Caps were taped in each canister and eluted linalool at a combined rate of approximately 1 mg every 6 hr. Six different treatments were used:

1. One bolt containing 25 male *I. paraconfusus*
2. One bolt with 25 male *I. paraconfusus* plus a linalool canister hung on the sticky basket
3. One bolt containing 50 male *I. paraconfusus*
4. One bolt containing 50 male *I. paraconfusus* plus linalool
5. One bolt without beetles but with linalool
6. One bolt without beetles or linalool

After each replication (one treatment in a given position for 6 daylight hr), the treatments were interchanged so that no treatment occupied the same site twice. Each Micro-Cap of linalool was refilled between replications. After four such replications, the linalool canisters were changed from 25- and 50-male bolts with linalool to those that had previously been without. The experiment continued for a further three replications. Beetles trapped during each replication were picked from the screens; placed in solvent, and later identified, counted, and sexed in the laboratory.

A similar design was used in the second linalool test (Miami Creek, August 23–28, 1975), but in this test the linalool was not switched between bolts. Each replication consisted of the trap catch for a 24-hr period. After each replication the treatments were interchanged so that no treatment occupied the same site twice.

In the third test (McCloud Flats, September 3–23, 1975) the treatments were alternated in a line, but were not interchanged. At the end of 20 days the traps were picked and counted. Each replication was one treatment in one site for the duration of the test. In both the second and third tests linalool was evaporated from a single open glass vial (2.6×0.6 cm ID) held vertical within the film canister. The elution rate was approximately 1.2 mg over 24 hr.

Inhibition of I. paraconfusus *by* I. pini

To evaluate the effect of California vs. New York *I. pini* in inhibiting *I. paraconfusus* attraction, 25 males of each were separately introduced into bolts already containing 25 male *I. paraconfusus*. Catches on traps containing these bolts were compared to those on traps with bolts of male *I. paraconfusus* alone. Control bolts contained no beetles. The entire bolts were screened to

prevent voluntary attacks and picked, as above, after each replication. All treatments were duplicated and changed in position every 24 hr for five days to give a total of 10 replications.

Inhibition of I. pini *by Ipsenol*

A preliminary experiment was conducted in 1975. A ponderosa pine was felled at McCloud Flats (August 25, 1975) and two 3-m lengths of the trunk were cut and separated by 70 m. They were raised above the surrounding surface on small logs to allow beetle access to all sides. Two clusters of six male *I. pini* were introduced into holes on each side of the upper midline of each log. The beetles were restrained in these holes for 24 hr by fine screen. The holes were marked with indelible black ink to identify these forced attacks during later analysis. After 24 hr the logs were checked to see if these beetles were active, i.e. producing frass.

Six stakes were placed around one log and film-canister elution devices taped on their tops. The canisters were about 5 cm to the side and 5 cm above the log surface, and they were positioned alternately along the log. The same arrangement was used in 1976 (Figure 1). Ipsenol was eluted from the same specification glass vials used in the second and third linalool tests, giving an elution rate for ipsenol of approximately 1.0 mg (1.0–1.05) every 24 hr in the field. This rate is based on that used by Wood et al. (1968) and Birch and Wood (1975).

After 30 days, the field appearance of the logs was described and the logs cut into four equal sections and brought back to Davis. For each log, one inner section and one outer section were totally dissected and the number of *I. pini* counted and sexed.

In 1976 the experiment was repeated with more replications. Similar

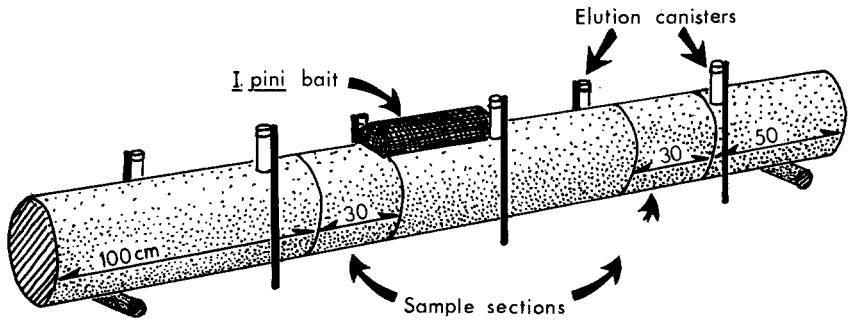

FIG. 1. Placement of ipsenol vials around a ponderosa pine log baited with male *I. pini* (McCloud Flats, California, June 1976).

methods were used, except that the bait beetles were introduced into slabs of ponderosa pine ($35 \times 10 \times 5$ cm) wrapped completely in fine wire screen and tied to the center of the log. This method was adopted to prevent the males from being pitched out of the freshly cut tree.

The elution system was similar to that used in 1975, except that the canisters were 2 cm longer than film canisters and the lower end of each was covered with fine-mesh wire screen. Two elution rates of ipsenol were evaluated. At the higher rate, the same vials and elution of ipsenol were used as in 1975 (i.e., ± 1.0 mg/day). At the lower rate small glass tubes were used, sealed at one end (3.0 cm $\times$ 2.5 mm ID). The elution rate, measured in the field, averaged 0.12 mg/24 hr period.

The treatments used were:

1. Log baited with *I. pini*
2. Log baited with *I. pini* and surrounded by six vials of ipsenol, each eluting ipsenol at 1.0 mg/day
3. Log baited with *I. pini* and surrounded by six tubes of ipsenol, each eluting ipsenol at 0.12 mg/day
4. Log with no *I. pini*, but surrounded by six vials of ipsenol eluting at 1.0 mg/day
5. Log with no *I. pini* bait and no ipsenol treatment

The experiment was set up at McCloud Flats on June 7, 1976. Treatments 1–5 were set out at each of two locations, and three each of treatments 1 and 2, and one of treatment 3 at a third location. The three sites formed an approximate equilateral traingle with a side length of 5 km. At each site, the treatments were arranged in a line at 50-m intervals.

After 22 days, the logs were examined in the field for evidence of attack. Two 30-cm bolts were removed from each log; one, starting 50 cm in from the smaller-diameter end of the log, the other starting 100 cm in from the large-diameter end (Figure 1). These bolts were brought back to the laboratory and dissected to determine the number of *I. pini* and the sex ratio of the attacking beetles.

Weather conditions in all tests, with linalool, New York *I. pini*, and ipsenol, were typical for northern California summers. Daytime high temperatures averaged 26–30°C, with nighttime lows of 4.5–5.5°C. Winds were very variable in direction and between 0 and 6 km/hr. There was no extensive cloud cover in any test.

RESULTS AND DISCUSSION

Inhibition of I. paraconfusus *by Linalool*

In the three tests with linalool there was no significant difference in trap

TABLE 1. EFFECT OF LINALOOL ON TRAP CATCHES OF *I. paraconfusus* AT BOLTS CONTAINING BORING MALE *I. paraconfusus*. MEAN NUMBER (RANGE IN PARENTHESES) AND SEX RATIO OF *I. paraconfusus* ARE LISTED BY TREATMENT IN EACH TEST

Test location and date	Treatment	*I. paraconfusus*[a] captured	♂:♀[a]	No. of replicates
McCloud Flats July 14–18, 1974	25 ♂♂ *I. paraconfusus*	45.0 (11–90)	1:3.0	14
	25 ♂♂ *I. paraconfusus* + linalool	44.1 (2–123)	1:2.2	14
	50 ♂♂ *I. paraconfusus*	60.7 (13–153)	1:3.1	7
	50 ♂♂ *I. paraconfusus* + linalool	35.7 (9–74)	1:1.9	7
	Control bolt, no beetles	0	—	7
	Bolt + linalool	0	—	7
Miami Creek August 23–28, 1975	25 ♂♂ *I. paraconfusus*	23 (8–50)	1:1.7	5
	25 ♂♂ *I. paraconfusus*	46 (23–81)	1:1.5	5
	25 ♂♂ *I. paraconfusus* + linalool	61 (6–119)	1:2.1	5
	25 ♂♂ *I. paraconfusus* + linalool	58 (21–110)	1:2.2	5
	Control bolt, no beetles	2 (0–8)	—	5
McCloud Flats September 3–23, 1975	25 ♂♂ *I. paraconfusus*	92 (78, 97, 101)	1:1.3	3[b]
	25 ♂♂ *I. paraconfusus* + linalool	96 (41, 151)	1:2.3	2
	Control bolt, no beetles	0	0	1
	Linalool alone, no bolt	0	0	1

[a] No significant differences (Student's *t* test) in beetles trapped between treatments with and without linalool in any of the tests. Sex ratio differences similarly not significant (χ^2 test) in any test.

[b] In this test each treatment was left in the same site for the duration of the test. Each treatment is, therefore, a single replicate.

catches between treatments, i.e., bolts containing male *I. paraconfusus* with or without linalool at McCloud Flats or Miami Creek (Table 1). This clarifies the equivocal results of the earlier test (Birch and Wood, 1975). In the current tests linalool treatments in only one instance caught fewer beetles than *I. paraconfusus* alone (50 male bolts, test 1). In test 2, the two linalool bolts caught considerably more *I. paraconfusus* than the other treatments. There was no significant difference in the sex ratios of beetles trapped between treatments in any test.

Linalool can be described as a natural chemical isolated from *I. pini* and female *I. paraconfusus* (Young et al., 1973), which was tested for biological activity based on deductive reasoning (Birch and Wood, 1975), but for which no behavioral effects have yet been demonstrated.

There is no discrepancy between these results and those of Birch and Wood (1975). Both illustrate the variability encountered in field evaluations of behavioral chemicals, particularly in a forest environment. They underline the necessity for using an experimental design that implicitly minimizes effects of trap location and day, and maximizes effects of treatments (Wood et al., 1976). For example, a test for significance between line positions 4, 5 and 6 vs. 1, 2, 3, 7, and 8 in test 1, showed a highly significant positional difference ($P < 0.005$: Student's t test) regardless of treatment or rotation.

Inhibition of I. paraconfusus *by California and New York* I. pini

The presence of either California or New York male *I. pini* with male *I. paraconfusus* reduced the catch of *I. paraconfusus* by 84% over the catch on male *I. paraconfusus* bolts alone (Table 2). There was no significant difference in the effect of male *I. pini* from either source to inhibit the response of *I. paraconfusus*.

The olfactory basis for the phenomenon of mass aggregation in Scolytidae was first proposed for *I. pini* (Anderson, 1948), but the pheromone has still not been identified. Although Lanier et al. (1972) demonstrated differences in the pheromone systems of California vs. New York populations of *I. pini*, it is apparent that the source of inhibition of *I. paraconfusus* does not reside in those differences. Further work on the phenomenon must await the chemical identification of the aggregation pheromone for *I. pini*.

Use of Ipsenol to Prevent Attack by I. pini

In the 1975 experiment, the log baited with *I. pini* was heavily attacked with 7.74 attacks/100 cm^2 (72/ft^2) (Table 3). The log baited with *I. pini* and also surrounded by ipsenol was attacked by only five additional males in the 50% of the surface area dissected, an attack density of less than 0.02/100

TABLE 2. EFFECT OF MALE *I. pini* FROM CALIFORNIA AND NEW YORK ON TRAP CATCHES OF *I. paraconfusus* IN RESPONSE TO MALE *I. paraconfusus*. MCCLOUD FLATS, CALIFORNIA, AUGUST 11–15, 1974. MEAN NUMBER (RANGE IN PARENTHESES) AND SEX RATIO LISTED BY TREATMENT. 10 REPLICATES OF EACH TREATMENT THROUGH TIME

Treatment (males in bolt)	*I. paraconfusus* captured	♂:♀
25 ♂♂ *I. paraconfusus*	14.3 (0–48)[a]	1:1.2[c]
25 ♂♂ *I. paraconfusus*+ 25 ♂♂ *I. pini* California	2.3 (0–20)[b]	1:1.9
25 ♂♂ *I. paraconfusus*+ 25 ♂♂ *I. pini* New York	2.4 (0–9)[b]	1:2.4
Control bolt, no beetles	0	—

[a] Trap catches on *I. paraconfusus* traps alone significantly different ($P > 0.025$, Student's t test) from catches at other treatments.

[b] No significant difference (Student's t test) between these means of trap catches.

[c] No significant difference (χ^2 test) between sex ratios in entire test.

TABLE 3. COMPARISON OF ATTACKS BY *I. pini* ON LOGS BAITED WITH MALE *I. pini*, WITH AND WITHOUT IPSENOL TREATMENT. MCCLOUD FLATS, CALIFORNIA, AUGUST 25–SEPTEMBER 24, 1975

Treatment	No. beetles dissected after 30 days[a] ♂	♀	Surface area[a] (cm^2)	Attack density[b] (per 100 cm^2)
3-m log baited with 12 ♂♂ *I. pini*	815	2147	10,533	7.74
3-m. log baited with 12 ♂♂ *I. pini* surrounded by 6 containers of ipsenol	5	9	8,856	0.01

[a] 50% of each log dissected. Each log divided into 4 equal sections, one inner and one outer section dissected. Surface refers to area dissected.

[b] Attack density/100 cm^2 based on no. of males located per surface area assuming 1 male/attack.

cm^2 ($0.1/ft^2$). The sex ratio of 1:2.6 (male/female) in the heavily attacked log indicates that the infestation was a normal breeding attack rather than a feeding attack where densities are high but the sex ratio is 1:1 (Sartwell et al., 1971). The attack density is, however, much greater than the $10.8ft^2$ average found by Sartwell (1970). There was no obvious difference in distribution of attacks in the heavily attacked log between central and end sections, but in the treatment log, only one of the 14 beetles recovered was from the end section. All the others were close to the bait males (an average of 6.5 cm from the nearest bait male).

In 1976 (Table 4), a similar reduction in attack density occurred in logs baited with *I. pini* and treated with ipsenol at the 1 mg/day level. The sex

TABLE 4. EFFECTIVENESS OF IPSENOL IN PREVENTING ATTACKS BY *I. pini* ON PONDEROSA PINE LOGS. MCCLOUD FLATS, CALIFORNIA, JUNE 7–29, 1976

Treatment	No. of replicates	Total no. *I. pini* on all sample sections	Sex ratio (♂:♀)	Attacks[a] (100 cm^2)
3-m log+ *I. pini* bait (no ipsenol)	5	700	1:3.5	1.44[f,g]
3-m log+ *I. pini* bait+ ipsenol (high rate[b])	5	5[c]	1:1.5	0.02[f]
3-m log+ipsenol (no *I. pini*)	2	0[d]	—	0.00
3-m log (no *I. pini*, no ipsenol)	2	0[e]	—	0.00
3-m log+*I. pini* bait+ipsenol (low rate[b])	3	952	1:3.6	3.11[g]

[a] Number of attacks taken to equal no. of males.
[b] The high elution rate of ipsenol was measured at 1.0 mg/canister/day over the 22-day period. The low rate was 0.12 mg/day.
[c] One log also lightly attacked by *D. brevicomis* (16 beetles in sample sections) and *D. ponderosae* (4).
[d] One log also attacked by *D. brevicomis* (74) and *D. ponderosae* (71).
[e] One log attacked by *I. latidens* (16).
[f] Difference lightly significant (χ^2: $P<0.005$)
[g] Difference not significant (χ^2 test).

ratios again indicate that these were breeding rather than feeding attacks. The two logs which were treated with ipsenol, but not baited, were not attacked at all by *I. pini*, nor were the control logs, without beetles or ipsenol. All the other logs were baited to ensure that they were attractive substrates for *I. pini*. *Ips pini* was very abundant in the McCloud area in the early summer of 1976, and we feel that our control logs would normally have been attacked under these conditions without baiting. However, there was also an unusual abundance of infested host material at the time of the test which would have provided competing sources of attraction for flying beetles.

No *I. paraconfusus* were found in any of the 34 sample sections that were dissected. However, *Dendroctonus brevicomis* LeConte and *D. ponderosae* Hopkins attacked two of the logs baited with ipsenol. One of these, without *I. pini* bait, was heavily attacked. *Ips latidens* LeConte lightly attacked one of the control logs (i.e., without ipsenol or *I. pini* as bait).

The three logs treated with a lower concentration of ipsenol were attacked to a surprisingly heavy extent by *I. pini*. Although all three logs were attacked, the high attack density (Table 4) results from only one of these logs which had attack densities of 7.3 and 8.0/100 cm^2 in the sample sections [comparable to the control log in 1975 (Table 3)]. Since the increase in attack density is not significant, there is no evidence that the lower level of ipsenol elution stimulates attacks by *I. pini*.

The results indicate that ipsenol could be used to protect vulnerable host material from attack by *I. pini*. It could be used in the same manner as is MCH (3-methyl-2-cyclohexen-1-one), which is deployed around fallen attractive Douglas fir trees to prevent attack by *Dendroctonus pseudotsugae* Hopkins (Furniss et al., 1974). With *I. pini*, an inhibitor could be an effective means of suppressing local populations since they build up in logging slash- and storm-damaged timber from which they can attack living trees (Sartwell et al., 1971). Ipsenol could also be applied in a suitable slow-release formulation, such as that developed for MCH by Rudinsky et al. (1974), to prevent attacks by *I. pini* until the potential host material had dried out and become unsuitable as a breeding substrate. Although the geographic variation in *I. pini* pheromones does not seem to affect their inhibition of *I. paraconfusus* the effect of ipsenol on different populations of *I. pini* is not yet known. The response of *I. pini* to pheromone also varies seasonally (Birch, 1972). Before ipsenol can be developed as a practical inhibitor, further experiments are needed to determine seasonal and geographical effects, the threshold of the inhibitory response, and optimum levels for deployment.

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MATING-STIMULANT PHEROMONE AND CUTICULAR LIPID CONSTITUENTS OF THE LITTLE HOUSE FLY, *Fannia canicularis* (L.)[1,2,3]

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Abstract—Chromatograms of the cuticular lipids washed from recently emerged female and male *Fannia canicularis* (L.) flies were nearly identical, but by the time the flies were 5 days old, the cuticular components of the two sexes differed considerably. A monoolefin which constituted 66% of the cuticular lipid from 5-day-old females stimulated a copulatory response from males and was identified as (Z)-9-pentacosene. The cuticular lipid of the same age males contained only 1% of this compound. Although all the major constituents of the cuticular lipid from mature females were hydrocarbons, 27% of the lipid washed from 5-day-old males was a nonhydrocarbon material that was represented by a single GLC peak. This material was identified as heneicosan-8-ol acetate.

Key Words—*Fannia canicularis* (L.), little house fly, pheromone, sex pheromone, (Z)-9-pentacosene, heneicosan-8-ol acetate.

[1] Diptera: Muscidae.
[2] A portion of a dissertation intended for submission by the first author to the Graduate School of the University of Maryland in partial fulfillment of the requirements for the Ph.D. degree.
[3] Mention of a proprietary or commercial product in this paper does not constitute an endorsement of this product by the U.S. Department of Agriculture or the University of Maryland.
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INTRODUCTION

The little house fly, *Fannia canicularis* (L.), breeds in poultry manure and often becomes a problem around poultry ranches (Axtell, 1970; Legner et al., 1973). Moreover, *Fannia* species have recently been implicated in the transmission of Newcastle disease of poultry (Rogoff et al., 1975). These flies, which have a world wide distribution, enter buildings, and it is estimated that they constitue 1–50% of the total fly population in the average house (Herms and James, 1961).

Sex pheromones or compounds that stimulate males to copulate have been reported for such Diptera as the house fly, *Musca domestica* L. (Carlson et al., 1971; Rogoff et al., 1973), the face fly, *Musca autumnalis* De Geer (Chaudhury et al., 1972; Uebel et al., 1975c), and the stable fly, *Stomoxys calcitrans* (L.) (Muhammed et al., 1975; Uebel et al., 1975b). A preliminary account of the sex pheromone of the little house fly was reported by Uebel et al. (1975a). We present here a more detailed report of the isolation and identification of the sex pheromone and of some of the major components in the female and male cuticular lipids.

METHODS AND MATERIALS

The Colony

The flies used for the study were obtained from the USDA Western Insects Affecting Man and Animals Laboratory in Fresno, California, where the colony had been maintained for many years. At Beltsville, the adult flies were fed a honey–water solution and a mixture of dry milk and sugar. Fermented CSMA medium was used for ovipositing and larval development. After pupation, the medium was dumped into a pan of water, and the floating pupae were scooped up and rinsed with water in a round-hole sieve (size 4.5/64 in., Seedburo Co., Chicago, Illinois). The pupae were dried with a stream of air from a fan and held for eclosion in a 1-liter plastic container.

Isolation of Cuticular Materials

For isolation of cuticular materials, the flies were immobilized by cooling when they were less than 24 hr old and were separated according to sex. The females and males were then held in separate cages. External lipids were collected by submerging the chilled flies in petroleum ether (boiling point 30–60°C) for ca. 0.5 min and filtering off the solvent. The solvent was removed on a rotary evaporator after the solution had been dried with anhydrous sodium sulfate.

External lipids from 5-day-old flies were used for the isolation of active materials. These lipids were fractionated by column chromatography on Florisil (deactivated with 5% water) and eluted from the column with hexane, 50% benzene in hexane, and 2% acetic acid in benzene. The hydrocarbons from the hexane fraction were separated into saturated and unsatured components by column chromatography on 20% silver nitrate-impregnated Florisil (Vroman and Cohen, 1967). The saturated hydrocarbons were eluted with hexane and the unsaturated hydrocarbons with benzene.

The number of double bonds in the unsaturated hydrocarbons and their geometric configuration were determined by comparison with standards through thin-layer chromatography (TLC) on 20% silver nitrate-impregnated silica gel G plates (Uniplate, Analtech, Inc.). At an application of 300 μg/spot, 2% of the trans in a mixture with the cis isomer would have been detected easily. The plates were developed with 15% benzene in hexane, sprayed with concentrated sulfuric acid, and heated.

Washes were prepared also from newly emerged, 3-day-old, and 15-day-old flies. These lipids and the fractions of the lipid from the 5-day-old flies obtained from the Florisil and silver nitrate columns were resolved by gas–liquid chromatography (GLC) with 5% SE-30 coated on Gas Chrom Q packed in a 0.64 (OD) × 91.4 cm glass column. The temperature was programmed from 180 to 280°C at a rate of 1°C/min. Unbranched monoolefins and unbranched saturated hydrocarbons were identified by GLC through coinjections of known standards. The percentages of material present were calculated from peak areas.

Monoolefins in the cuticular lipids of the males and females were trapped individually from the column of the gas–liquid chromatograph and converted into aldehydes by the method described by Beroza and Bierl (1967). A Supelco ozonizer (Supelco, Inc., Bellefonte, Pennsylvania) was used to convert the monoolefins to ozonides which were reduced with triphenylphosphine. The aldehydes were identified by GLC by coinjection with known aldehydes. Monoolefins (>94% cis isomer) were synthesized for confirming tests by a Wittig reaction under the conditions described by Sonnet (1975).

Bioassay for Mating Stimulants

The bioassay used for testing the possible stimulating materials was similar to that reported by Uebel et al. (1975c). Ten newly emerged male flies were confined within a quart mason jar with food and water. When they were between 3 and 9 days old they were exposed to pseudoflies prepared by applying hexane solutions containing 100 or 200 μg of the test materials to khots of black yarn. The treated pseudoflies (usually 4) were inserted into the quart jars, and numbers of copulatory attempts made on the pseudoflies

by the males were counted for a 5-min period. After a male had completed an attempt, he usually flew from the treated pseudofly; if not, he was flicked off. A jar of males was used only once each day. The same pseudoflies were used throughout a test, which consisted of a series of five 5-min trials.

RESULTS AND DISCUSSION

Cuticular Lipid from Mature Females

A comparison of the gas–liquid chromatogram of the cuticular lipid (Figure 1) from 5-day-old females with the chromatogram of the female hydrocarbon fraction revealed that all the major cuticular materials extracted by petroleum ether washing were hydrocarbons. After the saturated and unsaturated hydrocarbons were separated, the major saturated hydrocarbons were identified as straight-chain, odd-carbon-numbered paraffins by GLC with coinjections of standards.

When the unsaturated hydrocarbon fraction was chromatographed on silver nitrate-impregnated thin-layer plates, only a single spot of the same R_f value as a cis monoolefin standard was produced. The female monoolefins were identified as pentacosene and heptacosene by GLC by coinjections with standards. When the 2 alkenes were cleaved to aldehydes, nonanal and hexadecanal were obtained from the C_{25} monoolefin, and nonanal and octadecanal from the C_{27} monoolefin. Thus, the major unsaturated hydrocarbons of the female were (Z)-9-pentacosene and (Z)-9-heptacosene (Table 1).

Unlike the other species of flies that we have investigated, the major portion of the cuticular material from the females of the little house fly is made up of just one unsaturated hydrocarbon.

Cuticular Lipid from Mature Males

The cuticular lipids obtained from 5-day-old males were similar to those obtained from females in that they contained straight-chain paraffins and monoolefins (Figure 1). However, the saturated hydrocarbon fraction from the males contained branched hydrocarbons with retention times different from those of straight-chain paraffins. Branched hydrocarbons with more than 29 carbon atoms were not found in the lipid from 5-day-old females. The three major monoolefins from the males were found to be a mixture of (Z)-7- and (Z)-9-monoalkenes (Table 1).

A large peak of the male cuticular lipid between the C_{23} and C_{25} alkanes (Figure 1) was not present in the chromatogram of the male hydrocarbon fraction. This material consituted approximately 27% of the male cuticular lipid. Thirty mg of pure material was isolated by washing 3-day-old flies

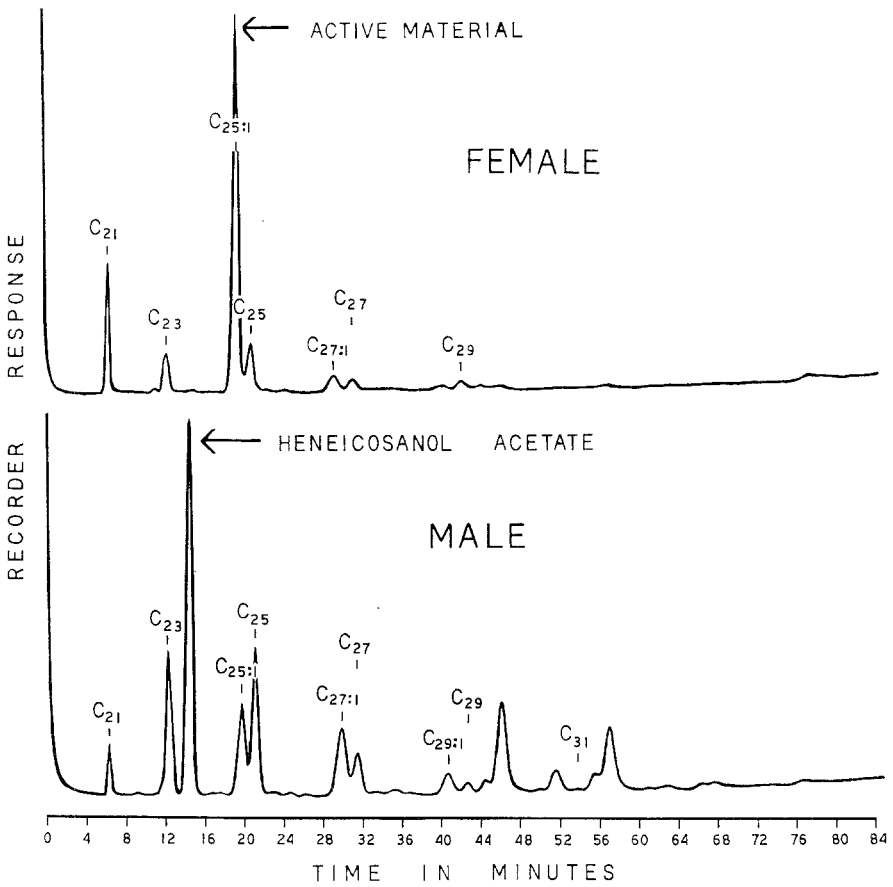

FIG. 1. Gas–liquid chromatograms of the cuticular lipid from 5-day-old female and male little house flies (temperature programmed from 180°C at a rate of 1°C/min). C with subscript identifies the straight-chain saturated hydrocarbon and straight-chain monoolefins.

(males and females) with petroleum ether, eluting the material with 50% benzene–hexane from a Florisil column, and trapping it from the gas–liquid chromatograph. It migrated between dodecyl ether and methyl oleate standards on a silica gel thin-layer sheet (1B2 Baker-flex, J.T. Baker Chemical Co.). When ozonized or treated with bromine, it retained its original GLC retention time; thus, it did not have a carbon–carbon double bond.

The IR spectrum of the material as a 3% solution in carbon tetrachloride showed a carbonyl absorption at 1735 cm^{-1}, which suggested an ester; NH or OH absorptions were not observed. An 100-MHz NMR spectrum in

TABLE 1. PERCENTAGES OF THE *cis*-MONOOLEFINS IN THE TOTAL CUTICULAR LIPID OF 5-DAY-OLD FEMALE AND MALE *F. canicularis*

Monoolefin	Female cuticular lipid (%)	Male cuticular lipid (%)
(Z)-7-pentacosene		6.5
(Z)-9-pentacosene	66.5	0.3
(Z)-7-heptacosene		5.5
(Z)-9-heptacosene	3.4	2.1
(Z)-7-nonacosene		0.7
(Z)-9-nonacosene		1.9

$CDCl_3$ with tetramethylsilane as an internal standard on a Varian XL-100 instrument revealed a broad triplet at $\delta 0.88$, indicating the presence of a chain terminating with CH_3 groups. A singlet at $\delta 2.06$ was assigned to an acetyl CH_3 group; a quintuplet at $\delta 4.92$ was consistent for a proton on an acetate-bearing carbon. The areas of the acetyl methyl group and the terminal methyl groups of the carbon chain were in a 1:2 ratio. These data suggested that the compound was a secondary acetate of a straight-chain saturated alcohol. Approximately 5 mg was saponified with 400 μl of 1 N sodium hydroxide in ethanol at 75°C for 1 hr. After the addition of 0·5 ml of water, the alcohol was extracted with petroleum ether and found to have a retention time (5% SE-30) close to that of a primary C_{20} alcohol. This secondary alcohol, therefore, probably contained 21 carbons. Consequently, a C_{21} secondary acetate (heneicosan-8-ol acetate) was synthesized which did indeed cochromatograph with the acetate.

The male material and the alcohol obtained from its saponification were analyzed by mass spectrometry (Dupont 21 491 B mass spectrometer). Isobutane chemical ionization of the acetates produced the ions expected for an ester (Pescheck and Buttrill, 1974) at *m/e*: 355 $(M+H)^+$, 100%; 295 $(M+H-\text{acetic acid})^+$, 86%; and 61 $(\text{acetic acid} + H)^+$, 64%. These MS data confirmed the structure as that of an acetate with the molecular weight of 354. Chemical ionization of the alcohol yielded the ions expected for a secondary alcohol (Field, 1970; Jelus et al., 1975) at *m/e*: 295 $(M+H-H_2O)^+$, 100%; 311 $(M-H)^+$, 27%; and 351 $(M+39)^+$, 2.5%. These ions confirmed a molecular weight of 312 for the alcohol.

Electron impact ionization of the alcohol produced diagnostic ions at *m/e*: 129 $(C_7H_{15}CHOH)^+$, 32%; and 213 $(C_{13}H_{27}CHOH)^+$, 18%. The

presence of these ions and the absence of other ions characteristic for a secondary C_{21} alcohol provided evidence that the hydroxyl group was located on the 8th carbon. Thus, the material from the males was identified as heneicosan-8-ol acetate.

The presence of an acetate among the cuticular constituents of a dipteran, especially in such a large amount, is interesting, and, so far, unique to the males of this species of fly.

Cuticular Lipid from Flies of Different Ages

Chromatograms of the cuticular lipid from newly emerged flies (Figure 2) showed that the young females and males produce nearly identical materials. These are vastly different from those of mature flies since they contain a large amount of long-chain and branched-chain hydrocarbons that are not present in the lipid from older flies.

Table 2 shows the percentages of the major cuticular components of mature flies found in the cuticular wash of newly emerged, 3-day-old, and

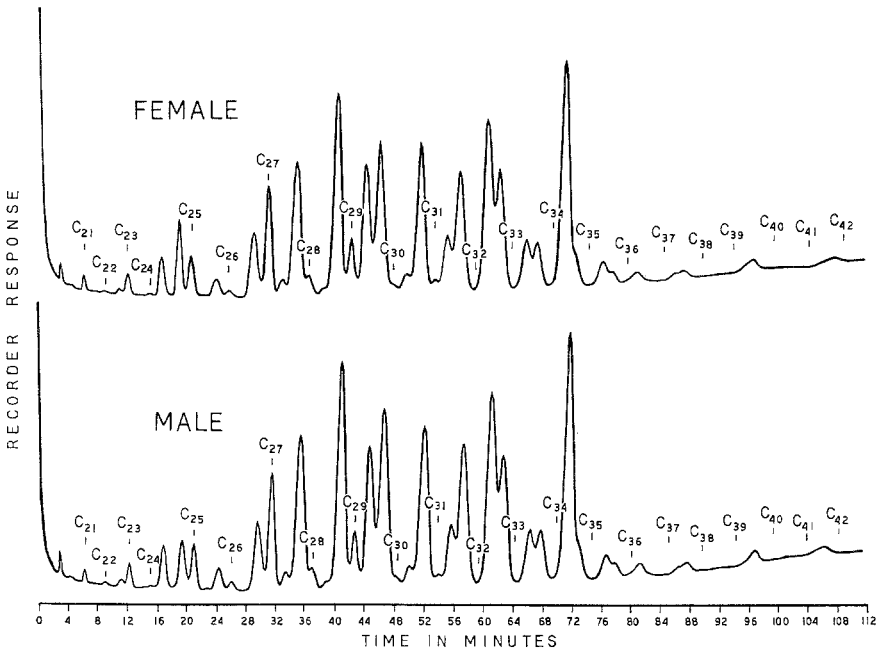

FIG. 2. Gas–liquid chromatograms of the cuticular lipid from newly-emerged female and male little house flies (temperature programmed from 180 to 280°C at a rate of 1°C/min). C with subscript indicates the retention time of saturated hydrocarbon standards.

TABLE 2. PERCENTAGES OF MAJOR COMPONENTS IN CUTICULAR LIPID FROM *F. canicularis* FEMALES AND MALES OF DIFFERENT AGES

	Percentage in lipid from flies of indicated ages					
	0 days		3 days		15 days	
Material	♀	♂	♀	♂	♀	♂
C_{21}	0.2	0.1	10.1	1.6	9.4	2.0
C_{23}	0.4	0.4	3.8	5.5	5.0	10.6
C_{21} acetate	0.0	0.0	0.0	30.8	0.0	19.4
$C_{25:1}$	1.9	1.6	64.6	5.9	66.3	5.6
C_{25}	0.9	1.2	8.1	9.5	11.7	20.3
$C_{27:1}$	2.5	2.6	1.6	4.7	1.3	7.0
C_{27}	3.7	3.3	2.0	5.1	2.3	7.0
C_{29} branched[a]	12.7	14.7	2.1	15.7	0.3	7.9
C_{31} branched[a]	9.3	9.3	0.0	9.7	0.0	9.6

[a] Tentatively identified as methyl and dimethyl branched hydrocarbons.

TABLE 3. NUMBER OF COPULATORY ATTEMPTS BY *F. canicularis* MALES AT PSEUDOFLIES TREATED WITH CUTICULAR LIPID OR HYDROCARBON FRACTIONS FROM 5-DAY-OLD FEMALE AND MALE FLIES

	Total number of copulatory attempts[a]	
Test material	100 μg/pseudofly	200 μg/pseudofly
♀ Cuticular lipid	34	73
♂ Cuticular lipid	0	0
♀ Hydrocarbon	42	51
♂ Hydrocarbon	0	0
♀ Saturated hydrocarbon	0	0
♀ Unsaturated hydrocarbon	55	49

[a] Totals are for 20 replicates of 5 min each.

15-day-old flies. Dramatic changes occurred in the makeup of these lipids during the first 3 days. After the 3rd day, the relative amounts of the major components in the female lipid became more or less stable. However, changes continued to occur in the male lipid, and as the male flies became older, the

amounts of heneicosanol acetate decreased, while the amounts of tricosane and pentacosane increased.

Identification of a Mating Pheromone

The numbers of attempted copulations with the pseudoflies treated with cuticular lipids and hydrocarbon fractions from 5-day-old female and male flies are reported in Table 3. The cuticular lipid, the hydrocarbon, and the unsaturated hydrocarbon from 5-day-old females all produced copulatory responses; the female saturated hydrocarbon, the benzene–hexane, and the acetic acid–benzene fractions of the female lipid from the Florisil column did not. The cuticular lipid and hydrocarbon from the males were also inactive.

Since both (Z)-9-pentacosene and (Z)-9-heptacosene are found in the unsaturated hydrocarbons of the females, they were synthesized and tested. Two other olefins having a cis-9 double bond, (Z)-9-tricosene and (Z)-9-nonacosene and two other isomers of the C_{25} monoolefin, (Z)-6- and (Z)-10-pentacosene, were also synthesized and evaluated.

Table 4 shows the results of the bioassays with six synthetic olefins. Only (Z)-9-pentacosene elicited as much response as the female unsaturated hydrocarbon fraction, thereby confirming that the copulatory stimulant produced by the females of the little house fly must be (Z)-9-pentacosene. This monoolefin, which is used by the males as a species- and sex-recognition pheromone, appears to be more specific for eliciting a copulatory response

TABLE 4. NUMBER OF COPULATORY ATTEMPTS BY *F. canicularis* MALES AT PSEUDOFLIES TREATED WITH SYNTHETIC *cis*-MONOOLEFINS

	Total number of copulatory attempts[b]	
Test materials[a]	100 μg/pseudofly	200 μg/pseudofly
(Z)-9-Tricosene	0	1
(Z)-9-Pentacosene	38	49
(Z)-9-Heptacosene	2	0
(Z)-9-Nonacosene	0	0
(Z)-6-Pentacosene	0	0
(Z)-10-Pentacosene	0	0

[a] Monoolefins contained $\leq 6\%$ of the trans isomer.
[b] Totals are for 15 replicates of 5 min each.

than the sex pheromones of some of the other flies (e.g., face fly), which respond to several structurally related compounds.

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A STUDY OF FACTORS THAT CONTROL BIOSYNTHESIS OF THE COMPOUNDS WHICH COMPRISE THE BOLL WEEVIL PHEROMONE[1,2]

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Abstract—Biosynthesis of the four monoterpene compounds that comprise the pheromone of the male boll weevil, *Anthonomus grandis* Boheman, [I, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; II, (Z)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III, (Z)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; and IV, (E)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde] was studied. More pheromone was biosynthesized in the summer months, somewhat less was produced by males in the presence of females than by isolated males, and the pheromone was concentrated in the gut and surrounding tissue of the abdomen of the males. Incubation of abdomens with the pheromone alcohols yielded related hydrocarbons, aldehydes, and alcohols, which suggested the presence of several enzyme systems. Boll weevils were able to allylically oxidize myrcene and limonene to alcohols, a capability that suggests pheromone precursors may be at least in part inhaled rather than ingested. The hemolymph may then transport the precursor to the gut or some alternative site where allylic oxidation to the pheromone occurs.

Key Words—boll weevil, pheromone biosynthesis, allylic biooxidation, *Anthonomus grandis* Boh.

INTRODUCTION

After the four monoterpene compounds [I, (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; II, (Z)-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III, (Z)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; and IV, (E)-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde] that comprise the sex pheromone of the male

[1] Coleoptera: Curculionidae.
[2] In cooperation with the Mississippi Agricultural and Forestry Experiment Station, Mississippi State, Mississippi 39762. Received for publication.

boll weevil (*Anthonomus grandis* Boheman) were identified (Tumlinson et al., 1969), efforts were begun to describe the factors and conditions that contribute to capability of the male weevil to produce the pheromone. Thus, Hedin et al. (1974a) showed that the total content of pheromone in the frass during 1 day is 1·3 μg and that the lifetime production is at least 40 μg. Peak productivity does not occur until the 7th–10th day of adult life, but the insect then maintains about that level of production for at least 3 weeks. Later (Hedin et al., 1975), it was shown that cotton (*Gossypium* sp.) buds, the natural food of the insect, promoted a higher level of biosynthesis than did the laboratory diet, mostly because weevils that ate the laboratory diet produced lesser amounts of the aldehydes (compounds III and IV). Also, male weevils fed the chemosterilants apholate and busulfan produced markedly less of the alcohols (compounds I and II) than did insects fed cotton buds or diet without chemosterilants. Furthermore, the chemosterilants caused a proportional decrease in the formation of II relative to I and III.

Even though male weevils must feed, preferably on cotton, if they are to produce the pheromone that attracts females, Mitlin and Hedin (1974) found that the biosynthesis of the pheromone compounds may be *de novo*: When the steam-distilled feces of adult males that had been injected with [1-^{14}C]acetate, [2-^{14}C]acetate, [2-^{14}C]mevalonic acid, or [U-^{14}C]glucose were fractionated by column and gas chromatography, approximately 0·02% of the administered radioactivity was incorporated into the volatile fraction, a percentage incorporation of these precursors into monoterpenes that is typical of plants. Moreover, the four pheromone compounds accounted for 57–80% of the radioactivity of the volatile fraction but only 39% of the total content of volatiles. Thus, the boll weevil, although it is essentially an obligate insect of cotton, does not appear to require any specific component in cotton for biosynthesis of the pheromone. The insect may nevertheless convert some constituent of the cotton to the pheromone since weevils fed buds produce more pheromone than those fed artificial diet (Hedin et al., 1975). Because of this possibility, Tumlinson et al. (1970) devised a hypothetical scheme by which a myrcene precursor such as geraniol could be converted into all four active components. [Myrcene and *trans*-β-ocimeme are major constituents of the cotton bud essential oil, and ten other monoterpene hydrocarbons, several monoterpene alcohols, and myrtenal have also been found in cotton buds (Hedin et al., 1973).]

Gut microorganisms may also affect pheromone biosynthesis. Brand et al. (1975) showed that *Bacillus cereus*, an isolate from the gut of *Ips paraconfusus*, converted α-pinene to *cis*- and *trans*-verbenol. On the other hand, Gueldner et al. (1976) found that in bacterially contaminated male boll weevils (220,000/insect) the pheromone was decreased 7-fold from that of males with an average contamination of 1400/insect. The prevalent bacteria

were *Streptococcus* sp., *Micrococcus varians* Migula, and *Enterobacter aerogenes* Hormaeche and Edwards.

In the present study, several additional aspects of pheromone biosynthesis were investigated in an effort to answer some questions. For example, it has been observed that male weevils are not equally attractive throughout the year. There also has been speculation about whether the male synthesizes less pheromone in the immediate presence of females than when isolated. Likewise, the site of biosynthesis has been assumed to be the gut because the best source of pheromone is the frass, but this has not been established. Therefore, buffered homogenates were incubated with the pheromone alcohols to obtain an enzymatically promoted equilibrium of precursors and product that would define the biosynthesis capability of the enzyme systems present in abdominal tissue. Some of the metabolites could conceivably be precursors in the normal sequence. Finally, because Hughes (1974) showed that males of *Ips* sp. allylically oxidized inhaled myrcene to the pheromones ipsdienol and ipsenol, boll weevils were exposed to myrcene, a hypothetical precursor of this insect's pheromones (Tumlinson et al., 1970), and to several other hydrocarbons to determine whether these insects had the same oxidative capability. The demonstration of such a capability could alter the present hypothesis that the insect converts some convenient food constituent to the pheromones in the gut.

METHODS AND MATERIALS

Handling of Insects and Frass

Newly emerged insects (500–1000) from the Mississippi State University Robert T. Gast Boll Weevil Rearing Laboratory were sexed, and the males and females were held separately at 29°C in cages with screened bottoms. They were fed fresh debracted cotton buds and/or diet plugs daily. The frass that fell through the screens was collected each day and stored at −20°C until analyzed. Counts of living insects were made daily to facilitate calculations. For the analyses that encompassed one year, the frass produced in 24 hr from 10-day-old insects (average) was used.

Incubation of Pheromones with Insect Abdomens

In a separate test, abdomens of feeding 10-day-old insects were ground in pH 7.0 phosphate buffer (0.1 mole) to which 10 μg of compound I or II was added. After the homogenate was incubated in a shaking water bath overnight at 37°C, it was extracted with pentane. The pentane concentrate or TLC fractions therefrom were analyzed by GLC-MS. Boiled homogenates

and homogenates to which no pheromone was added were included as controls.

Inhalation of Hydrocarbons

For the test of respiration, a small wick of absorbent cotton was impregnated with 20 mg of each hydrocarbon and placed overnight with 50 feeding, 10-day-old weevils of the appropriate sex in an enclosed petri dish. The insects were then ground in pentane for analysis. Control tests with unexposed insects were also performed.

Gas Chromatographic and Mass Spectral Analysis

The insect and the frass essential oils were prepared by grinding insects of each sex with water and steam-distilling for 1 hr in a continuous extraction apparatus with water and Et_2O (Bleidner et al., 1954; JE-1820, SGA Scientific Inc., Bloomfield, New Jersey). The Et_2O phase was dried with anhydrous Na_2SO_4. Alternatively, the insects were extracted overnight in a Soxhlet apparatus with pentane. For the final stage of the solvent evaporation, the ethereal oil was transferred to a 6-mm (ID) glass tube that had one end drawn out to capillary size and sealed. The length was adjusted to about 3 cm so a syringe needle inserted into the tube could reach the bottom. After the transfer, the liquid volume was reduced to 20 μl with the aid of a jet of nitrogen. Frass samples were handled in the same manner except that grinding was not necessary. The production of attractant was calculated for each insect each day, and insect and frass weights were recorded.

For quantitation, a stock solution was prepared containing 4 μg/μl each of dodecane, tridecane, and pentadecane in heptane; further dilution of 1 μl of this solution with 19 μl of heptane provided an external standard solution containing 200 ng/μl of the three hydrocarbons. Then, the addition of 1 μl of the stock solution to each of the insect oils (adjusted to a nominal volume of 20 μl) permitted calculation of the concentration of attractant by reference to the ratio of the GLC detector responses to internal and external standards. The GLC column was a 250-ft $\times$ 0·03-in. capillary column coated with OV-17 and used at 160°C, 5 ml/min N_2. The concentrations were determined by comparison of the products of the peak heights $\times$ retention times with those of the internal and external standards. The Kováts indices (Kováts, 1961) were: compound I, 1363; compound II, 1383; compound III, 1428; and compound IV, 1439.

A 250-ft $\times$ 0.03-in. capillary column coated with OV-17 was interfaced with a Hewlett–Packard 5930 quadrupole mass spectrometer. The column temperature was 160°C, and the helium flow was 8 ml/min. Mass spectra were

obtained at 70 eV. The spectra obtained for the insect oils were compared with standards when they were available and with compilations of standard spectra (Stenhagen et al., 1969).

RESULTS AND DISCUSSION

Total pheromone biosynthesis fluctuated throughout the year; it was at its peak from May until September and then slowly decreased to a minimum during December and January (Figure 1). Biosynthesis of compound I was also minimal until April. The fluctuations in the levels of III and IV (data not shown) were similar, but the total amounts were much less. Although insects from the rearing facility do not attain a firm state of diapause (high lipid, atrophied sex organs), the data suggest that a yearly endogenous rhythm may exist. Alternatively, the environmental conditions and diet of the insects may not be as favorable during the winter as during the summer.

The pheromone content of various insect body sections and organs is shown in Table 1. Less than 7% was found in the heads and thoraces; the remainder was present in the abdomens, as expected. The gut contained 75% of the pheromone found in the tissues or organs of the abdomen, the abdomen shells contained an appreciable 23%, and the gonads contained only 2%. The presence of pheromone in the abdomen shells suggests that glands or ducts attached to the gut remained with the abdomen shells when the gut was dissected out. It is also possible that biosynthesis occurs in fat bodies of

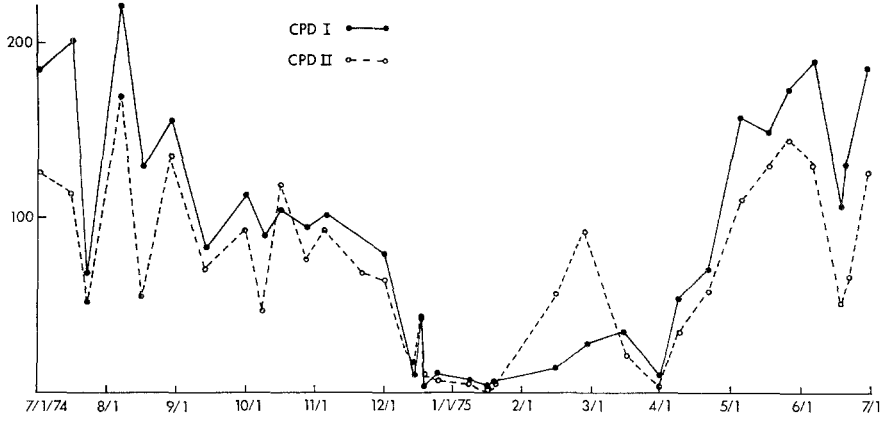

FIG. 1. Biosynthesis of compounds I and II by males (ng/insect per day in the frass) from July 1, 1974, to June 30, 1975. Production in one day by insects that were approximately 10 days old.

TABLE 1. PHEROMONE CONTENT OF MALE BOLL WEEVILS DISSECTED AT 10 DAYS AFTER EMERGENCE (NG/INSECT)[a]

Insect tissue	Compound I	II	III & IV	Total
Head and thorax	13.0	4.7	0	17.7
Abdomen	125.4	108.6	14.2	248.2
Gut	88.0	72.4	3.5	163.9
Gonads	1.5	1.1	0.5	3.1
Abdomen shell	24.7	20.8	5.8	51.3

[a] Four replicates of at least 20 insects/test. See Table 3 for chemical identification of the pheromone compounds.

TABLE 2. PHEROMONE BIOSYNTHESIS BY MALES IN SEXED AND UNSEXED COLONIES, CUMULATIVE FOR 20 DAYS (NG/INSECT IN THE FRASS)[a]

Insects	Compound I	II	III & IV	Total
Isolated males	1807.3	969.0	430.7	3207.0
Males with females	1125.9	1142.4	189.9	2448.2
Females	122.4	81.2	40.9	244.5

[a] Average for 3 replicates of 200 males, 400 mixed insects, and 200 females.

the abdomen shells and that the components are then transported to the gut for subsequent excretion. More detailed work on the site of biosynthesis is in progress.

The isolated males produced somewhat more pheromone than did the same number of males in a mixed colony, as had been anticipated (Table 2). This increased production resulted from increased biosynthesis of compounds I, III, and IV relative to that of the males in mixed colonies, but the biosynthesis of compound II by isolated males appeared to be lower than that of males in the mixed colony. Previously Hedin et al. (1974b) had failed to find more than a trace of any of the pheromone components in female frass. However, in current studies, a compound with the same mass spectral

fragmentation pattern as II has been isolated. Compounds I, III, and IV appear at much lower concentrations. The modest decrease in pheromone biosynthesis by males in mixed colonies may result from some type of hormonal control, but there is no experimental basis at present for this assumption.

By GLC-MS analysis of the oils obtained from the incubation of weevil abdomens with the pheromone alcohols, and from the inhalation of myrcene

TABLE 3. COMPOUNDS IDENTIFIED FROM INCUBATION OF ABDOMENS WITH PHEROMONE ALCOHOLS AND FROM INHALATION OF MYRCENE AND LIMONENE BY LIVE INSECTS

Compound	MS fragmentation[a]	Sex	Figure[b]
(+)-*cis*-2-Isopropenyl-1-methylcyclobutaneethanol	68,67,41,109,55[c]	♂	2a
cis-1-Methyl-1-vinyl-2-isopropenylcyclobutane	68,67,41,109,69	♂	2b
(+)-*cis*-2-Isopropenyl-1-methylcyclobutane-acetaldehyde	68,67,41,108,69	♂	2c
1,1-Dimethyl-1-[2-methyl-2-vinylcyclobutyl]-methanol	69,81,139,111,59	♂	2d
(Z)-3,3-Dimethyl-$\Delta^{1,\beta}$-cyclohexanethanol (compound II)	69,41,93,136,121[c]	♂	3a
1-Vinyl-3,3-dimethylcyclohex-1-ene	69,93,41,121,136	♂	3b
(Z)-3,3-Dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (compound III)	41,69,55,139,81[c]	♂	3c
(E)-3,3-Dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde (compound IV)	41,69,55,137,152[c]	♂	3d
3,3-Dimethylcyclohexaneethanol	69,81,95,67,123	♂	3e
Myrcene	93,69,41,77,53[c]	—	4a
Neral	69,41,84,39,94[c]	♂	4b
Geranial	69,41,84,94,109[c]	♂	4c
2-Methyl-6-methylene-2,7-octadien-1-ol	93,43,79,67,119[c]	♀	4d
Limonene	68,67,93,79,136[c]	—	5a
1,8(10)-*p*-Menthadien-9-ol	93,79,91,79,41[c]	♂	5b
1,*p*-Menthen-9-ol	67,79,93,94,68	♂	5c
1,8-*p*-Menthadien-7-ol	68,67,93,91,152,121[c]	♂,♀	5d
1-*p*-Menthen-7-ol	68,67,95,41,123	♂	5e

[a] Most abundant fragment ion values in descending order.
[b] See Figures 2–5 for structures. Nomenclature of the compounds identified from the inhalation and incubation studies.
[c] Standard spectra; see Stenhagen et al. (1969) or Tumlinson et al. (1969).

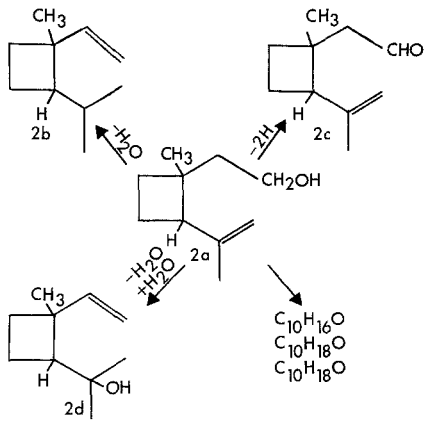

FIG. 2. Compounds biosynthesized by male boll weevil abdomens in pH 7.0 phosphate buffer incubated with compound I (see Table 3 for nomenclature and spectral data).

and limonene, a fairly large number of volatiles were observed. Similar analyses were performed on incubates to which pheromones were not added, incubates that were heated to inactivate the enzymes before the pheromones were added, and on insects not exposed to myrcene or limonene. Table 3 lists only the original terpenes and terpenoid metabolites that were formed as a result of the incubation and inhalation studies. These metabolites were not formed in the control tests. Schemes suggesting how each material may be formed are portrayed in Figures 2–5. Homogenates of male boll weevil abdomens appeared to possess three major enzymatic capabilities: (1) oxidation of the alcohols (including the pheromone alcohols) to aldehydes by a dehydrogenase, (2) dehydration of the alcohols to hydrocarbons by a hydrase, and (3) conversion to other alcohols by an isomerase (Figures 2 and 3). The most significant activity is the dehydrogenase, which can explain the presence of compounds II, III, and IV. However, the aldehyde of compound I is not normally present, nor is the trans form of compound II.

Because of Hughes' report (1974) that various bark beetles possess the capability of allylically oxidizing plant hydrocarbons to related pheromone alcohols, a search was made for the precursors which would have to be $C_{10}H_{18}$ hydrocarbons. Only $C_{10}H_{16}$ hydrocarbons were found. Also, the terpene hydrocarbons, myrcene, limonene, α-pinene, β-pinene, camphene, and β-caryophyllene, were used in the respiration study to determine whether boll weevils possess the allylic oxidation capability of beetles (Hughes 1974).

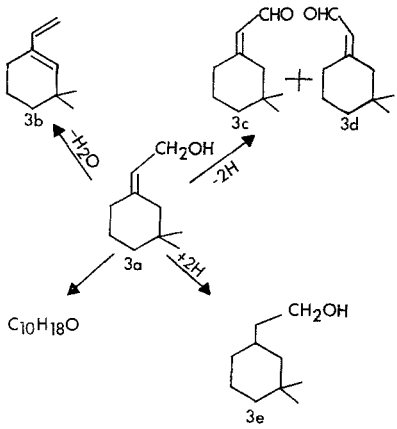

FIG. 3. Compounds biosynthesized by male boll weevil abdomens in pH 7.0 phosphate buffer incubated with compound II.

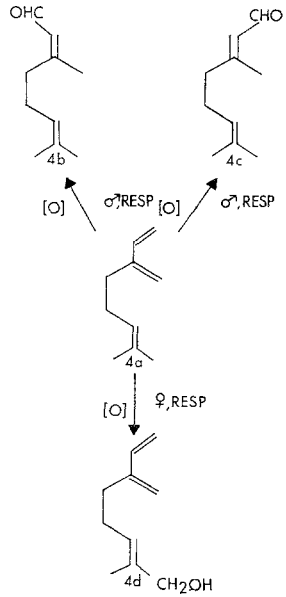

FIG. 4. Oxidation reactions by boll weevils inhaling (respiring) myrcene.

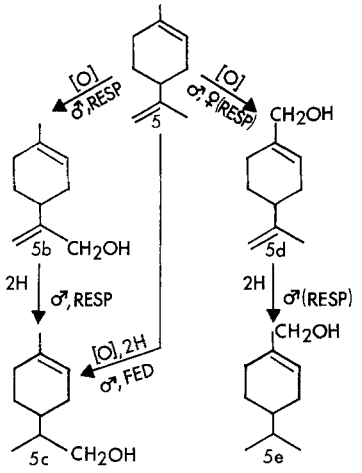

FIG. 5. Oxidation reactions by boll weevils inhaling (respiring) limonene.

Inspection of the pentane extracts of the beetles, before and after a mild acid hydrolysis (in HCl, 37°C, 1 hr), by TLC revealed that visible amounts of oxygenated terpenes were formed only with myrcene and limonene. Furthermore, this capability was much more pronounced with males than with females (Table 3). By GLC-MS, the identity of several of these metabolites was established (Figures 4 and 5). One of several potential allylic oxidation alcohols, 2-methyl, 6-methyl-ene,2,7-octadien-1-ol, was biosynthesized from myrcene. In addition, geranial and neral were formed. With limonene, allylic oxidation by males occurred both on the ring methyl and the isopropenyl functions; females were able to oxidize only the ring methyl group. Males also possessed the capability of partially hydrogenating cyclic, unsaturated terpenes or the corresponding alcohols, but no compounds were isolated that would suggest the boll weevil is capable of cyclizing myrcene or related species such as geranial, neral, geraniol, nerol, or their pyrophosphates (Tumlinson et al., 1970). However, from the boll weevil's established capability to allylically oxidize myrcene and limonene, the insect should be able to oxidize 1-ethylidene-3,3-dimethylcyclohexane to 3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol (compound II). In somewhat similar fashion, the male may be able to oxidize *cis*-1-ethyl-1-methyl-2-isopropenylcyclobutane to (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol (compound I). The demonstrated capability of the insect to oxidize inhaled hydrocarbons also suggests that the precursor(s) is not necessarily ingested; it may be transported by the

hemolymph to the gut, or converted to the pheromone elsewhere, and then transported to the gut.

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PHEROMONE-MEDIATED BEHAVIOR OF THE GYPSY MOTH[1,2]

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Abstract—The pheromone-mediated behavior of gypsy moth males was studied in both natural and simulated populations in central Pennsylvania. Feral males released into 50-m-diam plots, each with 2 feral females around the perimeter, oriented initially to trees and not to females. Neither exposure to virgin females nor exposure to wicks baited with approx 6 mg disparlure affected the subsequent sexual activity of males released into the 0.2-hectare plots. Males released into untreated plots, following 24 hr exposure in an area treated with approx 37 g/hectare of microencapsulated disparlure, located and mated with feral females within 4 min after release. None of the released males was caught in disparlure-baited Delta traps. In the disparlure-treated plot none of the females was mated. Males within this treated plot continued to search actively but did not settle down on the bark surface and initiate short-range (< 15 cm) search behavior. In plots testing the effect of various ratios of baited wicks to virgin females on disruption, there was no evidence of mating disruption due to point-source confusion. There were no significant differences in the responses of feral males to either virgin females or the various portions of Hercon wicks placed out in 0.2-hectare plots. In a series of tests using feral virgin females given various treatments to alter their physical and chemical characteristics (i.e., removed wings, denuded abdomen, washed in xylene, etc.), all females elicited the full range of sexual behavior responses of the male moths in natural populations. Apparently, males stimulated by pheromone are capable of using a number of different additional stimuli to initiate and terminate short-range sexual behavior patterns.

Key Words—*Lymantria dispar*, gypsy moth, sex pheromone, sexual behavior, disparlure.

[1] *Lymantria dispar* (L.); Lymantriidae: Lepidoptera.

[2] Authorized for publication on April 19, 1976 as paper no. 5066 in the journal series of the Pennsylvania Agricultural Experiment Station, Experiment Station Project No. 2044.

INTRODUCTION

Disparlure, the synthetic sex pheromone of the gypsy moth, *Lymantria dispar* (L.), is currently being developed as a pest management tool. This effort is evidenced in papers describing the identification and synthesis of disparlure (Bierl et al., 1970, 1972), the sexual behavior of the gypsy moth (Granett, 1974; Granett and Doane, 1975; Cardé et al., 1973, 1974; Richerson and Cameron, 1974; Richerson et al., 1976a,b), and mating-disruption field tests of various formulations of disparlure (Beroza et al., 1971a,b, 1973, 1974a,b, 1975a,b; Cameron, 1973; Cameron et al., 1974; Stevens and Beroza, 1972).

As part of this development program, Richerson et al. (1976a,b) reported the sequences of the long-range sexual communication behavior of gypsy moths and proposed a hypothetical mode of action for disparlure as a disruptant of this long-range behavior. This paper continues the evaluation of that and other hypotheses and examines aspects of the short-range sexual communication behavior. The specific objectives during the 1975 field season were to determine if: (1) pheromone-stimulated males, in 0.2-hectare plots containing 2 virgin females per plot, oriented directly to females or initially to trees; (2) previous exposure to natural or synthetic pheromone affected the numbers of pheromone-stimulated feral males caught in disparlure-baited Delta traps (Tardif et al., 1976) or the numbers of feral females mated; (3) 24-hr exposure to microencapsulated disparlure in the field affected the response of feral males to disparlure-baited traps or virgin females in untreated plots; (4) mating of tethered feral females in 0.2-hectare plots could be disrupted by placing various numbers of synthetic pheromone sources (Hercon wicks baited with approx 6 mg disparlure) in the plots; (5) the new Hercon dispensers (Beroza et al., 1974b, 1975b) were as attractive to feral males as feral virgin females in the field; and (6) feral virgin females, given various treatments to alter their physical and chemical characteristics, would elicit similar intensities of male response and whether such treatments affected the short range communication behavior of males.

Source of Insect Material

Feral moths were collected as pupae from areas with moderate to heavy defoliation in Centre, Clinton, Schuylkill, and Union counties, Pennsylvania. Laboratory-reared (lab) material was obtained from the USDA Gypsy Moth Methods Development Laboratory, Otis, Massachusetts.

Feral pupae were segregated by sex and placed in 480-ml cardboard cartons (No. 2186 Dixie Cup). Male pupae were held at approx 22°C under 16:8 L/D photoperiod. Females were held under same photoperiod at 13°C

to retard their rate of emergence. Emergent moths were collected daily, further segregated by age into 480-ml cartons, and held at ca 13°C under a 16:8 L/D photoperiod. They were allowed to acclimatize to field temperatures for approx 24 hr prior to release in field plots.

METHODS AND MATERIALS

Experimental Plots

Except where otherwise stated, all tests were conducted during July and August when native moths were active in infested forests in central Pennsylvania. Thirteen 0.2-hectare circular plots (50 m diam) were established, approx 0.8 km apart, in an 800-hectare area in Rothrock State Forest in Huntingdon Co., Pennsylvania, in an area with no detectable endemic gypsy moth population.

A 16-hectare block in the Rothrock State Forest, 0.6 km from the untreated plots described above, was treated with an aerial application of the 1974 formulation of microencapsulated disparlure at a rate of 37.1 g/hectare. Four 0.2-hectare plots were established within this treated block.

Orientation to Vertical Silhouette

In each of six 0.2-hectare plots, all trees >10 cm diam were banded to a height of approx 1 m with brown wrapping paper coated with Tack-Trap. Two feral 2-day-old females were tethered diametrically opposite one another as described by Richerson et al. (1976a) 2 m from the ground on trees in a 5-m zone outside the perimeter of each plot. There were no females within the perimeter of any plot. One hundred feral males were released from the center of each plot in each of 6 replicates.

All females were observed continuously for 1 hr after the male releases. Any male attempting to mate with a female was killed once physical contact was made with the female. The total number of attempted matings and the numbers of males caught on the sticky bands after 1 hr were recorded. After 24 hr the total numbers of males caught on the sticky bands were recorded, and all females and any egg masses present were collected. Fertility determinations were made using the technique of Stark et al. (1974).

Mating and Pheromone Exposure History

In six 0.2-hectare plots, 4 Delta traps each baited with a Hercon wick containing approx 6 mg disparlure, and 4 tethered feral virgin females 1–2 days old were placed alternately 19.6 m apart inside the perimeter of each

plot. Test insects were 3 groups of 100 1–4-day-old feral males with mating and pheromone exposure histories as follows: (1) virgin males with no previous exposure to natural or synthetic pheromone, (2) virgin males exposed in a 30-cm^2 wire-screen cage from eclosion up to time of release (24 hr) to pheromone released from 10 Hercon wicks (each containing 6 mg disparlure) suspended in the cage, and (3) males that had mated with virgin females within 6 hr of release and held in 480-ml cartons with the females until released in the plots.

Four replicates were run, 2 replicates for each group of males/day. Females were observed for 30 min after males were released. The numbers of females depositing an egg mass (=mated females) and the numbers of males caught in traps were recorded at 1/2-, 1-, 4-, 8-, and 24-hr intervals. All mated females and females not exhibiting calling behavior were replaced at the end of each time period. All females were dissected to determine fertility, and egg masses were held for evidence of embryonation.

Air-Permeation Tests

Over a 5-day period 1000 feral 1–4-day-old males were held in the treated block for 24 hr in 30.5 × 30.5 × 30.5-cm wire-mesh cages. Another 1000 feral males were held for 24 hr in an untreated area in identical cages. Within the perimeter of each of the 4 treated and 4 untreated 0.2-hectare plots, 4 Delta traps baited with Hercon wicks containing 6 mg disparlure, and 4 tethered feral 2-day-old virgin females were placed 19.6 m apart. In each of the treated and untreated plots, 100 males previously exposed to the disparlure treatment were released. After male release, traps and females were checked at 1/2-, 1-, 4-, and 8-hr intervals. The numbers of females mated and the numbers of males caught in traps were recorded. Missing or mated females were replaced at the end of each time period. After a 4-day period (to minimize interference from previously released males), the test series was repeated with males not previously exposed to the disparlure treatment. Each plot was considered a replication.

Point-Source Confusion

Three feral virgin females 1–2-days-old were tethered 19.6 m apart within the perimeter in each of six 0.2-hectare plots. Fifteen feral females were tethered in a 7th plot. Hercon wicks (Beroza et al., 1974b), baited with approx 6 mg disparlure, were pinned to trees within each plot with a No. 2 insect pin to give wick-to-female ratios of 0:3, 1:1, 5:1, 20:1, and 50:1. Two female densities, 3 and 15, were used at the 1:1 female-to-wick ratio to evaluate the effect of increased female density on the disruptant potential

of an equal number of point sources of synthetic pheromone. In each of 4 replications for each ratio (and female density), 50 feral males were released from the center of each plot. During a 30-min observation period immediately following male release, the numbers of mating attempts were recorded. An unquantified evaluation of whether or not males oriented to and made contact with the Hercon wicks was also made. Males were killed once contact was made with a female. After 24 hr, all females were collected and fertility determinations made. Any eggs deposited were held for evidence of embryonation.

Hercon Wick "Attractiveness"

All of the following pheromone sources were placed in each of 4 plots: (1) a feral 2-day-old virgin female, (2) a whole Hercon wick containing 6 mg disparlure, (3) 1/2 of a Hercon wick, (4) 1/4 of a Hercon wick, (5) 1/8 of a Hercon wick, and (6) a feral 2-day-old virgin female plus a whole Hercon wick pinned adjacent to the female. Tests were conducted between 1000 and 1400 hr EDT. Fifty feral males were released from the center of each plot. The total numbers of males orienting to trees, orienting to the pheromone sources, and numbers of mating attempts were recorded for each source during 5 consecutive 30-min observation periods.

Short-Range Communication

Feral virgin females, 1–7 days old, with various treatments altering their physical and chemical characteristics were placed individually on trees approx 50 m apart in areas of light to moderate defoliation in Centre and Clinton counties, Pennsylvania. Each of the following treatments was tested in a series of consecutive 30-min observation periods (each 30-min period = 1 replicate):

1. untreated (live)
2. freeze-killed (dead)
3. painted white (live)
4. painted red (live)
5. painted blue (live)
6. denuded abdomen (live)
7. washed in technical-grade hexane (dead)
8. washed in technical-grade xylene (dead)
9. surgically removed wings (apterous) (live)
10. 1/4 Hercon wick with each of the above treatments using 6-day-old females only
11. 1/4 Hercon wick with 1–7-day-old untreated females only

The water-based paints (Rich Art, Rich Glo Acryl-acrylic daylight fluorescent white AD14 and red AD51, and Liquitex Modular Color ultra marine blue 380) were applied with a Scientific Products Universal Multi-Mist chromatographic sprayer. Solvent-washed females were immersed for 10 min in the solvents and air-dried.

During each 30-min observation period, the total numbers of males (1) orienting to trees with test females, (2) orienting to and contacting test females, and (3) attempting to mate with test females were recorded. All males attempting to mate with the females were killed. Any live female that failed to exhibit normal calling behavior within 5 min was replaced. A subjective decision was made to run more than 4 replicates of each treatment on test days when male flight activity was depressed by either environmental conditions or when tests were run on days after the peak emergence of the native population. All treatments of a given age group were run on the same day. The numbers of native virgin females on trees where test females were placed were also recorded. All tests series were begun at ca 1000 hr EDT.

An index of male response (IMR) was designed for use in analysis of variance. This index was calculated as follows: IMR = (0.15) (no. of males orienting to a tree in 30 min)+(0.35) (no. males orienting to and contacting test females in 30 min)+(0.50) (no. mating attempts to test female in 30 min). The male responses were so weighted because of the sequentially heirarchical nature of the 3 responses (Richerson et al., 1976a,b).

RESULTS

Orientation to Vertical Silhouettes

In each of the 6 replicates, no males were observed to orient directly to females. Search behavior was directed initially to trees and other understory vegetation. During the 1-hr observation periods, 1.8% of all males released attempted to mate with the females and 9.5% were caught on the sticky bands on the trees. After 24 hr, 75% of the females were mated and a total of 11% of the males released were caught on the sticky bands.

The index of activity (Richerson et al., 1976b) for males (IA = % males attempting mating in 1 hr + % males caught on sticky-banded trees after 24 hr) released in 0.2-hectare plots with 0 and 5 females was 9.5 and 15.5, respectively. The calculated IA for males released in plots containing 2 virgin females was 12.83. This IA is in accord with the predicted activity level from 1974 and conforms to the hypothesis that the intensity of the short-range components of male sexual activity in an untreated gypsy moth population is proportional to the number of pheromone sources.

Observations of pheromone-stimulated males in the test plots revealed

that they were actively searching trees and vegetation over a much broader area of the plots than would be expected if males restricted their sexual activity to discrete odor plumes or pheromone trails as described from laboratory bioassays for a number of insects.

Mating and Pheromone Exposure History

There was no significant difference (χ^2 test) in the number of females mated by any of the variously experienced feral males at any of the 4 observation periods (Table 1). The 24 Delta traps in this series caught only 1% of the 1200 males released. Moths were observed flying around the traps but did not enter. Apparently, the majority of males released had flown out of the untreated area after 1 hr. However, at the 8- and 24-hr periods, a few males were observed actively searching trees within the 0.2-hectare plots.

Air-Permeation Tests

In the treated plots, none of the females were mated during the test and no males were caught in the 16 Delta traps (Table 2). During each 30-min observation period immediately after release of the males and at each of the subsequent check periods, moths were observed flying up and down trees but not searching in the walking–wing-flutter behavior patterns on the bark of trees.

In the untreated plots, females were mated during each time period by

TABLE 1. NONACCUMULATIVE MATING ACTIVITY OF FERAL MALES WITH DIFFERENT MATING AND PHEROMONE-EXPOSURE HISTORIES RELEASED IN 50-M-DIAM PLOTS IN UNTREATED UNINFESTED AREAS[a]

Mating and pheromone exposure histories of males	Percent of 16 feral females mated (hr)[b]			
	1/2	1	8	24
Virgin, naive	43.75	31.25	18.75	12.50
Virgin, Hercon wick with 6 mg disparlure	37.50	25.00	25.00	12.50
Mated with 2-day-old feral female	50.00	25.00	18.75	12.50

[a] For each treatment group, 100 feral males released in each of 4 replicates.
[b] Based on total number of feral females exposed in 4 replicates for each time period.

TABLE 2. MATING ACTIVITY OF NAIVE FERAL MALES AND "HABITUATED" FERAL MALES EXPOSED TO MICROENCAPSULATED DISPARLURE FOR 24 HR IN A SERIES OF 50-M-DIAM PLOTS IN DISPARLURE-TREATED AND UNTREATED AREAS

	Mean no. feral females mated per replicate[a]							
	Treated plots (hr)				Untreated plots (hr)			
Male treatment	1/2	1	4	8	1/2	1	4	8
Naive	0	0	0	0	2.00	1.75	1.25	0.75
"Habituated"	0	0	0	0	1.25	1.25	0.75	0.50

[a] In each of 4 replications for each male treatment, 100 feral males were released into plots each containing 4 feral virgin 1–2-day-old females. Disparlure-exposed males were held in plot treated with microencapsulated disparlure applied at a rate of 37.1 g/hectare.

habituated or naive males (Table 2). The first mating after release by a "habituated" male was at 3.5 min ($\bar{X}$ = 4.8, range = 3.5–10.1 min) and for the naive male was 3.1 min ($\bar{X}$ = 4.3, range = 3.1–8.9 min). There was no significant difference in the mean number of females mated by either of the 2 groups of males at each of the 4 time periods. Only one male, habituated, was caught in one of the Delta traps. As in previous tests, most males had left the untreated plots within 1 hr; very few remained after 8 hr.

TABLE 3. SEXUAL RESPONSE OF FERAL MOTHS (N = 50/REPLICATE; 4 REPLICATES) RELEASED IN 50-M-DIAM PLOTS CONTAINING VARIOUS RATIOS OF HERCON WICKS TO FERAL 2-DAY-OLD VIRGIN FEMALES

No. pheromone sources placed in plot		Percent of total no. released males attempting to mate in four 30-min periods	$\bar{X}$ no. and % females mated per replicate after 24 hr	
Wick	Female		$\bar{X}$	%
0	3	4.00	2.25	75.0
3	3	4.50	2.50	83.3
15	3	3.00	2.00	66.7
30	3	2.50	2.00	66.7
60	3	2.50	1.75	58.3
150	3	2.00	2.00	66.7
15	15	14.00	11.00	73.3

Point-Source Confusion

There was no significant reduction of mating activity (χ^2 test) at any of the wick-to-female ratios tested (Table 3). The decrease in the numbers of attempted matings in plots with the greater number of wicks to females was not significant. Males did not persist at wicks for longer than approx 3 min ($\bar{X}$ = 2.4, range = 0.2–3.2). Few males were observed making copulatory actions toward wicks. The intensity of male search activity on trees appeared to be greater in plots as the number of pheromone sources (natural + synthetic) per plot increased.

Hercon Wick "Attractiveness"

There was no significant difference (χ^2 test) in the relative "attractiveness" of 2-day-old feral virgin females, Hercon wicks, and portions of these wicks as measured in the 0.2-hectare plots (Table 4). There were significantly more male orientations to trees bearing a whole wick or a virgin female + a whole wick than to other sources tested (χ^2 test). However, the same proportion of males contacted the other pheromone sources. Only the virgin females and the whole wick elicited copulatory behavior from the males. The copulatory attempts toward wicks generally terminated within 3 min after initial contact.

TABLE 4. SEXUAL RESPONSE OF FERAL MALES RELEASED INTO 50-M-DIAM PLOTS CONTAINING A SERIES OF SYNTHETIC AND NATURAL PHEROMONE SOURCES

Pheromone source[a]	Total no. of orientations to trees by released males (N = 50)	Male orientation efficiency[b]
2-day-old feral virgin female	61	29.51
Hercon wick (approx 6 mg disparlure)	89	26.96
1/2 Hercon wick (approx 3 mg disparlure)	57	24.56
1/4 Hercon wick (approx 1.5 mg disparlure)	44	27.27
1/8 Hercon wick (approx 0.75 mg disparlure)	38	28.94
2-day-old virgin female + Hercon wick (approx 6 mg disparlure)	105	27.61

[a] Disparlure content of Hercon wicks based on amount required in USDA specifications to manufacturer, The Herculite Corp.

[b] Male orientation efficacy = $\frac{\text{Total no. males contacting source}}{\text{Total no. orientation to trees w/source}} \times 100$.

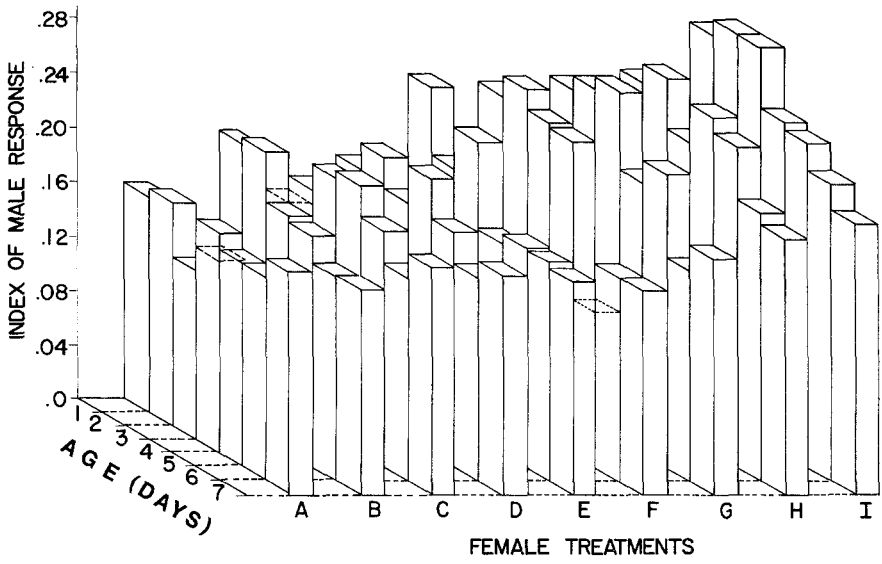

FIG. 1. The mean of the index of male response (see text) of feral males in infestations with light-to-moderate defoliation, to 1–7-day-old females given 9 treatments altering their physical and chemical characteristics. A = xylene-washed females, B = hexane-washed females, C = painted red, D = painted blue, E = freeze-killed, F = painted white, G = denuded abdomen, H = untreated, and I = apterous. Dotted lines indicate position of a column hidden by other columns.

Short-Range Communication

Neither age nor treatments given the feral females completely eliminated the sexual response of the native male moth population (Figure 1). As expected from the sequential and heirarchical nature of the sexual responses of gypsy moth, the proportion of males that attempted mating was lower than number of males orienting initially to trees. The addition of the 1/4 Hercon wick baited with disparlure only increased the number of males orienting to trees and contacting females (particularly 4–6-day-old females) but did not increase the relative number of mating attempts (Table 5).

In analysis of the IMR elicited by each of the 9 treatments with all ages pooled, the apterous females were significantly more "attractive" to feral males than all other females (Figure 1) (Fisher's LSD test, $P<0.04$). There was no significant difference in the IMR between the untreated females and the females with denuded abdomens. There was no significant difference between females with denuded abdomens and females painted white. Females painted red or blue, washed in xylene or hexane, and freeze-killed elicited a

TABLE 5. MEAN NUMBER OF MATING ATTEMPTS BY FERAL MALE GYPSY MOTHS TO 1–7-DAY-OLD FERAL FEMALES WHOSE PHYSICAL AND CHEMICAL CHARACTERISTICS HAVE BEEN MODIFIED[a]

Female treatments	Mean no. mating attempts ±SE[b,c]	
	Without 1/4 Hercon wick	With 1/4 Hercon wick
Apterous (live)	15.14±1.89 (a)	14.00±1.61 (a)
Untreated (live)	11.57±1.47 (b)	11.57±0.80 (b)
Denuded (live)	5.71±1.68 (c)	6.14±1.14 (c)
Painted white (live)	5.29±1.43 (c)	5.57±1.30 (c)
Painted blue (live)	6.57±1.50 (c)	5.29±1.51 (c)
Painted red (live)	2.71±1.31 (d)	2.29±1.13 (d)
Freeze-killed (dead)	2.71±1.27 (d)	2.57±1.41 (d)
Washed in hexane (dead)	2.71±1.27 (d)	2.43±0.97 (d)
Washed in xylene (dead)	2.43±1.38 (d)	1.88±1.26 (d)

[a] Mean no. mating attempts based on male activity during 4–7 consecutive 30-min observation periods in a gypsy moth infestation with light-to-moderate defoliation.

[b] The 1/4 Hercon wick contained approx 1.5 g disparlure (based on USDA specifications for the whole wick).

[c] Numbers followed by same letters within columns are not significantly different at the 5% level (Fisher's LSD test).

significantly lower IMR from native male moths than females that were untreated, apterous, painted white, and with denuded abdomen (Fisher's LSD test, $P<0.05$) (Figure 1).

In analysis of the IMR elicited by each of the 7 age groups with all treatments pooled, 1–4-day-old feral test females were significantly more attractive than older females tested (Fisher's LSD test, $P<0.01$) (Figure 1).

In analysis of the IMR as related to both treatment and age of the feral test females (Figure 1), apterous females were the most attractive of all treatments particularly in the 1–3-day-old females (Fisher's LSD test, $P<0.04$). Generally, the 1–3-day-old dead, white-painted, denuded abdomen, untreated, and apterous test females were significantly more "attractive" to native male moths than all the other treatments (and ages) (Fisher's LSD test, $P<0.05$). Of all treatments, the 1–3-day-old xylene-washed females were least "attractive" to native males than other tested females of similar age (Fisher's LSD, $P<0.05$).

The addition of a 1/4 Hercon wick (containing ca 1.5 mg disparlure) to (1) 6-day-old feral females given all 9 treatments and (2) untreated feral

females 1–6 days old did not significantly alter the relative "attractiveness" of these females compared to test females without the Hercon wick.

DISCUSSION

The air-permeation technique is a most promising use of sex pheromone to control pest insect populations (Beroza et al., 1973, 1974a,b, 1975a,b; Farkas et al., 1974; Kaae et al., 1972; McLaughlin et al., 1972). This technique requires a pheromone formulation to be dispersed over large areas, releasing sufficient pheromone to disrupt sexual communication. Air permeation implies a mode of action through the ill-defined and poorly understood phenomena of either habituation of the CNS or adaptation of the sensory receptors (Farkas et al., 1975). Other proposed mechanisms of disruption are: (1) inability of males to distinguish between pheromone emitted by females (or males) and synthetic pheromone sources (Shorey et al., 1972), (2) physical exhaustion of males that have repetitively responded to individual synthetic phermone sources (Beroza, 1971), (3) confusion due to overlapping scent plumes or odor trails (Marks, 1975), and (4) the blocking of the release of short-range premating behavior (Richerson et al., 1976a,b).

Numerous laboratory bioassays of insect pheromone systems demonstrate that males will fly upwind and orient to an emission source. Over longer distances (1 m), males are described as responding to either the presence or absence of pheromone in the environment. By keeping within an odor plume or trail they can get close enough to a source to orient along concentration gradients (Farkas and Shorey, 1975; Jacobson, 1974). At this point, the role of other stimuli (chemical, visual, tactile, etc.) is generally assumed to be subordinate to the response of the male to chemical gradients. The importance of visual stimuli in sexual communication has not, however, been completely overlooked (Brown and Cameron, 1976; Doane, 1968; Shorey and Gaston, 1970).

Most of the "modes of action" of disruption are symptomatic of the "dogma of immaculate perception" (Bertalanffy, 1968); i.e., the organism as a passive receptor of stimuli, responds in fixed, rigid behavior patterns to specific external stimuli. This concept of organisms as automatons is a basic tenet in the mechanistic stimulus–response (S–R) theory of behavior accepted by many pheromone researchers. This acceptance is evidenced by the emphasis placed on the chemical (pheromone) as the dominant, if not the only, stimulus affecting sexual communication behavior in insects (Beroza and Bierl, 1971).

Hodgson (1974) and Markl (1974), among others, suggest that the central nervous system of insects can play an important role in the integration of afferent impulses from the chemoreceptors. Bertalanffy (1968) contends that

even under constant external conditions and in the absence of external stimuli, the organism is not a passive but an active system in which internal activity rather than reaction to stimuli is fundamental to behavioral responses.

Within the framework of the S–R theory of behavior, habituation and adaptation are generally viewed as either reducing or terminating any sexual behavior responses (Farkas et al., 1975). Habituation is often assumed to be a relatively long-lasting influence, while adaptation is short-term. Laboratory bioassays using Tortricidae and Noctuidae have demonstrated that previous exposure to pheromone reduced the responsiveness of males to subsequent pheromone exposure (Bartell and Lawrence, 1973; Traynier, 1970). The responsiveness of *Trichoplusia ni* (Hubner) to subsequent pheromone exposure was reduced by a 30 sec preexposure to pheromone (Shorey and Gaston, 1964). Recovery from habituation is often less rapid. *T. ni* males in laboratory bioassays remained partially habituated for at least 15 min following approx 16 hr of preexposure to either continuous supply or repetitive pulses of pheromone (Farkas et al., 1975). Bartell and Shorey (1969) observed that the tortricid *Epiphyas postvittana* (Walker) fully recovered after 80 min. Payne et al. (1970) reported that *T. ni* male antennae totally regained their responsiveness to female sex pheromone within 60 sec following a prior pheromone stimulation. This rapid recovery is thought to reflect recovery from sensory adaptation rather than recovery from habituation (Farkas et al., 1975). In 1974 field tests using laboratory-reared gypsy moth males with different pheromone exposure and mating histories, no significant difference in the numbers of males caught at pheromone-baited traps after an 8-hr test was observed (Richerson et al., 1976b). In the 1975 field tests using feral males with the same pheromone exposure and mating histories as in 1974 tests, there was no reduction of sexual responses to virgin females when the males were released into untreated plots. Furthermore, males held for 24 hr in treated plots and released within 5 min into untreated plots containing virgin females and pheromone-baited traps were observed mating with females within 4 min after release. Males continued to search trees and understory vegetation in areas treated with a disparlure formulation that disrupted mating activity. Recovery from any habituation or adaptation is thus concluded to be rapid for the gypsy moth.

In 1-hectare plots treated with 18 g/hectare of microencapsulated disparlure applied by backpack sprayer, there was very little or no sexual activity observed within the treated plots or at the edge of the plots (Granett and Doane, 1975). These tests were conducted in dense collapsing populations, and the disparity of results in 1974 and 1975 tests makes comparisons impossible. Tests reported herein and by Richerson et al. (1976b) were made in simulated populations in uninfested forests.

Field tests conducted in 1974 and 1975 using simulated populations in

0.2-hectare plots (Richerson et al., 1976b) have shown that pheromone-stimulated gypsy moth males will search trees and other vertical silhouettes in the forest. Without the presence of pheromone above the behavioral threshold needed to initiate search activity, male flight is random and non-directed. Within an active space in disparlure-treated or untreated plots containing virgin females, search behavior is initiated. This has been considered as part of the long-range sexual behavior pattern, which includes upwind anemotactic flight behavior (Richerson et al., 1976b). Short-range flight behavior occurs when males exhibiting vertical search flight settle down on the bark or other substrate and begin a walking–wing-flutter search activity. Apparently, this behavior always precedes any mating activity or entry into a baited trap. The assumed habituating or adapting concentrations of microencapsulated disparlure in treated plots does not reduce the long-range sexual behavior but does somehow prevent the initiation of the short-range sexual behavior. Removal of males from this "disruptant" influence results in the rapid "recovery." In the presence of pheromone-baited traps, disparlure dispensers, or virgin females such males respond with both long- and short-range sexual behavior patterns. Therefore, it appears that failure of gypsy moth males to locate females in disparlure-treated areas is not due to either habituation or adaptation, at least not in the context of total "shut-down" of the sexual responses of the insect.

The concept of point-source confusion or numerical confusion (Beroza and Knipling, 1972) assumes that the receptors of the males are not adversely affected by the pheromone and that males continue to search for females but orient to synthetic pheromone point sources instead. These point sources may be pheromone-baited traps, cork granules, paper chips, microcapsules, or any other pheromone dispenser (Beroza et al., 1973). It is assumed that these point sources are: (1) at least as attractive as the female and (2) present in the environment in greater numbers than females by several orders of magnitude (Beroza and Knipling, 1972). If the individual point sources are more attractive than individual females, the greater the ratio of point sources to females the greater the expected confusion of the male response. Field tests using various ratios of disparlure-baited Hercon wicks (containing approx 6 mg disparlure/wick) to feral virgin females demonstrated that at wick-to-female ratios of 50 : 1 there was no significant reduction of mating.

Laboratory assays on the emission rates from the Hercon wicks indicate that at room temperature the emission rate is approx 0.22 μg/hr and at 27°C it is 0.24 μg/hr (J.R. Plimmer, personal communication). Field temperatures during my tests were within that temperature range. Feral females emitted approx 1 μg/day in a laboratory study (Richerson and Cameron, 1974). Males were sexually stimulated by the lure from both females and Hercon wicks. However, in the field, males have not been shown to orient

directly to point sources of either natural or synthetic pheromone (Richerson et al., 1976a,b). It is unlikely that the point-source-confusion method is the mode of action of disparlure as a disruptant of the sexual activity of gypsy moths.

None of the alternations of the physical and chemical characteristics of feral virgin females terminated the complete sexual activity of males. The pheromone-stimulated males were able to utilize a number of different stimuli (chemical, visual, and tactile) in order to initiate short-range behavior and to locate and be stimulated to attempt to mate with the test females. This flexibility of male moth behavior to respond to a variety of combinations of stimuli, under the influence of pheromone, does not conform to the predicted behavioral response of an organism operating under the S–R theory of behavior. In light of published data on adult gypsy moth behavior (Cardé et al., 1974; 1975; Doane, 1968; Granett and Doane 1975; Richerson et al., 1976a,b) this insect does not appear to be a passive automation. It is possible that pheromone-mediated behavior can best be interpreted through the general system theory principal of equifinality; i.e., the same goal may be reached from different initial conditions and from different pathways in organismic processes (Bertalanffy, 1968). In the tests reported here, and in previous tests (Richerson and Cameron, 1974; Richerson et al., 1976a,b), males were able to arrive at the "goal" of mate location and mating activity under a number of different pheromone concentrations in spite of previous exposure to pheromone and alteration of the "natural" characteristics of virgin females.

Irrespective of the theoretical framework used in analyzing behavioral data, the hypothetical mode of action of disparlure proposed by Richerson et al. (1976b) continues to be supported by data presented here. Disparlure in the microencapsulated formulation appears to block the initiation of the short-range sexual behavior of the males. Field tests of this formulation of disparlure have shown that this disruption breaks down after a few weeks (Beroza et al., 1973, 1974a,b, 1975a,b; Stevens and Beroza, 1972). It is not known why disruption breaks down. It is possible that the formulation itself deteriorates or that breakdown of disruption coincides with the peak emergence of the adult population. Theoretically, disparlure disrupts mating best only in sparse populations (Beroza and Knipling, 1972). Since pheromone-stimulated males are able to utilize a number of stimuli from females to locate and mate with them, it may be a matter of relative proportion of stimuli that is critical for short-range disruption to occur. In disparlure-treated plots in sparse populations, the synthetic and natural pheromone concentrations would be relatively high for the actual density of moths. Pheromone-activated males would not perceive other stimuli in sufficient quantity or quality to release short-range behavior. As the peak emergence of moths occurs there

would be sufficient stimuli from the available females to release short-range behavior. The mode of action of the olefin precursor and the optical isomers of disparlure (Cardé et al., 1975; Iwaki et al., 1975) may be quite different from that of the disparlure formulations presently being tested. Whatever the mode of action of these compounds, it may be necessary to modify the current view of behavior as comprising a series of performance units of allegedly equal status, and each successive unit having a higher concentration threshold level than the preceding unit (Bartell and Shorey, 1969).

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Syn- AND *Anti*-PHENYLACETALDEHYDE OXIME TWO NOVEL TESTOSTERONE-DEPENDENT MAMMALIAN METABOLITES

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Abstract—Domestic guinea pigs having high levels of circulating testosterone (i.e., males and castrate males receiving testosterone propionate injections) excrete in their urine equal amounts of both the *syn* and *anti* isomers of phenylacetaldehyde oxime, while those guinea pigs having low testosterone titers (i.e., females, castrate males, and juveniles) excrete neither. These aldoximes were also detected in the urine of wild male guinea pigs. Radiolabeling studies in the domestic guinea pigs strongly suggest that these aldoximes are derived from phenylalanine. To our knowledge this is the first observation and isolation of phenylacetaldehyde oxime from a mammalian source. The significance of these components in phenylalanine metabolism as well as in guinea pig chemical communication is discussed.

Key Words—chemical communication, phenylalanine metabolism, guinea pig urinary metabolites, phenylacetaldehyde oxime.

INTRODUCTION

Chemical signals are now known to be an important means of communication for a wide variety of animals including mammals and primates (for review see: Birch, 1974; Doty, 1976; Preti et al., 1976). In higher animals urine is often utilized as a medium for such chemical communication. For example, Beauchamp demonstrated in 1973 that given a choice, male domestic guinea pigs consistently prefer urine collected from female guinea pigs, regardless of their estrus stage, over: (1) urine from male conspecifics, (2) urine of other

mammals, or (3) water. This investigator also observed that urine of males castrated several weeks prior to collection, or urine from immature male and female donors is preferred to the same extent as is urine from adult females.

Additional work from these laboratories demonstrated that recognition of and preference for female urine by male conspecifics is communicated by an array of components having remarkably diverse chemical properties and molecular weights (Berüter et al., 1973; Smith et al., 1975). It was while investigating which factors differentiate male from female urine that two novel testosterone-dependent metabolities, namely the the *syn-* and *anti-*phenylacetaldehyde oximes were identified. Although these aldoximes are well-established metabolic intermediates, known to arise from phenylalanine, and important in plant biosynthesis of glucosinolates (mustard-oil glucosides), to our knowledge this is the first observation and isolation of these aldoximes from a mammalian source (Conn, 1973; Underhill et al., 1973).

METHODS AND RESULTS

Analytical Methods

Gas chromatographic (GC) analyses were performed on a Perkin-Elmer Model 990 chromatograph equipped with a flame ionization detector and a 3.5 m × 4 mm (OD) glass column packed with either 1% OV-101 or 2% Carbowax 20 M on Chromosorb GHP 80/100 mesh (Applied Science Laboratories, Inc.) operated at a helium (He) flow rate of 25 ml/min and at 100° for 4 min followed by temperature programming to 230° at 3°/min. Gas chromatographic fractions were collected in glass capillary tubes (30 cm × 2 mm OD) utilizing a thermal gradient collector (Brownlee and Silverstein, 1968).

Infrared (IR) spectra were recorded on a Perkin-Elmer Model 237 spectrophotometer fitted with focusing beam condensers and 4-μl microcells containing the isolated components dissolved in spectroquality carbon tetrachloride. Low resolution mass spectra (MS) were obtained on a Hitachi/Perkin-Elmer RMU-61 mass spectrometer interfaced with a Perkin-Elmer 990 gas chromatograph employing the OV-101 glass column. All mass spectra were recorded at an ionization potential of 70 eV. The 220-MHz NMR spectra were recorded in spectroquality CCl_4 on a Varian HR 220 NMR spectrometer employing the Fourier transform mode of operation.

Radioactive decay was determined in 10-ml aliquots of Bray's solution, employing a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 2425). Disintegrations per minute (dpm) were calculated from counts per min (cpm) using the known efficiency of the instrument.

Isolation and Identification of Syn- *and* Anti-*Phenylacetaldehyde Oxime*

Four gonadally intact adult male and four intact adult female domestic guinea pigs were housed individually in metabolism cages while their urine was collected in flasks cooled to 0°. Every 3 h the urine was pooled according to sex, frozen, and stored under nitrogen at −76° until required. For analysis the urine was thawed and centrifuged at 12,100*g* for 10 min to remove particulate matter. The resultant clear solutions were then treated with solid $(NH_4)_2SO_4$ (20% w/v) and extracted three times with one-third the volume of CH_2Cl_2. After drying over Na_2SO_4, the solvent was removed by rotary evaporation at reduced pressure to yield the concentrated extract.

This extract was chromatographed on preparative thin-layer silica gel plates (Anisil GF, 250 μm; Analabs, Inc.). Elution over a distance of 15 cm with benzene ethanol (9 : 1 v/v) followed by ultraviolet (λ = 180–300 nm) detection, revealed a complex array of components. The thin-layer plate was arbitrarily divided into four unequal bands or regions, 5, 7, 9, and 15 cm above the origin. Removal of the silica gel and elution with methanol gave four TLC fractions designated as 1–4, respectively. Previous observations in our laboratories indicated that domestic male guinea pigs could discriminate between male and female fractions 2 and 3. That is, male guinea pigs in a two choice preference test show a consistent preference for female TLC fractions 2 and 3 when compared to similarly prepared male fractions (Smith et al., 1975).

After removal of the methanol via distillation through a 10-cm Vigneaux column, the components arising from each of the four male and female TLC fractions were examined by gas chromatography employing a 1% OV-101 and a 12% Carbowax 20M column. The only obvious consistent difference between the various male and female fractions was the presence of two additional volatile components in TLC fraction 2 arising from male urine (Figure 1).

Analysis by combined gas chromatography–mass spectrometry (GC-MS) revealed that both components had mass spectra which were identical in all respects to that obtained from authentic benzyl cyanide. *However, neither component displayed GC retention properties consistent with those of benzyl cyanide.*

The unknown components were next isolated by micropreparative GC and then derivatized with 2 μl of trimethylchlorosilane (TMCS), 10 μl *N,O*-Tris (trimethylsilyl)trifluoroacetamide (BSTFA), and 12 μl of pyridine (Gehrke and Leimer, 1971; Stalling, et al., 1968). After 20 min examination of the reaction mixture via GC-MS revealed the presence of two monosilyl ethers, both having parent ions at *m/e* 207. The two components were therefore isomeric, and their actual molecular weights before derivatization could now

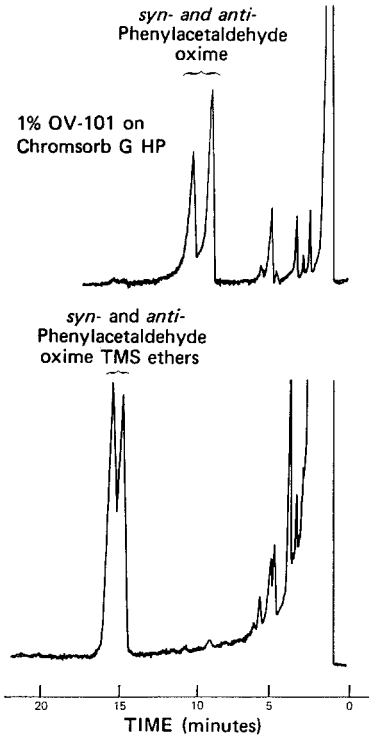
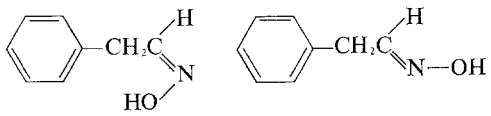


FIG. 1. Gas chromatogram of TLC fraction 2.

be assigned as 135 rather than the 117 (i.e., benzyl cyanide) which had been derived from the mass spectra of the underivatized samples. The difference of 18 atomic mass units between the actual molecular weight and the ion observed at *m/e* 117 is explained by the loss of H_2O. With this information at hand, the two components were tentatively assigned as 1a and 1b, the *syn* and *anti* isomers of phenylacetaldehyde oxime.

Structures 1a and 1b were confirmed by comparison of the IR, 220-MHz NMR, and mass spectra as well as the GC retention properties with those derived from authentic *syn*- and *anti*-phenylacetaldehyde oxime (Doffus,



(1a) (1b)

1892). In addition, the mass spectra and GC retention times of the trimethylsilyl ether derivatives of 1a and 1b were identical in all respects to the corresponding synthetic derivatives.

In retrospect these oximes, upon electron impact in the ion source of the mass spectrometer, would be expected to lose H_2O to yield the radical cation of benzyl cyanide as the first fragment. The unusually high efficiency of this process in this case prevents observation of a parent ion at *m/e* 135 (Goldsmith et al., 1966; Mika, 1973).

During the course of our isolation studies, it became clear that the underivatized isomers of phenylacetaldehyde oxime undergo isomerization during silica gel chromatography. Thus, in order to determine the amount and relative concentration of the oximes present in urine from intact male guinea pigs, the oximes were extracted with CH_2Cl_2 without the aid of $(NH_4)_2SO_4$ or silica-gel chromatography. Direct examination of this extract via calibrated GC on an OV-101 column indicated that the oxime isomers were present in approximately equal amounts, namely 0.05–0.53 μg/ml, depending on the total volnme of urine produced by the animal per unit time.

Via a similar protocol the *syn* and *anti* isomers of phenylacetaldehyde oxime were found to be present in the urine of wild male guinea pigs. The concentration range for the oximes was approximately the same as in urine of domestic males.

Radiolabeling Studies

Two intact adult domestic male and two intact adult domestic female guinea pigs were placed individually in metabolism cages three days prior to intraperitoneal injection with 6 μCi of randomly labeled [^{14}C]phenylalanine (New England Nuclear). After injection, urine was collected from individual animals each day for five consecutive days and analyzed for radioactivity by liquid scintillation counting. The radioactivity content of the excreted urine was highest during the first 24 h and then decreased significantly over the next several days. At the end of the 5-day period the urine was pooled according to individual and the oxime isomers isolated as before. Calibrated GC analysis and micropreparative GC collection revealed that TLC fraction 2 derived from the two male guinea pigs contained a total of 99.4 μg (♂376) and 105 μg (♂398) of the two oxime isomers. In the case of the two females (♀272 and ♀541) in which the aldoximes were not observed, a small region or window around the GC retention time of the aldoximes was collected. The observed radioactivity in dpm for each animal over the course of the isolation protocol (i.e., urine, CH_2Cl_2 extract, TLC fraction 2, and GC fraction containing the oximes) is given in Table 1.

Previous experience in our laboratories indicates that male and female

TABLE 1. RADIOACTIVITY OBSERVED DURING COURSE OF PHENYLACETALDEHYDE OXIME ISOLATION PROTOCOL

Animal	Intact urine (dpm)	CH_2Cl_2 extract (dpm)	TLC fraction 2 (dpm)	*Syn* and *anti* oximes (dpm)
398 ♂	754,000	8670	5010	803
376 ♂	905,000	9230	5410	867
273 ♀	711,000	1956	396	73
541 ♀	842,000	2360	342	44

domestic guinea pigs (weight 700–900 g) consume approximately 40 g of Purina chow/day. According to the manufacturers, this chow contains approximately 1.2% phenylalanine. Thus, over a 5-day period each animal consumed on the order of 2.4 g of phenylalanine. The molar conversion factors for phenylalanine to the aldoxime isomers were calculated to be 5.07×10^{-5} and 5.35×10^{-5}, respectively, for guinea pigs ♂376 and ♂398. If the same conversion factor applies for the [^{14}C]phenylalanine injected intraperitoneally and allowance is made both for the efficiency of the GC thermal gradient collector (i.e., 80%) and for loss of one carbon atom (i.e., phenylalanine to phenylacetaldehyde oxime), one would then expect 753 and 791 (dpm) for the oxime fractions derived from guinea pigs ♂376 and ♂398, respectively. The close agreement of the specific activity (e.g., 802 and 867 dpm) observed strongly suggests that phenylalanine is the predominant biosynthetic precursor of *syn*- and *anti*-phenylacetaldehyde oximes.

Testosterone Dependence of the Phenylalanine–Phenylacetaldehyde Oxime Metabolic Pathway

In a separate experiment four intact mature domestic male guinea pigs were placed individually in metabolism cages and after several days of adjustment to this environment, two of the animals were castrated and two received sham-castration operations. After the operation the urine of each animal was collected separately for a period of 24 h on days 2, 5, 7, 9, 12 and 14. Each urine sample was examined for the presence or absence of the oxime isomers via the previously described protocol. That is, the various underivatized and derivatized samples arising from each day's TLC fraction 2 were examined for the aldoximes by GC on an OV-101 column.

Twenty-nine days after the castration operations, the two castrated animals were given daily intramuscular injections of testosterone propionate

TABLE 2. BEHAVIORAL RESPONSES TO URINE AND PRESENCE OR ABSENCE OF OXIMES FOLLOWING CASTRATION AND AFTER SUBSEQUENT TREATMENT WITH TESTOSTERONE PROPIONATE

	Days postcastration														
	−7	0[a]	2	5	7	9	12	14	29[a]	31	34	36	39	41	43
Castrate urine and intact urine equally attractive[b]	+		+	−	0	0	−	0		0	+	0	+	+	+
Oximes present in castrate urine[c]			++	++	+	0	0	0		0	0	+	++	++	++

[a] On day 0, the experimental animals were castrated and controls (intact) given sham operations. Beginning on day 29, castrate animals were given daily intramuscular injections (1000 μg/animal in 0.2 ml) of testosterone propionate in sesame oil while intact controls were given oil only.

[b] A "+" means that urine from castrate and intact males is equally attractive to the test males. A "0" means that they are no longer equally attractive; instead, castrate urine is preferred to intact urine ($P<0.05$, Wilcoxon test). A "−" means the urine was not tested on this day.

[c] ++ means that the oximes are present in approximately normal amounts, + means they were detectable but in reduced amounts. 0 means they were undetectable.

(1000 μg/day), while the two sham-operated guinea pigs were injected with sesame oil as a control. Again, the urine of all four animals was collected over a period of 24 h at 2, 5, 7, 10, 12, and 14 days after the start of the testosterone propionate therapy and was assayed for oxime content.

These experiments revealed first that the amount of oxime isomers (0.17–0.53 μg/ml) present in the urine of the two castrated males and the two sham-operated males remained unchanged during the first 5 days post-surgery (Table 2). On day 7, the oxime content of urine from both castrate males was somewhat reduced with neither oxime being present on days 9 and 12 (e.g., <0.02 μg/ml). Second, after initiation of testosterone therapy, urine from the two castrate males showed none of the oxime isomers for the first 5 days. However, detectable amounts (0.05 μg/ml) did appear on day 7, with normal amounts observed 10 and 12 days after therapy initiation. Third, there appeared to be no detectable change throughout the experiment in the amount or ratio of the aldoxime isomers produced by the sham-operated animals.

During the course of the above experiment, a second group of 12 intact domestic adult males was given a series of behavioral preference tests in an

attempt to distinguish the time at which urine from castrate males loses its male identity and becomes like that of the female (i.e., more attractive than urine from intact males). Here urine isolated from castrate males and urine from sham-operated males was compared using a brief (4-min) 2-choice preference procedure where the time spent investigating each of the two samples was recorded. The testing methods are described in detail elsewhere (Beauchamp, 1973). These tests indicated that urine from castrated domestic males becomes attractive relative to urine from intact males on the seventh day after castration. Similarly, urine of castrates receiving testosterone therapy loses attractiveness and becomes "male-like" approximately 5–11 days after initiation of testosterone therapy.

Thus, production of the oxime isomers ceases in male animals around the seventh day after castration and begins again 7 days after initiation of testosterone therapy. Likewise, male guinea pig urine becomes more attractive than control male urine 7 days after male castration, while urine from castrates receiving testosterone becomes less attractive or more male-like at approximately the same time after initiation of therapy (see Table 2).

Behavioral Response to Syn- *and* Anti-*Phenylacetaldehyde Oxime*

To examine the possibility that the oxime isomers are responsible, at least in part, for the differential attractiveness of domestic male and female urine, the aldoximes were added to half of a pooled sample of female urine and to half of a pooled sample of castrate male urine. In these experiments the total concentration of the oxime varied from that observed in normal male urine to ten times that amount (0.17–1.7 μg/ml). When these samples were assayed behaviorally against the unaltered urine using the same 12 test males as employed above, there was no apparent change in attractiveness of the female urine or castrate male urine upon addition of the oximes. Thus, while the appearance and disappearance of the aldoxime isomers occur simultaneously with the as yet unidentified chemical communicants that influence urine attractiveness, the oximes themselves do not appear to influence this attractiveness.

DISCUSSION

Previous studies have demonstrated that urinary signals play an important role in regulating the social behavior of guinea pigs (Beauchamp, 1973; Beauchamp and Berüter, 1973; Beauchamp et al., 1976) as well as a wide variety of other mammalian species (for reviews, see Birch, 1974; Doty, 1976; Preti et al., 1976). One of the most significant messages transmitted among conspecifics is the sex of an individual. The *syn* and *anti*

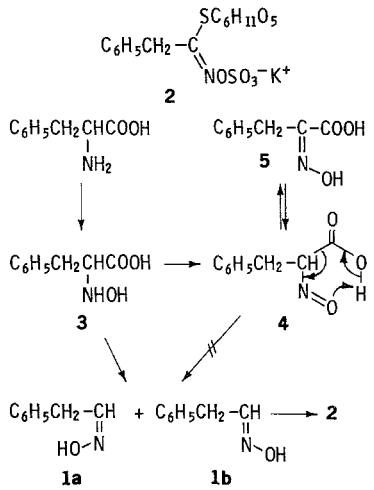


FIG. 2.

isomers of phenylacetaldehyde oxime present in urine of intact male guinea pigs could serve to distinguish males from females. However, our behavioral experiments have failed to produce evidence supporting this possibility even though we have noted a most remarkable parallelism between disappearance and reappearance of these aldoximes and alternations in attractiveness of urine following castration and replacement therapy with testosterone (Table 2).

A careful examination of the literature revealed that although *syn*- and *anti*-phenylacelaldehyde oximes are unknown as mammalian metabolic products, these aldoxime isomers are well-established intermediates in plant biosynthesis of glucotropaeolin (Figure 2, **2**), a mustard-oil glucoside. For example, the elegant studies of both Underhill and Kindl, and Tapper and Butler demonstrated that glucotropaeolin arises from the amino acid phenylalanine through the intermediacy of phenylacetaldehyde oxime (**1a** and **1b**) (Underhill, 1967; Tapper and Butler, 1967). The biosynthetic pathway, as proposed by these investigators, is illustrated in Figure 2. Refinement of this pathway allows for the initial formation of *N*-hydroxyphenylalanine (**3**). Evidence that this hydroxyamino acid was intermediate was provided by Underhill (Kindl and Underhill, 1968).

Subsequent conversion of this hydroxyamine to phenylacetaldehyde could, as suggested by Underhill, occur via decarboxylation of 2-nitroso-3-phenylpropanoic acid (**4**), the tautomeric isomer of 2-oximino-3-phenylpropanoic acid (**5**). However, [2-^{14}C]2-oximino-3-phenylpropanoic acid

failed to serve as a precursor of phenylacetaldehyde oxime. Further studies revealed that the conversion, *N*-hydroxyphenylalanine to phenylacetaldehyde oxime, requires one equivalent of molecular oxygen per equivalent of aldoxime produced. To explain these observations, Underhill suggested that the dehydrogenation–decarboxylation sequence may involve an enzyme bound substrate, as in the oxidative decarboxylation of amino acids to lower acid amides (Kindl and Underhill, 1968).

Although direct comparison of plant metabolism to that of higher mammalian systems is difficult at best, precedent has been set for the conversion of phenylalanine to phenylacetaldehyde oxime. The significance and generality of this novel pathway to mammalian physiology, as well as a clarification of the reason why only guinea pigs with high circulating levels of testosterone metabolize phenylalanine to these aldoximes, will require further study. Such studies are currently underway in our laboratory.

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QUINONES AND PHENOLS IN THE DEFENSIVE SECRETIONS OF NEOTROPICAL OPILIONIDS[1]

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Abstract—Three quinones (2,3-dimethyl-1,4-benzoquinone; 2,5-dimethyl 1,4-benzoquinone; and 2,3,5-trimethyl-1,4-benzoquinone) and two phenols (2,3-dimethylphenol and 2-methyl-5-ethylphenol) were isolated from the defensive secretions of four species of opilionids (suborder Laniatores) from the Canal Zone. Phenols had not previously been reported from opilionids, and 2-methyl-5-ethylphenol was not known as a natural product. The secretions, which are mixed with regurgitated enteric fluid upon discharge, are administered by the animals by leg dabbing.

Key Words—Quinones, phenols, defensive secretions, Arachnida, Opiliones

INTRODUCTION

All three suborders of opilionids (Arachnida: Opiliones), the Cyphophthalmi, the Palpatores, and the Laniatores, produce defensive secretions. Chemical studies have so far been restricted to a few species of Palpatores, which produce short-chain acyclic compounds, including several ketones and an alcohol (Blum and Edgar, 1971; Jones et al., 1976; Meinwald et al., 1971), and to two species of Laniatores, which produce methylated quinones (Eisner et al., 1971; Estable et al., 1955; Fieser and Ardao, 1956). We here

[1] Paper no. 53 of the series *Defense Mechanisms of Arthropods.*

report on the chemistry of the secretion and on the defensive behavior of four species of Laniatores from Central America.

METHODS AND MATERIALS

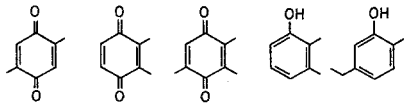
The animals were taken at night, on the ground or above ground on the walls of buildings, on Barro Colorado Island, Panama Canal Zone. They were shipped to our Cornell laboratories, where they were maintained on pieces of freshly killed cockroaches and *Tenebrio* larvae. They survived with little mortality for several weeks, during which they were "milked" of secretion by the technique previously used with other opilionids (Eisner et al., 1971). Three of the species were identified as belonging to the family Cosmetidae (*Cynorta astora, Paecilaemella eutypta, P. quadripunctata*), and one to the family Gonyleptidae (*Zygopachylus albimarginis*). Given the rather poor state of the taxonomy of neotropical Laniatores, the identifications should be considered tentative. We are depositing specimens under our label (R.E.S.) and experiment numbers (T.E. 853, 855–857) in the collection of the National Museum of Natural History, Smithsonian Institution, Washington, D.C. Upward of a dozen live specimens were available of the first two species, and single specimens of the last two.

Gas chromatographic analyses were made with a Varian 2100 gas chromatograph equipped with a flame ionization detector, using either column A (2.4 m × 2 mm, 5% OV-1 on Gaschrom Q) or column B (1.5 m × 2 mm, 3% FFAP on Chromosorb W). Mass spectra were obtained using column A in a Finnigan 3300 gas chromatograph–mass spectrometer coupled with a Systems Industries 150 computer. Infrared spectra were taken in carbon disulfide on a Perkin-Elmer 237 recording spectrophotometer. Nuclear magnetic resonance spectra were recorded at 60 MHz on a Varian A-60A instrument and at 100 MHz on a Varian XL-100 Spectrometer.

The scanning electronmicrographs were made with a JEOL-23 instrument at the Marine Biological Laboratories, Woods Hole, Massachusetts. The specimens were critical-point dried in preparation for electronmicroscopy.

Chemistry of the Secretion

Paecilaemella eutypta. Gas chromatographic analysis on column A of a sample of secretion from several *P. eutypta* showed three components in a 1:8:2 ratio. The first component had a mass spectrum identical to that reported for 2,5-dimethyl-1,4-benzoquinone (Figure 1, Structure I) (Stenhagen et al., 1974). The second had a mass spectrum which shared characteristics of the published spectra, as well as of those obtained from authentic samples,



	I	II	III	IV	V
Cosmetidae					
Paecilaemella eutypa	1	8	2		
P. quadripunctata		4 2	1 1		
Cynorta astora				1	16
Gonyleptidae					
Zygopachylus albimarginis		3			1 (?)

FIG. 1. Quinones (I–III) and phenols (IV, V) in defensive secretions of species shown. The numbers give ratios of components as determined by chromatographic peak-area comparisons.

of both ethyl-1,4-benzoquinone and 2,3-dimethyl-1,4-benzoquinone (II) (Stenhagen et al; 1974). Coinjection and retention-time analysis on column B showed that this component was quinone II. The third component had a mass spectrum identical to that published (Stenhagen et al., 1974) and observed from an authentic sample of 2,3,5-trimethyl-1,4-benzoquinone (III).

Paecilaemella quadripunctata. Gas chromatographic and mass spectral analysis (as described above) of two milkings of the defensive secretion from the single available individual of this species showed the presence of two main components, identified as quinones II and III, in ratios of 2 : 1 and 4 : 1.

Cynorta astora. Gas chromatographic analysis on column A of a sample of secretion of several *Cynorta astora* showed two components in a 1 : 16 ratio. The first had a mass spectrum identical to that reported for 2,3-dimethylphenol (IV) (Stenhagen et al., 1974). The second had a mass spectrum suggestive of a methyl ethyl phenol [*m/e* 137(5), 136(56), 135(6), 122(11), 121(100), 107(19), 103(14), 91(34), 77(38), 65(13), 53(11), 51(16)]. The 100-MHz NMR spectrum of the total secretion (CD_2C1_2) showed absorptions at δ 7.09, 7.01 (1H), 6.74, 6.56 (2H) (2,5-dialkyl aromatic substitution), 2.59 (2H, *q*, $J = 7.5$ Hz, $CH_2\phi$), 2.21 (3H, *s*, $CH_3\phi$), and 1.21 (3H, *t*, $J = 7.5$ Hz, $CH_3CH_2\phi$). The infrared spectrum of the total secretion was identical to that published for 2-methyl-5-ethylphenol (V) (Shrewsbury, 1960). An authentic sample of phenol V was prepared (Morgan and Pettet, 1934) and showed infrared, NMR, and mass spectra identical to those of the natural product.

Zygopachylus albimarginis. Gas chromatographic analysis on column A of a single milking of secretion from the one specimen of *Zygopachylus*

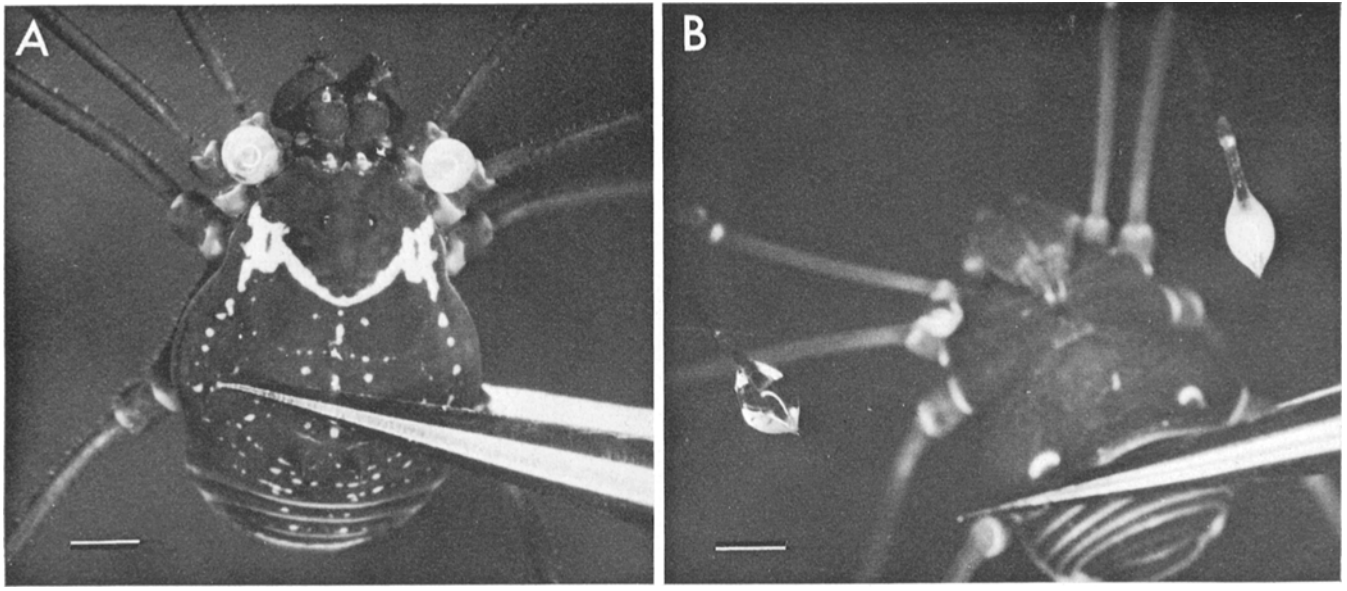
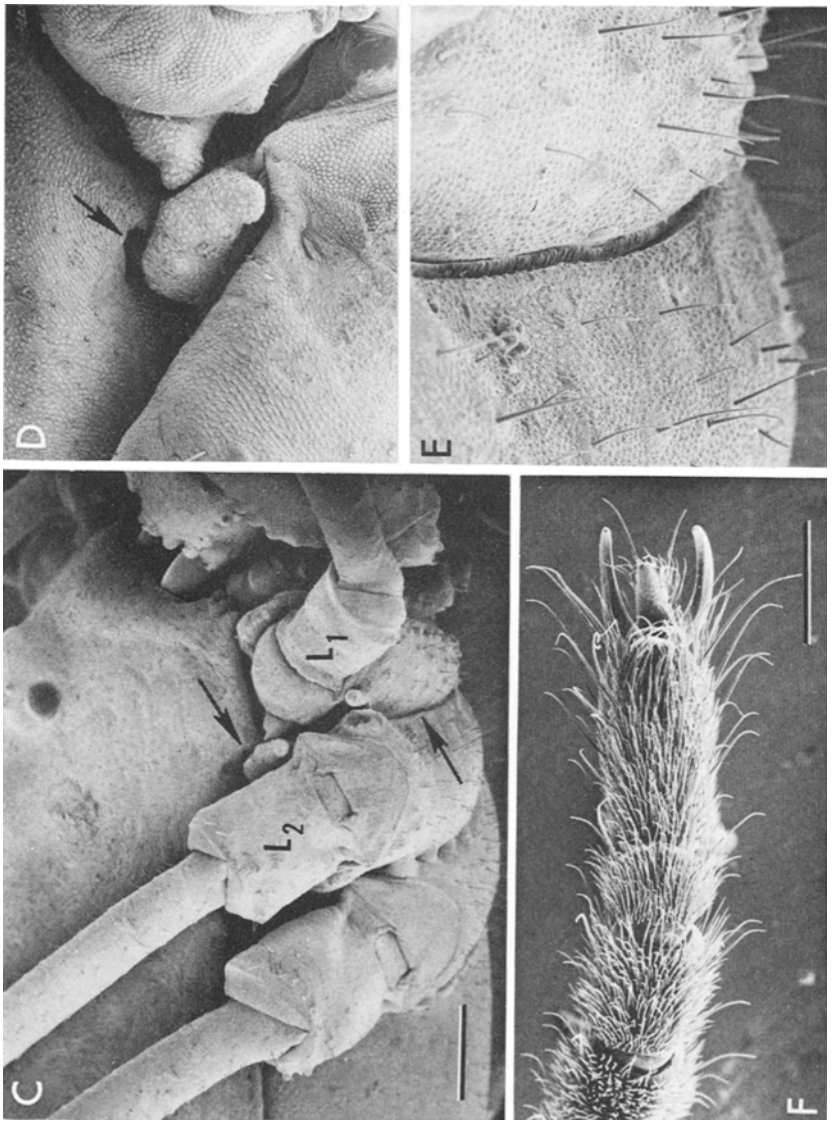


FIG. 2. (*A*) *Cynorta astora* (dorsal view), held in forceps, with droplets of defensive fluid at anterolateral margins of carapace. Phenolic secretion has already been discharged into the droplets, accounting for their white coloration. (B) *Paecilaemella eutypa* (ventral view), with tips of front legs laden with fluid, at moment of initiation of leg dabbing. Only the droplet on the right bears secretion-containing fluid. The droplet on the left is clear and consists of enteric fluid alone, having been picked up by the leg before secretion was discharged into the droplet. (C) *C. astora*: right lateral view of anterior portion of body. Upper arrow points to gland opening, lower arrow to groove along which oral fluid is conveyed (L_1, leg 1; L_2, leg 2). (D) Same, enlarged view of gland opening (arrow) and surrounding area. (E) Same, enlarged view of groove. (F) Hairy tip of foreleg of *P. eutypa*. Reference bars A, B = 1 mm; C = 0.5 mm; F = 0.25 mm.


D
E
C
L_1
L_2
F

albimarginis available showed two components in a 3 : 1 ratio. The first had a mass spectrum identical to that of 2,3-dimethyl-1,4-benzoquinone (II). Because of the small amount of material available, the mass spectrum of the second component was weak, but suggestive of a methyl ethyl phenol [*m/e* 136(66), 121(100), 91(50)].

Defensive Behavior

Previous observations on a species of Laniatores, the cosmetid *Vonones sayi*, had shown that this animal administers its defensive fluid by leg dabbing. When disturbed, *Vonones* discharges quinonoid secretion from its two glands, dilutes it with aqueous enteric fluid emitted from the mouth, and effects dosaged delivery of the mixture by brushing it against the enemy with its forelegs (Eisner et al., 1971).

The same behavior was found to occur in *P. eutypa*, *P. quadripunctata*, and *C. astora*. The first noticeable response to disturbance of these animals, which was effected in the laboratory by pinching them or seizing them with forceps, was the appearance at the anterolateral margin of the carapace, directly opposite the gland openings, of two droplets of clear enteric fluid. Next, the droplets were seen to discolor, as secretion was injected into them. In the case of the two quinone-producing *Paecilaemella*, the droplets took on a golden brown coloration, while in the phenol-producing *C. astora* they became white (Figure 2A). The animals then dipped their front legs into the droplets and, by means of deliberate and well-aimed strokes, proceeded to wipe the wetted leg tips against the forceps.

The discharges from the two glands did not always occur simultaneously. In fact, discharges were commonly asynchronous, and occasionally even exclusively unilateral. It sometimes happened, therefore, that during leg dabbing only one of the legs bore secretion-laden fluid (Figure 2B). Animals depleted of secretion by repeated milkings still responded by regurgitating (provided they had fed and had replete guts), and sometimes even with leg dabbing. Although one might think that such brushings would be defensively useless, it is conceivable that the enteric fluid is in itself to some extent a deterrent and therefore useful as a weapon of last resort.

The scanning electron micrographs revealed some of the structural features of the cosmetid defensive apparatus. The mouth of cosmetids is midventral, and the regurgitated fluid seeps to the sides of the carapace along two linear clefts formed between the bases of the first and second legs (Eisner et al., 1971). The appearance of such a cleft, and its relationship to the site near the gland opening where enteric fluid and secretion mix, is shown in Figures 2C–E. The tips of the front legs are densely hirsute and admirably adapted to serve as brushes (Figure 2F).

No behavioral observations were made on the single specimen of *Zygopachylus albimarginis* available. It was therefore not determined whether leg dabbing occurs also in the Gonyleptidae.

DISCUSSION

One of the first defensive secretions of arthropods to be studied chemically was that of the gonyleptid *Heteropachyloidellus robustus*. The investigation was carried out collaboratively by Clemente Estable and his Uruguayan associates, who first studied the animal in its native land, and by Louis Fieser of Harvard, in whose laboratory the active principles of the secretion were identified. Gonyleptidine, as the secretion was named, was found to contain quinones I, II, and III (Estable et al., 1955; Fieser and Ardao, 1956). Production of quinones is probably widespread in the Laniatores. One of us (T.E.), who grew up in South America and has had an opportunity to observe a diversity of Laniatores in Uruguay and the Canal Zone, has noted that these animals quite commonly discharge a brownish fluid of quinonoid odor. Production of phenols, either exclusively, as in *C. astora*, or in conjunction with quinones, as in *Z. albimarginis*, might therefore be of exceptional occurrence. Because of their white coloration and characteristic odor, defensive fluids containing only phenols should be preliminarily identifiable in the field as distinct from quinonoid fluids.

Although quinones themselves are extraordinarily widespread in the defensive secretions of arthropods (Altman and Dittmer, 1973), the methylated quinones found in opilionids are of more restricted occurrence. Compounds I and III are known from no other natural source, while II is known only from a tenebrionid beetle and some carabid beetles (Eisner et al., 1974; Eisner and Meinwald, unpublished observations on *Brachinus* and *Ozaenini*). Phenols also occur in the secretions of arthropods (Altman and Dittmer, 1973), but IV and V do not. In fact, V was not known as a natural product, and IV was reported only from a single plant (Irvine and Saxby, 1968).

Previous work with the quinone-producing *Vonones sayi* had demonstrated the protective effectiveness of leg dabbing vis-à-vis real predators such as ants (Eisner et al., 1971). To what extent the phenols of Laniatores match the quinones in effectiveness remains unknown, although there is evidence from other studies that phenols as such are also powerfully deterrent (Eisner et al., 1963).

Leg dabbing is not restricted to the Laniatores, but occurs also, albeit in a behaviorally somewhat different form, in the Cyphophthalmi (Juberthie, 1961a). Other opilionids have a diversity of ways of administering secretion.

The fluid may be sprayed or it may merely spread from the gland openings along special integumental grooves or over the surface of the body (Bishop, 1950; Blum and Edgar, 1971; Juberthie, 1961b; Lawrence, 1938). A deliberate mixing of secretion with enteric fluid appears to be restricted to the Laniatores. Much remains to be learned about opilionids, an altogether too often neglected group.

Acknowledgments—We thank Dr. W.A. Shear, who identified the opilionids and pointed out the tentative nature of the identifications, and Ms. A. Aiello, who assisted in the collection of specimens. The study was supported in part by the National Institutes of Health (grants AI-12020 and AI-02908, and Fellowship 1-F32-CA05139 to T.H.J.) and the National Science Foundation (grant BMS75-15084).

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A SEX PHEROMONE FOR MALE BOLL WEEVILS[1] FROM FEMALES

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Abstract—Steam distillation of frass of the female boll weevil, *Anthonomus grandis* Boheman, yielded an extract that was more attractive to males than to females in the laboratory. Extracts purified by TLC were attractive to males only. The active components appear to be alcohols and hydrocarbons.

Key Words—boll weevil, *Anthonomus grandis* (Boheman), sex pheromone.

INTRODUCTION

Keller et al. (1964) reported that trapped volatiles from the male boll weevil, *Anthonomus grandis* Boheman, attracted females in the laboratory. Tumlinson et al. (1969) later identified the pheromone as a mixture of two terpene aldehydes and two terpene alcohols [I = (+)-*cis*-2-isopropenyl-1-methylcyclobutaneethanol; II = *cis*-3,3-dimethyl-$\Delta^{1,\beta}$-cyclohexaneethanol; III = *cis*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde; IV = *trans*-3,3-dimethyl-$\Delta^{1,\alpha}$-cyclohexaneacetaldehyde]. Field activity of the synthesized mixture, which was named grandlure, was confirmed, and both sexes responded to it in the field (McKibben et al., 1971; Hardee et al., 1972).

[1] Coleoptera: Curculionidae.
[2] In cooperation with the Mississippi Agricultural and Forestry Experiment Station, Mississippi State, Mississippi 39762.
[3] Mention of proprietary products does not constitute an endorsement by the USDA.

In laboratory bioassays, male boll weevils were not attracted to females significantly more than to other males (Hardee et al., 1967). Likewise Tumlinson et al. (1969) reported that neither males nor females were responsive to extracts or steam distillates of females.

Cross and Mitchell (1966) reported that males were not observed to respond to females over distances greater than about 5 cm, and they speculated that the female emitted a weak secondary pheromone that attracted the male over very short distances. Cross (personal communication) also observed male weevils in an excited state as though near a female weevil when in fact a female was on the opposite side of a cotton leaf. Activity of the male was assumed to be due to olfactory perception. We therefore undertook the present study to learn whether female weevils produce a pheromone that is attractive to males.

METHODS AND MATERIALS

Laboratory-reared boll weevils (Gast and Davich, 1966) were separated by sex within 2–3 days after emergence. Females were then maintained in $30 \times 30 \times 30$-cm. stainless-steel screen cages and fed fresh cotton squares supplemented with artificial diet plugs. Frass accumulating on wrapping paper under the cages was collected twice daily and stored in stoppered flasks at 0°C.

The volatiles were removed by steam distillation of the frass for 1 hr. Then the distillate was extracted exhaustively with 20% ethyl ether in pentane. The extract was concentrated in a rotary evaporator at 50°. A volume that contained approx 1000 female–day equivalent (FD eq) of frass per milliliter was used for GLC and bioassay. A volume that contained approx 100,000 FD eq/ml was used for TLC. The GLC procedure was as follows: A Hewlett-Packard 5710 gas chromatograph with a flame ionization detector was used with a 2-mm × 305-cm stainless-steel column packed with 10% SP-2401® on 80/100 Gas Chrom Q®. The oven was operated isothermally at 135°. Nitrogen flow was 32 ml/min.

The TLC fractions were analyzed by applying 1 ml of the extract to 1 Silica Gel-G® TLC plate that was then developed with 20% ethyl ether in pentane. The plate was divided into 6 bands on the basis of its differing absorption of UV light. Each band was scraped from the plate and extracted with 20% ethyl ether in pentane. The laboratory bioassay procedure was that used by Hardee et al. (1967). Insects for the bioassay were separated by sex on the day of emergence and fed fresh cotton squares daily. Insects 4–6 days old were used for the bioassays.

RESULTS AND DISCUSSION

Generally, in the laboratory bioassay, the whole extract of female frass was attractive to male weevils and was slightly attractive to females (Table 1).

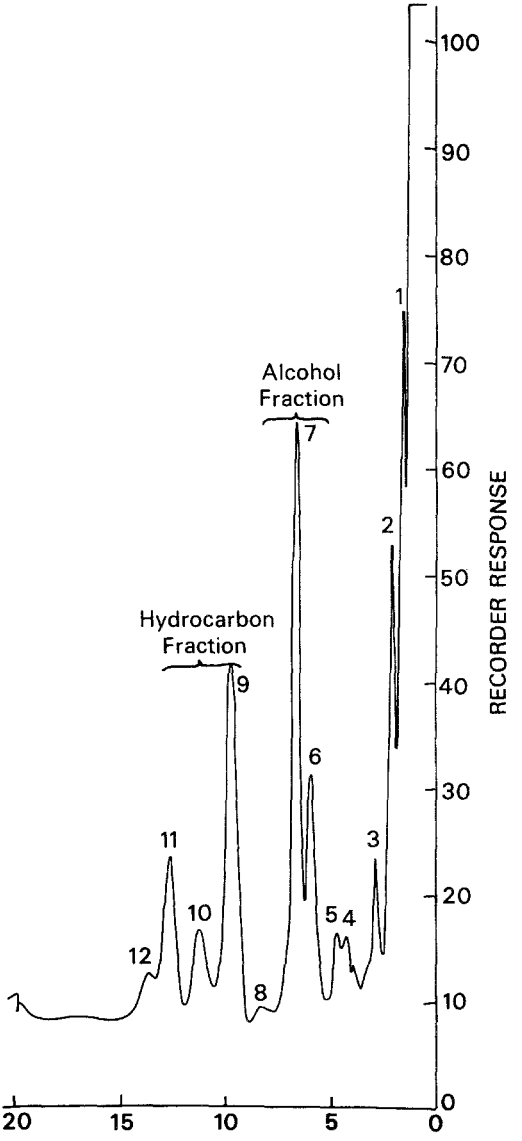

FIG. 1. Gas chromatogram of extract of female boll weevil frass.

TABLE 1. AVERAGE RESPONSE OF MALE AND FEMALE BOLL WEEVILS TO EXTRACTS OF FEMALE FRASS AND GRANDLURE

Treatment	Responding sex	Index of attraction[a]
Whole ♀ frass extract	♂	27±4
	♀	10±3
Active TLC[b] fractions of ♀ frass extract	♂	19±3
	♀	1±3
Grandlure	♂	1±3
	♀	46±5

[a] Index of attraction = (% responding to treatment − % responding to control) ± standard error.
[b] Alcohol and hydrocarbon fractions, 2 and 6, respectively.

On the basis of the response of both sexes to TLC fractions of the extract, fractions 2 and 6 appeared to contain the active components. These two fractions were the most attractive to males and the least attractive to females. A comparison with the response to grandlure indicates a true sex response to the purified extracts (Table 1). The TLC separations were designed so fraction 2 contained compounds such as alcohols and fraction 6 contained primarily hydrocarbons. Thus there is evidence that the active compounds may be alcohols and hydrocarbons.

Figure 1 shows a gas chromatogram of a typical extract of female frass. Peaks 6 and 7 represent TLC fraction 2 and peaks 9–12 represent fraction 6.

Male weevils may produce 1 μg or more of pheromone/day (McKibben et al., 1976). However, we estimated from the major GLC peaks of the extract of female frass that a female produces approx 0.01 μg of pheromone/day. Possibly this difference explains the previous suggestion that the female pheromone is weak as compared with the male pheromone. The relatively small quantity of female pheromone produced may also be responsible for the failure of the earlier workers to find such a pheromone.

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CHEMICAL BASIS FOR INTERSPECIFIC RESPONSES TO SEX PHEROMONES OF *Trogoderma* SPECIES (COLEOPTERA: DERMESTIDAE)

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Abstract—Interspecific responses among several *Trogoderma* species have been correlated with their pheromone components. The most important component emitted by four of the species is (Z)- or (E)-14-methyl-8-hexadecenal, which is not detectable in extracts of macerated beetles. The response to macerated beetles is probably due to the corresponding alcohol and ester. The recency of common origin of seven species is discussed.

Key Words—Coleoptera, Dermestidae, *Trogoderma granarium* (khapra beetle), *T. variabile*, *T. glabrum*, *T. inclusum*, pheromone, (Z)- and (E)-14-methyl-8-hexadecenal, interspecific response.

INTRODUCTION

Interspecific attraction among *Trogoderma* species was first studied in 1970 by Vick et al. and by Levinson and Bar Ilan, who measured the responses of male beetles to extracts of macerated female beetles. The results of the Vick

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TABLE 1. INTERSPECIFIC RESPONSES TO SEX PHEROMONES OF *Trogoderma* SPECIES[a]

Source of extract (♀)	Male responses (%)[b]				
	T. inclusum	*T. simplex*	*T. variabile*	*T. sternale*	*T. glabrum*
T. inclusum	100a	100a	95a	0a	18a
T. simplex	98a	90a	95a	0a	5a
T. variabile	85a	80a	88a	0a	0a
T. grassmani	98a	82a	95a	52ab	18a
T. granarium	70ab	76a	82a	0a	10a
T. sternale	80a	28b	5b	95b	12a
T. glabrum	42b	24b	2b	55ab	90b

[a] From Vick et al., 1970.

[b] Percents in any vertical column followed by the same letter are not significantly different at the 5% level as determined by Duncan's multiple-range test.

et al. studies are shown in Table 1. The present study was undertaken to establish a chemical basis for these responses.

Chemical studies of extracts of macerated female beetles of three species [*T. inclusum* Le Conte, *T. glabrum* (Herbst), and *T. variabile* Ballion] resulted in the identification of five compounds that showed biological activity, although the quantities required to elicit a 50% response varied widely (Rodin et al., 1969; Yarger et al., 1975; Cross et al., 1977). Cross et al. (1976) aerated females of four *Trogoderma* species (*T. inclusum*, *T. glabrum*, *T. variabile*, and *T. granarium* Everts, the khapra beetle) and collected the volatiles on Porapak Q (Byrne et al., 1975); from hexane extracts of the Porapak Q (Porapak extracts) they isolated the aldehyde, 14-methyl-8-hexadecenal, the most important component of the pheromone of each of the four species. This component was not detected in the extracts of beetles of any of the four species either because it is enzymatically destroyed during the maceration–extraction or because it is synthesized and released only "on demand." In Table 2, we report which of the six known pheromone components are actually emitted by each of the four *Trogoderma* species mentioned above. We have also included the occurrence of these components in the extracts of macerated beetles and beetle-contaminated filter paper.

The extensive cross attraction to extracts of crushed female beetles, shown in Table 1, correlates with the findings that these extracts have several active components in common (Table 2). Interspecific attraction in a natural setting, however, must be due to the pheromone components actually

emitted by the female. For *T. granarium* it has been determined that the airborne volatiles were 10^4 times more active than an extract of females and 10^3 times more active than an extract of the filter paper on which the females were held during aeration (Cross et al., 1976). Synthetic samples of the most active components from aeration collection and macerated beetles were quantitatively evaluated against *T. glabrum*, *T. inclusum*, *T. variabile*, *T. simplex*, *T. sternale*, and *T. grassmani*.

These data have led us to consider the degree of phylogenetic relatedness of the seven *Trogoderma* species that we have studied.

METHODS AND MATERIALS

Female *T. inclusum*, *T. glabrum*, and *T. variabile* beetles (1000 in number, 1–5 days old) were aerated at 2 liter/min for 3 days at 27–28°C in the apparatus described elsewhere (Cross et al., 1976). Female *T. granarium*[5] beetles (1000–2000 in number, 1–5 days old) were aerated at 3 liter/min for 28 days at 20–25°C. All the beetles were on a 16:8 light/dark photocycle. The volatiles were collected on Porapak Q,[6] which was subsequently extracted with redistilled hexane or pentane in a Soxhlet extractor for 24 hr. The solution was concentrated to 2 ml by solvent distillation at 1 atm through a column packed with glass beads. This solution contained the volatile components actually emitted by the female beetles.

The concentrated Porapak Q extract was fractionated without further purification on column A, 10% Carbowax 20 M on Chromosorb W 60/80 mesh, 2.4 m × 6 mm (OD) glass, 60 ml/min He flow rate at 125°C initial temperature for 6 min, then temperature programmed at 4°/min up to 200°C and held there for 50 min. Under these conditions, methyl palmitate had a retention time of 40 min.

The presence or absence of caproic acid and γ-caprolactone was determined by coinjection on column A of an aliquot of the Porapak Q extract and an authentic compound. The identification of these compounds was not pursued more rigorously in this survey, because they had been identified previously in extracts of macerated female beetles and they were much less active than the other compounds. The peaks with the retention times of (Z)- and (E)-14-methyl-8-hexadecenal (41.4 min), methyl (Z)-7-hexadecenoate (41.4 min), methyl (Z)- and (E)-14-methyl-8-hexadecenoate (50 min), and (Z)- and (E)-14-methyl-8-hexadecen-1-ol (71 min) were collected separately

[5] Rearing, aeration, and bioassay of *T. granarium* were carried out exclusively in Seewiesen, Federal Republic of Germany. *T. granarium* cannot be reared in the United States because of quarantine restrictions.

[6] Mention of a proprietary product does not constitute an endorsement by the USDA.

and coinjected with authentic compounds on column B, 5% SE-30 on Chromosorb C 60/80 mesh, 5 m × 6 mm (OD) glass, 60 ml/min He flow rate at 190° (methyl pentadecanoate retention time 65 min). Methyl (Z)-14-methyl-8-hexadecenoate from *T. inclusum* was also coinjected with the synthetic ester on column C, 15% SP-2340 on Chromosorb P, 60/80 mesh, 2.5 m × 6 mm (OD) glass, 50 ml/min He flow rate at 185°. Methyl (Z)-7-hexadecenoate from *T. granarium* was further identified by its mass spectrum obtained on a Finnegan Model 3300 quadrupole mass spectrometer with a gas chromatographic inlet.

All glass columns and solid supports were washed with acid and treated with dichlorodimethylsilane. Fractions from the Varian Model 204B gas chromatograph were collected in glass capillary tubes (30 cm × 2 mm OD) in a thermal gradient collector (Brownlee and Silverstein, 1968).

We estimated the quantity of each component (except methyl (Z)-7-hexadecenoate) of the Porapak Q extract by comparing the area under its peak on column A with that of a known quantity of the synthetic compound. Since (Z)- or (E)-14-methyl-8-hexadecenal and methyl (Z)-7-hexadecenoate had identical retention times on column A, the quantity of the latter was estimated on column B. The detector was assumed to respond equally to the aldehyde and the ester; the quantity of the ester was, therefore, equal to the quantity of the aldehyde times the ratio of the areas of the two peaks. Since analysis of methyl (Z)-7-hexadecenoate in *T. inclusum* was not pursued rigorously enough to make this calculation, a "+" is entered in Table 2. The limit of detection by the flame ionization detectors (Varian Model 2700 series gas chromatographs) was somewhat arbitrarily set at 25 ng for (Z)- or (E)-14-methyl-8-hexadecen-1-ol and methyl (Z)- or (E)-14-methyl-8-hexadecenoate. This quantity gave a peak with a a satisfactory signal-to-noise ratio. If no pen deflection occurred at the retention time of a particular compound, 25 ng was divided by the sample size in beetle-days and this value was entered in Table 2 with the symbol "<".

For chemical analysis of pheromone components in whole *T. variabile* females, 2500 female beetles about 14 days old, unaerated and held on filter paper in closed containers, were extracted with hexane in a Waring blender, and the hexane was distilled as above. The oily residue was subjected to short-path distillation (110°/0.5 mm Hg, 4 hr), and the distillate was dissolved in 2 ml of hexane. The filter paper was extracted with hexane in a Soxhlet extractor, and the volume was reduced to 2 ml by distillation through a 20-cm column packed with glass beads. The pheromone components were isolated from these extracts by GLC on the same columns used for the Porapak extracts.

In the United States, all bioassays were conducted in midafternoon at 25–28°C and 40–50% relative humidity (RH) according to the method of

Vick et al. (1970) for all natural and synthetic compounds, except 14-methyl-8-hexadecenal, for which the method of Greenblatt et al. (1976) was used. For *T. granarium*, the bioactivity of all compounds was determined by a modification of the method of Levinson and Bar Ilan (1970) at 30±0.1°C and 40–50% RH. Synthesized compounds were more than 99% pure by gas chromatography. Insects were from laboratory cultures raised according to the procedure of Hammack et al. (1973) and were 7–9 days after eclosion at the time of assay.

RESULTS AND DISCUSSION

At the time the first interspecific attraction studies were conducted in 1970, detailed information was available only on extracts of whole female beetles of *T. inclusum* (Burkholder and Dicke, 1966; Rodin et al., 1969). Subsequently, sex pheromone components from three additional *Trogoderma* species were identified, synthesized, and bioassayed. The method of isolation has been considerably improved by the development of a method to aerate female beetles and collect the pheromone on Porapak Q, a polymeric absorbent that removes organic chemicals from air (Bryne et al., 1975). This technique led to the isolation of a new and highly active sex pheromone component and made it necessary to reexamine those species whose sex pheromones had been isolated only from extracts of whole beetles.

Of the six pheromone components isolated from the four *Trogoderma* species examined, the aldehyde, 14-methyl 8-hexadecenal, is by far the most important component of the volatile components of each of four species [E isomer in *T. glabrum*, Z in *T. inclusum* and *T. variabile*, and 92% Z:8% E in *T. granarium* (Table 2)] and is active at extremely low levels (Figure 1). However, it was not found in any of the extracts of macerated beetles. The next most active compound is the corresponding alcohol, 14-methyl-8-hexadecen-1-ol (Figures 1 and 2), but it is either absent in the volatiles or is present only in very small amount (Table 2). It has been found in the extracts of macerated females of *T. inclusum* (Z isomer, Rodin et al., 1969), *T. variabile* (Z isomer, Cross et al., 1977), and *T. glabrum* (E isomer, Yarger et al., 1975). The third most active component, the corresponding ester, methyl 14-methyl-8-hexadecenoate (Figure 1), is emitted in detectable amounts only by *T. inclusum* (Z isomer, Table 2) and has been found in extracts of macerated females of *T. inclusum* (Z isomer, Rodin et al., 1969) and *T. glabrum* (E isomer, Yarger et al., 1975). Caproic acid, caprolactone, and methyl-7-hexadecenoate are active only at much higher concentrations.

From these data, we may conclude that the response of males to emitting, i.e., "calling," females in and between the four species in their habitats is

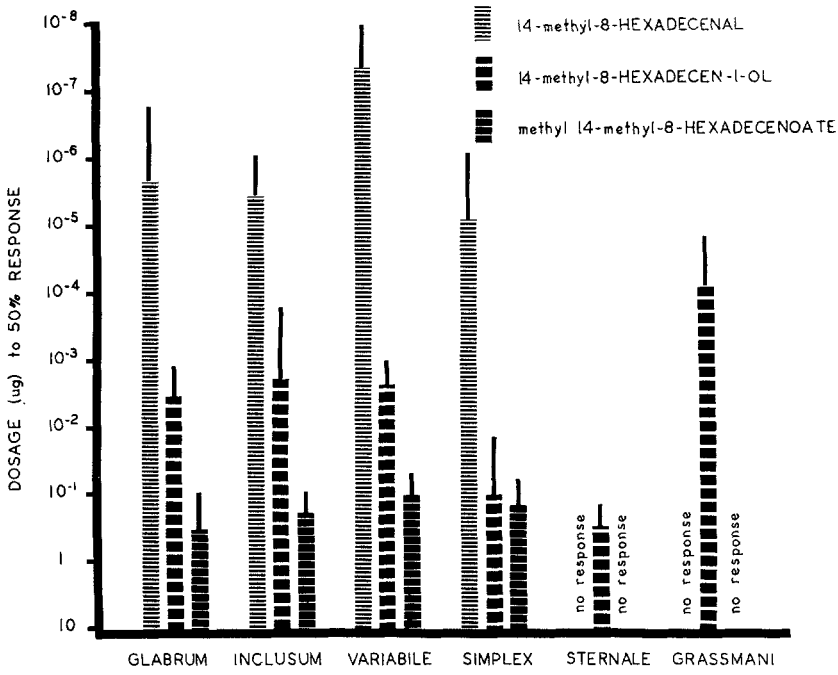

FIG. 1. Activity of synthesized 14-methyl-8-hexadecenal, 14-methyl-8-hexadecen-1-ol, and methyl 14-methyl-8-hexadecenoate in eliciting arousal in 6 *Trogoderma* species. Activity is measured as the quantity of compound required to elicit a response in 50% of the males tested, as determined by best linear fit to serial dilution bioassay data. Narrow bars represent one tail of the 95% confidence interval. The activity of the Z form of the aldehyde and alcohol is shown for all species except *T. glabrum*, for which the E form is shown. The ester was a mixture of 90% Z/10% E for all species.

largely a function of the aldehyde. The response to extracts of macerated female beetles can be attributed largely to the alcohol and, to a lesser extent, to the branched-chain ester.

We also note that the strong interspecific responses between *T. inclusum*, *T. variabile*, and *T. granarium* are a consequence of the presence of the Z isomer of each of the pheromone components, whereas the distinctly different response of *T. glabrum* lies in its use of the E isomer. *T. granarium* males respond to both isomers of the aldehyde but more strongly to the Z isomer.

Table 1 shows that *T. simplex* males respond strongly to extracts of *T. inclusum*, *T. granarium*, and *T. variabile* females, and males of the latter three

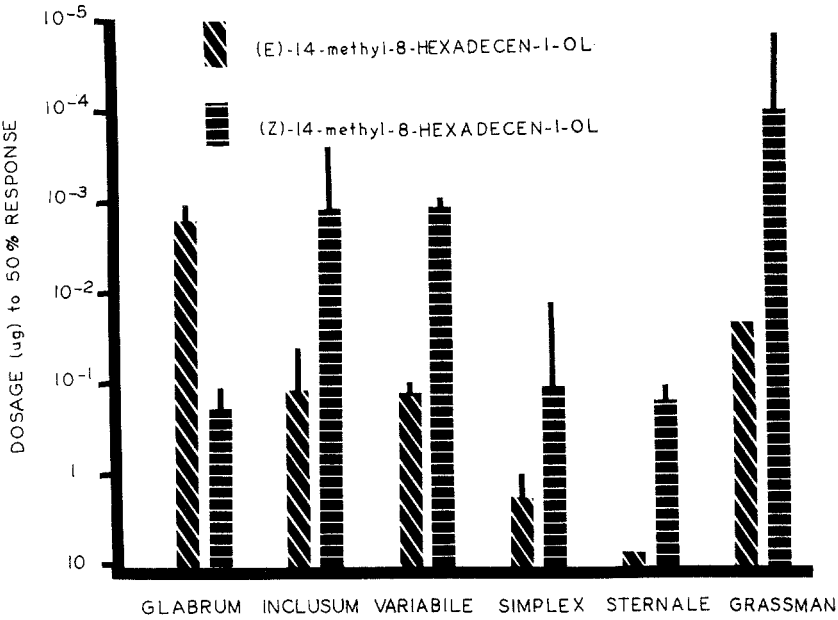

FIG. 2. Response of 6 *Trogoderma* species to pure (Z)- or (E)-14-methyl-8-hexadecen-1-ol. Activity is measured as the quantity of compound necessary to elicit arousal in 50% of the males tested, as determined by best linear fit to serial dilution bioassay data. Narrow bars represent one tail of the 95% confidence interval. The regression analysis for the activity of the E alcohol when tested on *T. sternale* and *T. grassmani* was nonsignificant and therefore no confidence interval is indicated.

species respond to very low concentrations of the Z aldehyde. It seems likely that these four species share one or more pheromone components and that *T. simplex* emits the Z aldehyde. On the other hand, *T. sternale* males show little cross attraction to any of the species except for a moderate attraction to *T. grassmani* and *T. glabrum* (Table 1). Neither *T. sternale* nor *T. grassmani* respond to the Z or E aldehyde (Figure 1).

An index of relative recency of common origin among 7 *Trogoderma* species that we have studied is suggested by the responses of each species to sex attractants. A cladogram for the relationships is presented in Figure 3 and supporting data are summarized in Table 3. Beal (1954) considered *T. inclusum*, *T. variabile*, and *T. glabrum* to be closely related on the basis of all observed morphological parameters; *T. sternale* and *T. grassmani* were placed together in another group, and *T. simplex* in a third more remotely related group. *Trogoderma granarium*, with an isomeric mixture of (Z)- and

TABLE 2. COMPONENTS IDENTIFIED IN EXTRACTS OF PORAPAK Q (AERATION), BEETLE-CONTAMINATED FILTER PAPER, AND MACERATED FEMALE BEETLES

Species	Component					
	14-Methyl-8-hexadecenal[a]	14-Methyl-8-hexadecen-1-ol	Methyl 14-methyl-8-hexadecenoate	Caproic acid	γ-caprolactone	Methyl 7-hexadecenoate
T. variabile	1. 35.66 (Z)[b]	<0·04[c]	<0.03	U	15.1	0.09
	2. +[d]	U	U	—	—	—
	3. U	+(Z)	U	—	—	—
T. inclusum	1. 2.00 (Z)	<0.03	0.39 (Z)	U	U	+
	3. U	+(Z)	+	—	—	—
T. granarium[e]	1. 1.47 (92Z:8E)	<0.01	0·02	2.98	1.83	0.01
T. glabrum	1. 28.3 (E)	<0.02	<0·02	9.32	7.74	0.031
	3. U	+(E)	+(E)	+	+	+(Z)

[a] 1. Aeration collection; 2. beetle-contaminated filter paper; 3. macerated female beetles.
[b] Quantities in ng/beetle/day; isomer in parentheses.
[c] The symbol < indicates that a component is not detectable by a flame ionization detector (FID). See Methods and Materials.
[d] + = detectable by FID; U = undetectable; — = not tested.
[e] Additional component; *p*-1,8-menthadiene.

TABLE 3. CHARACTERISTICS OF 7 *Trogoderma* SPECIES

Species	Morphological classification (Beal, 1954)	Geographical origin (Beal, 1954)	Pheromone components[a]				
			I	II	III	IV	V
T. glabrum[b]	5	Eurasian	+(E)	+	+	+	+
T. granarium[b]		Eurasian	+(Z+E)	+	+	+	−
T. variabile	5	Eurasian	+(Z)	+	−	−	+
T. inclusum	5	Eurasian	+(Z)	−	−	+	+
T. simplex	2	American	+(Z)[c]			+[c]	+[c]
T. sternale	4	American	−[c]			−[c]	+[c]
T. grassmani	4	American	−[c]			−[c]	+[c]

[a] + = present; − = absent or undetectable. I = 14-methyl-8-hexadecenal (isomer); II = γ-caprolactone; III = caproic acid; IV = methyl 14-methyl-8-hexadecenoate; V = 14-methyl-8-hexadecen-1-ol.
[b] Sterile hybrids when interbred.
[c] Bioassay only.

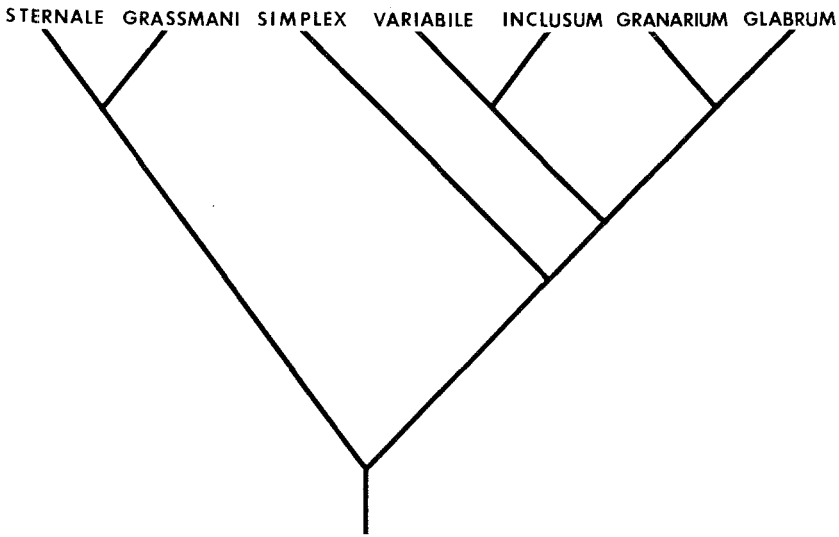

FIG. 3. Cladogram of 7 *Trogoderma* species suggested by pheromone components, morphology, and interbreeding responses. Recency of common origin is shown by the position of the branches in the diagram.

(E)-14-methyl-8-hexadecenal, has an intermediary position between *T. glabrum* (pure E) on the one hand, and *T. variabile* and *T. inclusum* (pure Z) on the other. *T. variabile* and *T. inclusum* differ by the presence of γ-caprolactone in the airborne pheromone of the former and methyl 14-methyl-8-hexadecenoate in that of the latter; the biological roles of these components remain to be elucidated. Cross activity between *T. grassmani* and *T. sternale*, as well as their common unresponsiveness to Z- or E-14-methyl-8-hexadecenal, are both consistent with the morphological similarity of these two species.

We believe that 14-methyl-8-hexadecenal plays the central role in the chemically mediated attraction and arousal during mating behavior in *T. glabrum*, *T. inclusum*, *T. variabile*, *T. granarium*, and *T. simplex*. For all but *T. simplex*, we have collected pheromone from extracts of female beetles, from extracts of beetle-contaminated filter paper, and from extracts of Porapak Q; the latter represents the pheromone as it is perceived at some distance from the female. The aldehyde is the most active compound isolated from any of these sources. We have observed mating behavior from an initial stage of arousal and search through a preliminary recognition stage leading to copulation. The aldehyde is active in one or more of these stages for each of the five species. The pheromones of *T. grassmani* and *T. sternale* are clearly different from those of the other species, and we have begun a detailed examination of these pheromones.

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ALARM PHEROMONE OF THE SPOTTED ALFALFA APHID, *Therioaphis maculata* BUCKTON (HOMOPTERA: APHIDIDAE)

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Abstract—An alarm pheromone, $C_{15}H_{24}$, was isolated from the spotted alfalfa aphid, *Therioaphis maculata*. The IR, PMR, and [^{13}C]NMR data showed that this alarm pheromone is germacrene A (structure IIIa). The negative plain curve in the ORD of the alarm pheromone implies that the pheromone is (−)-germacrene A (structure IIIb).

Key Words—spotted alfalfa aphid, *Therioaphis maculata*, alarm pheromone, sesquiterpene hydrocarbon, germacrene A.

INTRODUCTION

Aphids (Homoptera: Aphididae) are small, gregarious, plant-feeding insects of great economic importance. Damage by aphids is caused by imbibition of plant sap, injection of salivary phytotoxins, and/or transmission of plant viruses.

Aphid populations are often kept in check by numerous insect predators. In defense, aphids counter predator attack by secreting droplets from specialized structures called cornicles. The "gummy" droplets contain triglycerides which, when smeared on the predator, can result in release of the aphid (Dixon, 1958; Dixon and Stewart, 1975). Cornicle droplets also contain alarm pheromones which cause other aphids in the aggregate to escape by

walking, falling, or jumping away (Kislow and Edwards, 1972; Nault et al., 1973).

The first aphid alarm pheromone was identified as (E)-β-farnesene (TBF) (structure I) from a number of economically important species (Bowers et al., 1972; Edwards et al., 1973; Wientjens et al., 1973). Cross-reaction tests with several aphid species from four aphid subfamilies revealed that while TBF is widely utilized as an alarm pheromone, aphids possess other alarm chemicals (Nault and Bowers, 1974). The lack of response of most aphid species to cornicle droplets from the yellow clover aphid, *Therioaphis trifolii* (Monell) and the lack of responsiveness of this aphid to TBF clearly indicated the existence of a new alarm pheromone. Positive cross-reaction tests among *T. trifolii*, the spotted alfalfa aphid, *T. maculata* Buckton, and the sweetclover aphid, *T. riehmi* (Börner) indicated that members of the genus *Therioaphis* utilize a common alarm pheromone (L.R. Nault and M.E. Montgomery, unpublished data). We have identified the alarm pheromone of *T. maculata* as a sesquiterpene hydrocarbon, germacrene A (Bowers et al., 1977). In this paper we present the details of the isolation and structural elucidation of the alarm pheromone.

METHODS AND MATERIALS

Bioassay

T. maculata was cultured on alfalfa and *T. riehmi* on sweetclover. Rearing and testing conditions were $20° \pm 3°C$, 50–85% relative humidity and 16 hr light per day. Aphids feeding on the host plant were presented with the extracts, fractions, or compounds absorbed on a triangle of filter paper (8×2 mm). Methanol was used to dilute the test chemicals to the desired concentration. The triangle was held within 0.5 cm of a selected group of 3–10 adult or late-instar aphids. Falling, jumping, or walking of the aphids from the area within 1 min indicated a positive alarm response.

Collection of T. maculata

Populations of the spotted alfalfa aphid biotype "C" were reared in the field under two $12 \times 24 \times 6$-ft nylon screen cages at Tucson. Susceptible alfalfa plants of the variety *Caliverde* were grown in the cages and infested with aphids. Approximately three weeks after infestation, the plant stems were carefully harvested and brought into the laboratory. The aphids were then shaken off the stems and collected in methanol.

Extraction and Isolation

Approximately 2 liters of *T. maculata* were allowed to steep in methanol (1.5 liters) at room temperature for 20 days. The methanol extract was then filtered and concentrated in vacuo at 40°C. This concentrate (100 ml) was extracted in a separatory funnel with hexane. After washing the hexane extract with water it was dried over Na_2SO_4. The resulting yellow residue was chromatographed over Florisil (50 g) with petroleum ether to separate the hydrocarbons (57 mg). Bioassay with *T. riehmi* indicated that strong alarm activity was present in the hydrocarbon fraction. All other fractions were inactive. The hydrocarbon fraction was further purified by column chromatography over silicic acid (3 g) and eluted with petroleum ether. Fractions with alarm activity and showing a single VPC peak on 3% OV-225 were collected. These were combined and the solvent evaporated to give 9.0 mg of pure alarm pheromone.

Throughout the isolation, we were frequently dismayed by the exceptional lability of the active compound. A small amount of degradation was evident during gas chromatographic analysis on 3% OV-225 and on several other stationary phases in an all-glass system at 110°C. Breakdown was rapid on several adsorbents in metal columns even below 100°C. Although column chromatography over Florisil or silicic acid performed without delay did not result in noticeable change, prolonged contact with silicic acid promoted conversion to other hydrocarbons. To minimize conversion, exposure to temperatures over 40°C was avoided, and storage was maintained in petroleum ether at −15°C throughout this study.

Physical Data of the Pheromone and its Derivatives

Instrumentation. Infrared (IR) spectra were obtained as liquid films with a Perkin-Elmer 257 spectrometer. Mass spectra (MS) were measured on a Bendix Model 12 modified with a CVC Mark IV (for the pheromone) and on a Hitachi Perkin-Elmer RMU-6 (for the derivatives). Ionization was at 30°C and 70 eV. Proton magnetic resonance (PMR) spectra were recorded on a Varian HA-100 at 100 MHz with TMS as internal standard. Carbon-13 NMR (CMR) spectra were recorded on a JEOL PFT-100 at 25.03 MHz. Chemical shifts were measured from internal TMS. Optical rotatory dispersion (ORD) curve was obtained on a Cary 60 in a 3-mm cell.

Aphid Alarm Pheromone (*Structure IIIa*). $[\alpha]_D^{25} -26.8°$ (c = 1.0, CCl_4); MS: *m/e* 204 (M^+, $C_{15}H_{24}$); IR: 3060, 1780, 1650, 880, 850 cm^{-1}; PMR (in $CDCl_3$ at 30°C): 1.52 [6H, broad singlet (bs), half width (HW) = 5 Hz], 1.73 [3H, doublet (d), J = 1.5 Hz], 4.59 [1H, multiplet (m)], 4.66 (1H, m), 4.75–5.25 ppm (2H, m); PMR (in $CDCl_3$ at 50°C): Figure 1; CMR (in $CDCl_3$ at 54°C): Table 1; ORD (c = 1.0, CCl_4): Figure 2.

11,13-Dihydrogermacrene A. Hydrogen gas was passed through a mixture of the pheromone (2.5 mg) and 5% palladium–charcoal (11 mg) in methanol (2.5 ml) for 30 min with stirring at room temperature. The catalyst was filtered off and the filtrate concentrated to afford 11,13-dihydrogermacrene A (2.0 mg); MS: *m/e* 206 (M^+, $C_{15}H_{26}$); IR: 1650, 1375, 1385, 860 cm^{-1}.

β-Selinene (Structure V) from Alarm Pheromone. A solution of the alarm pheromone (6.0 mg) in hexane (5 ml) was stirred with silicic acid at room temperature for 8 hr. After filtration, removal of the hexane gave β-selinene (V) (5.0 mg); MS: *m/e* 204 (M^+, $C_{15}H_{24}$), 189, 161, 147, 133, 121, 109, 105, 93, 81, 77, 67, 55, 41 (base peak); IR: 3080, 1780, 1650, 890 cm^{-1}; PMR (CCl_4): 0.78 (3H, s), 1.78 (3H, s), 4.43 and 4.70 ppm (4H, bs). This identification was verified by direct comparison with an authentic sample of β-selinene from Dr. Nii. When the treatment with silicic acid was carried out for 15 hr, several isomers were produced from the pheromone. β-Selinene (V) was also afforded in quantitative yield when the pheromone was allowed to stand at 5°C for 25 days in a solution of $CDCl_3$ with TMS in a sealed tube.

RESULTS AND DISCUSSION

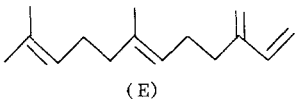
The molecular formula $C_{15}H_{24}$ was assigned to the isolated pheromone from the molecular ion peak at *m/e* 204 in the MS. This would correspond to a sesquiterpene hydrocarbon with four degrees of unsaturation.

The IR spectrum showed trisubstituted double-bond absorptions at 1780, 1650, and 850 cm^{-1}, and terminal methylene bands at 3060, 1650, and 880 cm^{-1}. In the IR spectrum of the dihydroderivative, terminal methylene bands disappeared while trisubstituted double-bond absorptions were still present. The IR spectrum also demonstrated a typical isopropyl doublet absorption at 1385 and 1375 cm^{-1} instead of a broad singlet at 1385 cm^{-1} in the spectrum of the original pheromone. These data imply the presence of an isopropenyl group in the pheromone. Typical isopropenyl signals were observed in the PMR spectrum of the isolated pheromone at 1.73 (vinyl methyl) and 4.59 and 4.66 ppm (terminal methylene protons). The mutual irradiation of both signals [1.73 and 4.62 ppm (center of terminal methylene proton signals)] confirmed the isopropenyl group.

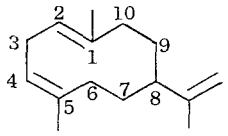
Two trisubstituted double bonds were indicated by a broad singlet signal at 1.52 ppm [half width (HW) = 5 Hz] due to two vinyl methyls and a multiplet signal at 4.75–5.25 ppm due to olefinic protons.

From these data emerged the structural picture for our isolated pheromone of a 10-membered ring sesquiterpene hydrocarbon bearing an isopropenyl group and two trisubstituted double bonds.

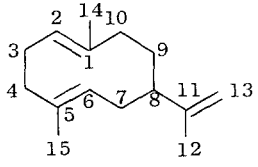
Although two germacrene-type structures, II and IIIa, might be proposed,



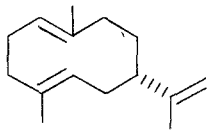
(E)
I



II



IIIa



IIIb

IIIa seemed to be more reasonable since the PMR spectrum of the pheromone did not possess any signal corresponding to the deshielded methylene protons at C-3 shown in II.

Since the broadened signals in the PMR at 30°C of the isolated pheromone seemed to show a mixture of conformers, we took the spectrum at 50°C and found that all signals were sharpened and several of them shifted slightly. In the spectrum at 50° (Figure 1), remarkable changes were observed

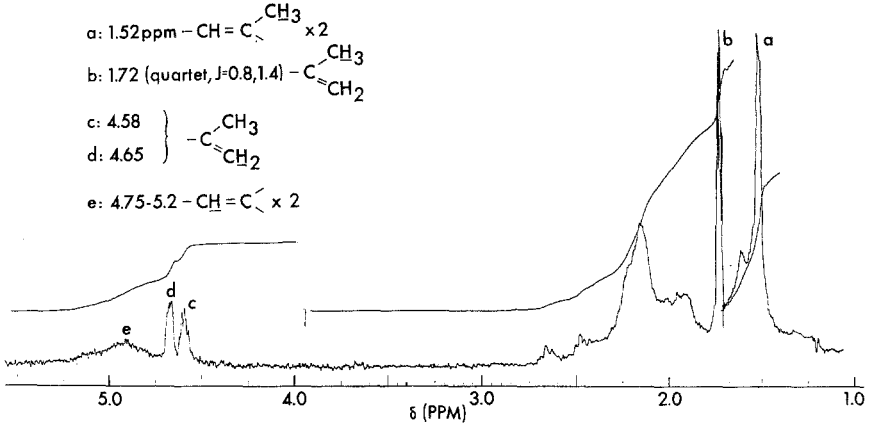


FIG. 1. PMR Spectrum of aphid alarm pheromone of *T. maculata* (in $CDCl_3$ at 50°C).

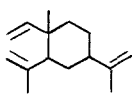
in the signal at 1.52 ppm which changed to a sharper singlet (HW = 3 Hz), while the signal at 1.72 ppm splits into a quartet with $J = 0.8$ and 1.4 Hz. The PMR and IR spectra, taken together with the labile nature of the isolated pheromone, matched well with the data for (−)-germacrene A (IIIb) reported by Weinheimer et al. (1970).

Germacrene A (IIIa) has been proposed as the biogenetic precursor of several mono- and bicyclic sesquiterpene hydrocarbons (Kulkarni et al., 1964; Morikawa and Hirose, 1969). However, its exceptional lability conspired to prevent its isolation until Weinheimer et al. (1970) isolated (−)-germacrene A (IIIb) from a gorgonian coral, *Eunicea mammosa* Labouroux. They reported that germacrene A isomerized to β-elemene (IV) through the Cope rearrangement. Morikawa and Hirose (1969) have also shown the rearrangement of germacrene C (VI) to bicyclic compounds by treatment with silicic acid. Similarly, we treated the isolated pheromone with silicic acid in hexane for 8 hr and obtained quantitatively an isomerized compound. This compound was stable even in metal columns during gas chromatography.

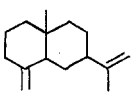
The IR spectrum of the isomerized compound (MS: M^+, *m/e* 204) indicated only hydrocarbon absorptions with terminal methylene bands at 3080, 1780, 1650, and 890 cm^{-1}. One tertiary methyl (0.78), one vinyl methyl (1.78) and four terminal methylene protons (4.43 and 4.70 ppm) were present in the PMR spectrum. The IR and PMR data and the gas chromatographic retention time were identical with those of an authentic sample of β-selinene (V).

The structure IIIa for the aphid alarm pheromone was finally confirmed by the CMR spectrum. To analyze the chemical shifts, the CMR studies with limonene (Jautelat et al., 1970) and laurenobiolide (Tori et al., 1976) were instructive. Chemical shifts and assignment of the signals shown in Table 1 were in accordance with the structure IIIa.

The ORD curve of the alarm pheromone demonstrated the negative-sign plain curve (Figure 2), indicating that the pheromone is the (−)-enantiomer of germacrene A (IIIb). From the curve, $[\alpha]_D^{25} -26.8°$ ($c = 1.0$, CCl_4) was established, which is in contrast with Weinheimer's value, $[\alpha]_D^{25}$



IV



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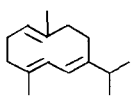
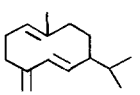


VI



VII

TABLE 1. CHEMICAL SHIFT AND ASSIGNMENT OF SIGNAL IN CMR SPECTRUM OF APHID ALARM PHEROMONE OF *T. maculata*

Chemical shift (ppm)	Assignment (carbon no. in IIIa)
16.4	14 or 15
21.0	14 or 15
21.7	12
27.0	3 or 7
29.8	3 or 7, 9
37.0	4 or 10
38.2	4 or 10
41.4	8
108.3	13
124.4–128.6[a]	2 and 6
135.0	1 and 5
150.5	11

[a] Multiplet in this range.

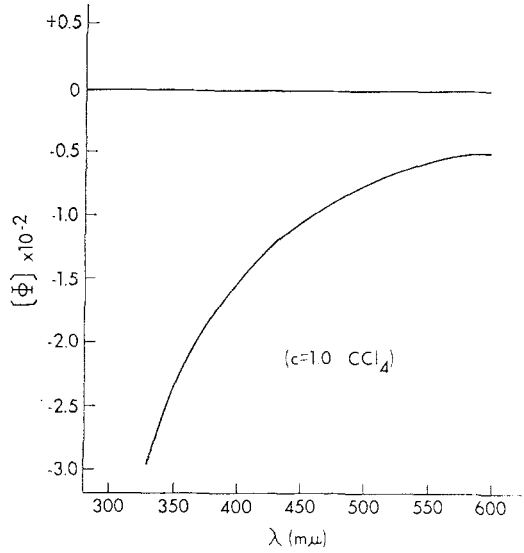


FIG. 2. ORD curve of aphid alarm pheromone of *T. maculata*.

-3.2 ($c = 14.4$, CCl_4) (Weinheimer et al., 1970). The disparity between our ORD value and that of Weinheimer's for germacrene A has caused us considerable concern. However, we repeated the ORD measurement several times and verified the purity of our sample before and after measurement by gas–liquid chromatography, finding no decomposition or rearrangement. Weinheimer (1970) speculated that most terrestrial sources of germacrene A will possess the (+)-enantiomer. Nevertheless the existence of the (−)-enantiomer in terrestrial sources was revealed in this study. Our identification of the alarm pheromone of *T. maculata* represents the first isolation of germacrene A from a terrestrial source.

11,13-Dihydrogermacrene A, β-selinene, and germacrene D (VII)[1] (Washio et al., 1976) did not show alarm activity against either *T. riehmi* or *T. maculata*. Thus, the structure of germacrene A (IIIa and/or IIIb) must be specific for the *Therioaphis* species.

An aphid alarm pheromone should decompose rapidly in the environment so that when the predator moves on, aphids can reinfest the feeding sites. Because of its lability, germacrene A is ideally suitable as an alarm pheromone since aphids would not be continually deterred from the host plant. On the other hand, the instability of the natural aphid alarm pheromone prevents practical use of it in the field. We have accordingly directed our efforts toward the development of more stable alarm pheromone analogs that can be used in aphid control (Nishino et al., 1976a,b; Nishino and Bowers, 1976).

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HOST SELECTION BY *Hylemya antiqua*[1] Laboratory Bioassay and Methods of Obtaining Host Volatiles[2]

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Abstract—An oviposition bioassay for the onion maggot, *Hylemya antiqua* (Meigen), is described in which females, in response to volatile stimulants, oviposit through small apertures onto moistened filter paper. Onion volatiles that act as attractants and oviposition stimulants were captured in Porapak Q from air passed over chopped onions in glass chambers. Pentane extracts from odor-impregnated Porapak Q induced ~30–50% of the oviposition that occurred in response to 15-g onion-slice stimuli. Extracts presented in pentane on waxed dental cotton wicks induced more constant oviposition over a 3-day period than extracts on unwaxed wicks. Extract of the Porapak Q–captured volatiles from bulbs of fresh, actively growing onions elicited a much stronger response than did stem and leaf extracts from the same onions. The bioassay techniques and chemical procedures developed in this study could be used in chemical isolation programs for host attractants or oviposition stimulants for *H. antiqua* or similar species.

Key Words—onion maggot, *Hylemya antiqua*, onion volatiles, host selection, oviposition.

[1] Diptera: Anthomyiidae.

[2] Research supported by National Research Council of Canada, Grant Nos. A3881 and A3706.

INTRODUCTION

Studies to determine the chemical basis for attraction to and oviposition on particular host plants have met with increasing success (Hedin et al., 1974). Oviposition stimulants have been isolated and/or identified for a number of economically important Diptera: *n*-propyl mercaptan and dipropyl disulfide for the onion maggot, *Hylemya antiqua* (Meigen) (Matsumoto and Thorsteinson, 1968); sinigrin, allyl isothiocyanate, β-phenylethylamine, and carbon disulfide for the cabbage maggot, *H. brassicae* (Bouché) (Traynier, 1967); and methyl isoeugenol for the carrot rust fly, *Psila rosae* F. (Berüter and Städler, 1971).

For *H. antiqua*, known or suspected volatile constituents of *Allium cepa L.* (Niegisch and Stahl, 1956; Carson and Wong, 1961; Matsumoto and Thorsteinson, 1968) have been tested for activity. Because there have been no systematic chemical isolation programs, however, no conclusions can be reached as to whether *n*-propyl mercaptan and dipropyl disulfide, the active compounds identified (Matsumoto and Thorsteinson, 1968), represent the entire range of oviposition stimulants present in onions. Recent studies on the capture of small amounts of insect pheromones in Porapak Q chromatograph packing (Byrne et al., 1975; Peacock et al., 1975) suggest that this technique would also be effective in capturing small amounts of host-plant volatiles in a chemical isolation procedure.

Our objectives were to devise a reliable and efficient technique for capturing onion volatiles, and to develop a simple bioassay for testing candidate attractants and oviposition stimulants for *H. antiqua.* These techniques were considered necessary prerequisites for the systematic identification of all possible attractants and oviposition stimulants for *H. antiqua.*

METHODS AND MATERIALS

Capture and Extraction of Host Volatiles

Onions (varieties unknown, ~2.5 kg) were peeled, quartered, and placed in one to four sterilized, borosilicate-glass chambers. The two-piece, cylindrical chambers (15.5 cm inside diameter (I.D.) × 27 cm) were fitted with a 1.5-cm-wide ground-glass flange about 9 cm from the top. The two pieces were held together by two plastic rings that rested on the flange and were drawn together by four screws. The top and bottom of the chamber were fitted with centered S-19 female and male spherical ground joints.

Porapak Q (50/80 mesh, Applied Science Laboratories, Inc.) was conditioned by extraction with anhydrous, reagent-grade ether in a Soxhlet

extractor for a minimum of 15 h and, after evaporation of residual ether, was stored in glass-stoppered bottles in the dark. Porapak Q–filled glass tube traps (2.4 cm outside diameter (O.D.) × 20 cm) were fitted with S-19 female and male ground joints on the top and bottom, respectively. A coarse, sintered-glass disk was sealed inside each tube at the bottom. Approximately 26 g of Porapak Q could be packed into each trap, and a plug of glass wool was inserted in the short tubing connecting the top joint to the trap body. A similar Porapak Q–filled trap (2.4 cm O.D. × 12 cm) was attached to the top of the chamber to serve as an air scrubber. To avoid Porapak contamination, stopcock grease was not applied to ground-glass joints, which sealed firmly and did not leak if kept clean.

The chambers were supported in a stand so that the large trap could be connected vertically to the bottom of the chamber. Air was drawn through the chamber by a suction pump connected to the bottom of the trap through a manifold constructed of polyethylene tubing. Airflow through the chamber (measured at the inlet with a Brooks flowmeter) was 1.6–1.8 liters/min.

In most aerations, four chambers were used. Low room temperature and small onion slices were avoided, as they resulted in onion juice flowing into the traps. If fungal growth on the onions became severe, the aeration was halted. After 1–7 days, the traps were removed and processed immediately, or plugged with rubber stoppers and stored in a freezer.

Pentane (Eastman Kodak Co. or Caledon Laboratories Ltd.) was purified by distillation through a 30-cm glass Dufton column. Volatiles were recovered by extracting the Porapak Q in a Soxhlet extractor with purified pentane for at least 8 h. The extract was concentrated to about 5 ml by distilling the pentane through a 30-cm Dufton column. The solution of onion volatiles was then placed in screw-cap vials with Teflon-lined lids and stored under refrigeration until used in bioassays or chemical analysis. Odor-contaminated Porapak Q was washed with distilled water and distilled acetone, and then conditioned as described above.

Dipropyl disulfide was synthesized by modification of the procedure for the synthesis of dibutyl disulfide (Stutz and Shriner, 1933), and *n*-propyl mercaptan was purchased from Eastman Kodak Co. Both chemicals were used as reference stimuli in bioassay procedures.

Bioassay Procedures

H. antiqua were reared by the combined methods of Allen and Askew (1973) and Tiechler (1971) (A. Syed, unpublished report).[5] Fifteen 10–45-day-old, gravid females [age range used was that in which active and con-

[5] Preliminary report of insect-rearing facilities at Simon Fraser University, May 31, 1974. 17 pp.

sistent oviposition occurs (R.S. Vernon, unpublished data)] were maintained with five males in $25 \times 25 \times 45$ cm wooden-frame, screen cages with Plexiglas fronts. Usually, five such cages were equally positioned in each of two walk-in growth chambers (internal dimensions $2.7 \times 1.7 \times 2.3$ m) maintained at 23°C, 30–40% RH, and a 16-h light, 8-hr dark photoperiod regime. The cages were evenly spaced over a 1×2.3 m shelf, 60 cm below three banks of fluorescent lights. Dead females were replaced daily with flies from an identically aged stock culture kept in one of the chambers. A petri dish containing food (skim milk, sugar, soya flour, yeast hydrolysate, and yeast) and a similar dish filled with water-soaked vermiculite were maintained in each cage. New cultures were ~2 weeks old and verified to be in an active ovipositional stage prior to use in experiments.

The bioassay apparatus[6] is shown in Figs. 1 and 2. A 14×1 cm glass petri dish contained five 12.5-cm No. 1 Whatman filter papers stapled together and moistened. A 100-ml Nalgene beaker with 10–15 regularly cut triangular nicks around the lip was inverted on the filter paper. These nicks enabled ovipositor insertion (Fig. 2), but were small enough to prevent fly entry. Extracts or solvent controls to be tested were injected into 3-cm-long pieces of dental cotton. Early experiments used unwaxed wicks, whereas later experiments employed paraffin-dipped wicks in an attempt to prolong and control the release of volatiles. The wicks were suspended from the bottom of the inverted beaker. Onion slice stimuli (~15 g) were suspended in a similar manner in cheesecloth sacks or taped directly to the beaker.

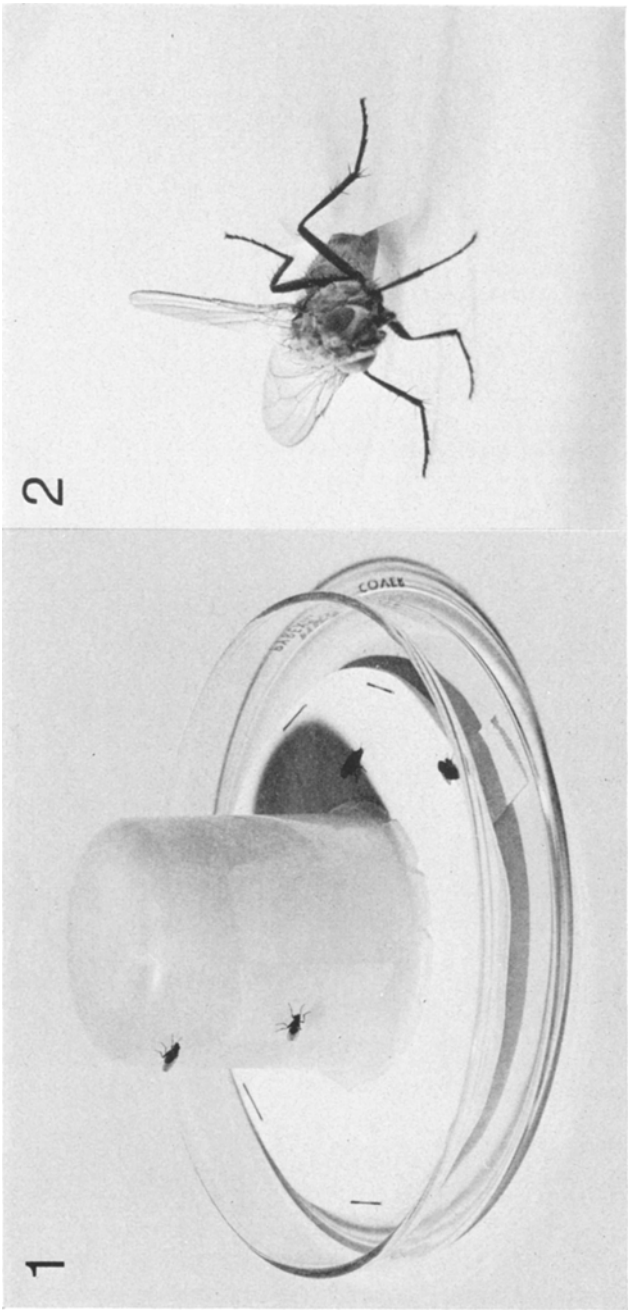
Testing of chemicals or extracts was confined to one growth chamber, and onion slice stimuli were tested simultaneously in the other chamber. Only one chemical or extract was tested at any time. These procedures minimized contamination or confusion caused by conflicting stimuli. Solvent controls were tested in the same cages as extracts or chemicals, and blank controls were tested in the same cages as the onion slice stimuli.

Most experiments were run for 3 days. Eggs were removed and counted on each day, and the filter papers and petri dishes replaced. Prior to experiments, the flies were held without host odor for 24 h to ensure flushing of residual odor in the chambers, and to allow the females to achieve a state of ovipositional readiness (R.S. Vernon, unpublished data).

RESULTS AND DISCUSSION

The bioassay procedure (Figs. 1 and 2) proved to be an effective means of determining qualitative and quantitative ovipositional response of *H.*

[6] Design adapted from that used to obtain eggs for rearing by D.G. Finlayson and C.J. Campbell, Agriculture Canada Research Station, Vancouver, B.C.



FIGS. 1 and 2. (1) Apparatus used in oviposition bioassays. (2) Female *H. antiqua* ovipositing through aperture in Nalgene beaker in response to the odor of onion slice. Note egg in beaker aperture.

TABLE 1. OVIPOSITIONAL RESPONSE BY *H. antiqua* FEMALES TO ONION AND STORED PENTANE EXTRACT OF ONION VOLATILES CAPTURED FOR 4 DAYS IN PORAPAK Q. ALL EXTRACTS ON WAXED DENTAL COTTON WICKS. $N = 5$ REPLICATES OF 15 FEMALES PER CAGE FOR 3 DAYS FOR EACH TREATMENT. ONE KG-H = EXTRACT EQUIVALENT TO 1 KG ONIONS AERATED FOR 1 H

Culture no. and age	Stimulus description	Total no. of eggs laid	% of response to onion slice
1, 16 days old	Pentane extract, 100 μl = 5.18 kg-h, tested after storage at −20°C for 25 days	1095	33.0
	Pentane control, 100 μl	11	0.3
	Onion slice, 15 g	3316	100.0
	Blank control	0	0.0
2, 17 days old	Pentane extract, 100 μl = 5.18 kg-h, tested after storage at −20°C for 122 days	1776	52.1
	Pentane control, 100 μl	13	0.4
	Onion slice, 15 g	3406	100.0
	Blank control	0	0.0

antiqua to host odor. Over a 3-day period, consistent numbers of ~600 eggs per 15-females in one cage were obtained from 15–25-day-old females in response to the odor of onion slices. Slightly fewer eggs were produced by 26–45-day-old females.

The apparatus yields free eggs which are easily counted, an advantage over other assays in which eggs must be laboriously separated from a sub-

TABLE 2. OVIPOSITIONAL RESPONSE BY *H. antiqua* FEMALES TO ONION AND TWO KNOWN OVIPOSITION STIMULANTS OFFERED IN 100 μL OF PENTANE ON WAXED DENTAL COTTON WICKS[a]

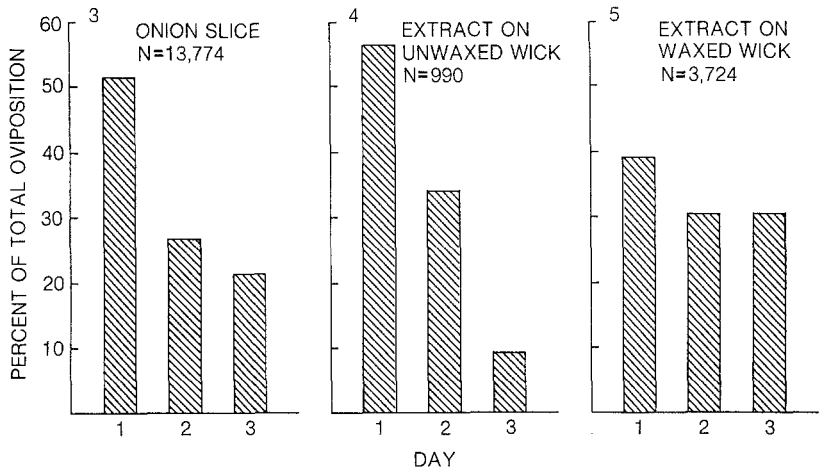
Culture no. and age	Stimulus description	Total no. of eggs laid	% of response to onion slice
1, 20 days old	*n*-Propyl mercaptan, 1 μl	467	17.4
	Pentane control, 100 μl	4	0.0
	Onion slice, 15 g	2676	100.0
	Blank control	0	0.0
2, 35 days old	Dipropyl disulfide, 1 μl	356	17.7
	Pentane control, 100 μl	19	0.9
	Onion slice, 15 g	2014	100.0
	Blank control	1	0.0

[a] $N=5$ replicates of 15 females per cage for 3 days for each treatment.

strate such as moistened sand prior to counting (Matsumoto and Thorsteinson, 1968). Very little oviposition is obtained in response to blank or solvent controls (Tables 1 and 2). Matsumoto and Thorsteinson's (1968) assay, which employs moistened sand as a control, may yield hundreds of eggs per replicate, possibly because of hygrotactic and/or thigmotactic responses. This effect makes precise comparisons of control and experimental stimuli quite difficult. An additional difference is that no contact with the test stimulus is allowed in the new bioassay. Thus, the ovipositional response of *H. antiqua* must be primarily mediated by olfaction.

Low-level responses to solvent-control stimuli (Tables 1 and 2) are considered to represent a thigmotactic response to an acceptable physical site by flies stimulated by threshold levels of host odor generally present in the cages. For consistency, bioassays were considered valid only if the blank or solvent controls contained less than 1% of the total number of eggs present in the onion-baited treatments.

As with pheromones (Byrne et al., 1975; Peacock et al., 1975), Porapak Q is a good adsorbent in which to capture onion volatiles. The response of females to the pentane extracts of odor-impregnated Porapak Q was always positive, and persisted even after long storage periods at −20°C. Earlier experiments with pressed onion juice or pentane extracts of whole or chopped



FIGS. 3–5. Effect of waxed and unwaxed dental cotton wicks on the 3-day ovipositional response of *H. antiqua* females to onion and pentane extract of onion volatiles captured for 3 days in Porapak Q. Extracts presented at concentration equivalent to volatiles from 5.18 kg of onion for 1 h; onion slice stimuli weighed 15 g. Data summed from different experiments for each figure; N = total number of eggs.

onions had not always produced positive responses, possibly due to interaction of chemical components, or enzymatic breakdown of active compounds in the onion juice.

Unlike the response to onion slices, responses to Porapak Q extracts were not always constant, and they did not approach the same response level. For example, in Table 1 the flies in culture 2 responded to the extract stored for 122 days at a much greater level than flies in culture 1 responded to the same extract after 25-days storage. However, more likely causes of response variation than storage or a change in fly culture may be that active constituents in the extract were nearer to threshold levels and thus were subject to very slight changes in ambient conditions, as was fly behavior, or that specific components of the natural odor complex were inefficiently captured in, or extracted from, the Porapak Q.

In the bioassay procedure, *n*-propyl mercaptan and dipropyl disulfide,

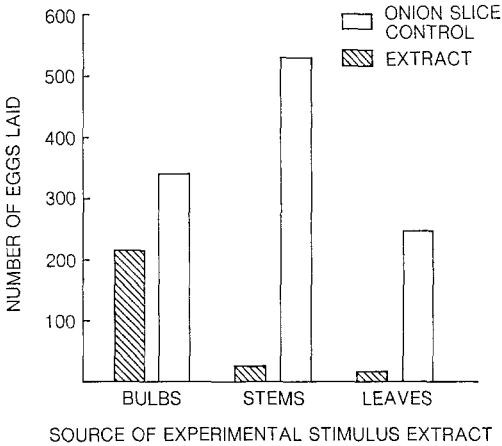


FIG. 6. Ovipositional response by *H. antiqua* females to 15 g of onion and pentane extract of volatiles from three regions of the same 24 onion plants. Volatiles were captured from bulbs (455.6 g), stems (71.0 g) and leaves (258.2 g) for 3 days in Porapak-Q. All extracts were presented in 100 μl of pentane on unwaxed dental cotton wicks at concentrations equivalent to volatiles from 17.3 onions for 1 h. $N = 3$ replicates of 15 females per cage for 1 day. The t test probabilities between onion slice and extract stimuli for bulbs, stems, and leaves, respectively, were <0.40, <0.20, and <0.025.

when tested at concentrations known to stimulate oviposition by *H. antiqua* (Matsumoto and Thorsteinson, 1968), effectively induced moderate oviposition (Table 2). However, neither was as competitive with onion slices as was the Porapak Q extract (Table 1). This lack of competitiveness suggests that additional active compounds are yet to be identified. Only when all such compounds are known will it be possible to evaluate host compounds accurately in applied pest-management procedures.

Waxed dental cotton wicks were clearly superior to unwaxed wicks (Figs. 3–5). They evidently were able to retain volatiles longer and to release them at more constant rates. Thus, there was a much more constant oviposition rate in response to extract on waxed wicks (Fig. 5) than to onion slices or extract on unwaxed wicks, both of which lost attraction rapidly after the first day (Figs. 3 and 4). In all cases, oviposition on the first day was greatest, apparently a characteristic response of females that had been denied exposure to host odor for 24 h. The superiority of waxed wicks suggests that they could be used reliably in behavioral experiments or in chemical isolation programs which rely on standardized bioassays of chemical fractions or candidate compounds.

Extract of onion bulbs stimulated far more oviposition than did the extracts of stems and leaves from the same plants (Fig. 6). Compared with the response to onion slice stimuli in each case, the extracts of bulbs, stems, and leaves elicited 63.1%, 3.7%, and 4.2% responses, respectively. The response pattern in Fig. 6 effectively eliminates aboveground parts of actively growing onions as the source of oviposition stimulants. It does not totally eliminate the possibility of long-range chemical attractants emanating from the aboveground regions of the plant. However, it does indicate that isolation programs for close-range attractants and oviposition stimulants should concentrate on onion bulbs.

We conclude that the bioassay and chemical techniques developed in this study can be used in a systematic program designed to identify host attractants and oviposition stimulants for *H. antiqua*. Moreover, they should be applicable to other insect species with similar oviposition habits on other host plants.

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SEX PHEROMONE COMPONENTS OF THE VARIEGATED LEAFROLLER MOTH, *Platynota flavedana*[1,2]

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Abstract—Female tip extracts of the variegated leafroller moth, *Platynota flavedana* (Clemens), were analyzed and found to contain a mixture of (*E*)-11-tetradecen-1-ol and (*Z*)-11-tetradecen-1-ol (9:1), as well as a mixture of (*E*)-11-tetradecenyl acetate and (*Z*)-11-tetradecenyl acetate (2–3:1). Small amounts of tetradecen-1-ol and tetradecyl acetate probably are also present. In the field, a mixture of the two alcohols (84:16, *E*:*Z* ratio) attracted significantly more male moths of this species than any other mixture tested. The data indicate that the acetates are probably not used as pheromone components.

Key Words—sex pheromone, sex attractant, *Platynota flavedana*, variegated leafroller moth, *Platynota idaeusalis*, tufted apple bud moth, (*E*)-11-tetradecen-1-ol, (*Z*)-11-tetradecen-1-ol, (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenyl acetate.

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INTRODUCTION

The variegated leafroller moth, *Platynota flavedana* (Clemens), is one of a sympatric complex of leafrollers occurring in the northeastern United States for which apple trees are a primary host (Chapman and Lienk, 1971). This report is part of our continuing efforts to identify the sex pheromones of these species (Cardé et al., 1977; Roelofs et al., 1974, 1975, 1976; Miller et al., 1976; Hill et al., 1974; Roelofs and Tette, 1970) as a step toward defining the role of specific pheromone blends in the reproductive isolation of these species. In addition, traps baited with pheromones have become an important part of pest-management practices.

METHODS AND MATERIALS

GLC columns (glass, 1.8 m × 2 mm, 3.6 m × 2 mm, or 1.8 m × 4 mm) were packed with 3% OV-1 (methyl silicone) on 100–120 mesh Gas-Chrom Q, 3% PDEAS (phenyldiethanolamine succinate, Hi-Eff 10BP) on 100–120 mesh Chromosorb W-AW-DMCS, or 10% XF-1150 (50% cyanoethyl methyl silicone) on 100–120 mesh Chromosorb W-AW-DMCS. Hydrogen flame ionization detection was used. The mass spectrometer was a Finnigan 3300 dual quadrupole EI interfaced with an OV-101 (methyl silicone) column (Cornell University Mass Spectrometry Center). (*Z*)-11-Tetradecenyl acetate (Z11-14:Ac) and (*E*)-11-tetradecenyl acetate (E11-14:Ac) were purchased from Farchan Chemical Co. (*Z*)-11-Tetradecen-1-ol (Z11-14:OH) and (*E*)-11-tetradecen-1-ol (E11-14:OH) were prepared from the acetates by saponification, using a 4 M excess of sodium hydroxide in refluxing aqueous ethanol for 1 h. GLC analyses showed that these four compounds were 98.8% pure or better.

Insects were reared continuously on fava bean plants in a greenhouse (Glass and Hervey, 1962). Female moths were collected at random times, their sex pheromone glands were manually extruded and snipped, then were soaked in redistilled methylene chloride for at least 24 h at freezer temperatures ($< -5°C$), and the extract was separated from the glands prior to use. Extracts prepared in this way were essentially free of interfering peaks in the GLC region of interest (where 10- through 18-carbon acetates elute), as is common for such extracts from the Tortricinae (Tortricidae) (Roelofs et al., 1974, 1976; Hill and Roelofs, 1975; Miller et al., 1976). Analytical procedures and chemical microreactions have been described (Hill et al., 1974; Roelofs et al., 1971), and were carried out with samples collected in glass capillary tubes from the OV-1 GLC column. GLC-collected samples

were recovered by washing out the capillary tube with carbon disulfide and evaporating the solvent in a stream of nitrogen. Microacetylation was carried out by the addition of 2–5 drops of acetyl chloride and, after 10 min at room temperature, evaporation of excess acetyl chloride in a stream of nitrogen. Microsaponification was carried out by addition of 0.5–1.0 ml of ~1% sodium hydroxide in aqueous ethanol, heating gently on a steam bath for ~1 h, and dilution with water and extraction with redistilled Skelly B to recover the products; this entire procedure was carried out in 1-dr vials with Teflon®-lined screw caps. Electroantennograms (EAG's) were carried out, and the data were normalized as described previously (Roelofs, 1976). Field tests were carried out in Idaville, Pennsylvania, using Pherocon® 1C traps (Zoecon Corp., Palo Alto, California) with test chemicals placed in polyethylene caps (OS-6 natural polyethylene closures, Scientific Products). Traps were set out in a randomized complete block design. Data were transformed to $(\chi+0.5)^{\frac{1}{2}}$ and submitted to an analysis of variance.

RESULTS

Pheromone Identification

Crude female tip extract was collected from OV-1, 170°, in timed fractions, for EAG assay. Good male antennal responses were found with fractions taken at 5–6.5 and 9–10.5 min (5.4 and 2.2 mV, respectively, with responses to other fractions of 0.4–1.4 mV). On OV-1, 170°, the crude extract showed two major peaks at 5.8 and 9.8 min, which coincided with the retention times of E11-14:OH and E11-14:Ac, respectively. The ratio between these two peaks showed some variability from sample to sample, being in the range 15:1 to 3:1 (alcohol–acetate).

To confirm the presence of the alcohol functionality in the earlier peak material, the 5–6.5-min fraction was recovered, acetylated with acetyl chloride, and collected from OV-1, 170°. EAG activity was evident only with the 9–10.5-min fraction (0.8 mV; with 0.2–0.5 mV for other fractions). A peak at 9.8 min (the retention time of E11-14:Ac) was present, and a peak at 5.8 min, present prior to acetylation, was absent. After the acetylated material was recovered and saponified, activity was found at 5–6.5 min (2.5 mV; with background activity of 0–0.1 mV). These data indicate the presence of a 14-carbon alcohol in the female tip extract. Similar treatment of the 9–10.5-min fraction from the crude extract indicated that a 14-carbon acetate also was present.

A mass spectrum of the 14-carbon alcohol fraction from a female extract

was the same as that of E11-14:OH, indicating the presence of one double bond and an unbranched 14-carbon chain.

Microozonolysis of the alcohol fraction from female tip extract and of E11-14:OH yielded 11-hydroxyundecanal (5.7 and 5.65 min, respectively, on OV-1, 160°). The acetate fraction from female extract and E11-14:Ac produced 11-acetoxyundecanal from microozonolysis (9.85 and 9.8 min, respectively, on OV-1, 160°). Thus, the double bond in both the alcohol and the acetate materials isolated from this insect must be located at the 11 position.

On XF-1150, 170°, the OV-1-collected alcohol fraction from female extract appeared as two peaks present in the approximate ratio of 9:1, with retention times of 12.15 and 13.3 min, respectively, compared to 12.25 and 13.5 min for E11-14:OH and Z11-14:OH, respectively. On PDEAS, 172°, these alcohols had retention times of 12.2 and 13.0 min, respectively, compared to 12.25 and 13.05 min for E11-14:OH and Z11-14:OH, respectively, and were present in the same 9:1 ratio. A small peak at 10.45 min corresponding to tetradecyl alcohol (10.35 min) also was evident, and amounted to ~3% of the mixture of 14-carbon alcohols. After acetylation of the alcohol fraction and collection of the acetates from OV-1, the retention times of the two major peaks on XF-1150, 170° (3.6 m column), coincided with those of E11-14:Ac and Z11-14:Ac (11.35 and 12.45 min, respectively). When the acetate fraction from crude female extract was examined on XF-1150, 150° (1.8 m column), peaks at 7.35 and 8.05 min were evident, corresponding to E11-14:Ac and Z11-14:Ac (7.25 and 8.0 min, respectively). The retention time of a third peak was coincident with that of tetradecyl acetate (14:Ac) (6.55 min). The *E*:*Z* ratios of the acetates from two different samples were 26:74 and 32:68, and the amounts of 14:Ac were 5 and 14% of the mixture of 14-carbon acetates.

Further confirmation of the presence of both *E* and *Z* isomers of the alcohols was obtained by TLC on silver nitrate–impregnated silica gel-G using development with benzene. The alcohol fraction from female tip extract was acetylated prior to TLC. The materials from four areas of the plate were recovered and collected from OV-1, 170°, for EAG assay. EAG activity was recovered only from the two areas corresponding to the *E* and *Z* acetates. For each of these, the GLC-collected materials showed EAG activity only at the retention of 14-carbon acetates (9–10.5 min); 2.3 mV at 9–10.5 min with 1.3–1.4 mV for the other GLC fractions from the TLC-E area, and 1.3 mV at 9–10.5 min with 0.7–0.8 mV for the other GLC fractions from the TLC-Z area. On XF-1150, 170°, materials from the *E* area and the *Z* area each produced one major peak, at 11.25 and 12.35 min, respectively, which corresponded to E11-14:Ac (11.35 min) and Z11-14:Ac (12.5 min). The two other TLC areas showed no peaks at either of these retention times.

The normalized EAG responses of male *P. flavedana* antennae to a series of monounsaturated 12-, 14-, and 16-carbon straight-chain acetates, alcohols, and aldehydes, when (*Z*)-6-tetradecenyl acetate is used as the reference standard, show that the greatest responses were obtained from E11-14:OH (23) and Z11-14:OH (22), followed by Z11-14:Ac (19) and E11-14:Ac (15). The involvement of each of these materials in the sex pheromone system of *P. flavedana* was investigated further in the field.

Attractancy Tests in the Field

In a preliminary field test carried out in Virginia in 1973,[5] neither E11-14:OH nor Z11-14:OH in rubber septa attracted male *P. flavedana* into traps (total catches of 1 and 0, respectively), but mixtures of the two in which E11-14:OH predominated were quite attractive (total catches of 64–199 moths for *E*:*Z* ratios from 95:5 to 50:50). Addition of an 83:17 mixture (2 and 5 mg) of E11-14:Ac and Z11-14:Ac to an 86:14 mixture (5 mg) of E11-14:OH and Z11-14:OH resulted in much lower trap catches (totals of 7 and 3 moths, respectively) than that obtained with just the alcohols (a total of 78 moths). The acetates alone (5 mg, *E*:*Z* ratio of 83:17) also were unattractive (total catch of two moths). A mixture of the alcohols (5 mg, *E*:*Z* ratio of 86:14) approximating that found in the female extract caught considerably more males when dispensed from a polyethylene cap (total catch of 273 moths) than from a rubber septum (total catch of 78 moths).

A more definitive test was carried out in Pennsylvania in 1974 (Table 1). The greatest trap catch was obtained with 5 mg of an 84:16 mixture of E11-14:OH and Z11-14:OH in a polyethylene cap. Again, neither isomer alone attracted males into the traps, and the corresponding acetates decreased trap catches of the alcohols.

The combined laboratory and field data clearly show that both E11-14:OH and Z11-14:OH are primary sex pheromone components for *P. flavedana*, and that these two compounds are most effective as lures in the field at an *E*:*Z* ratio of, or close to, 84:16. The corresponding acetates do not attract males of this species into traps and, on admixture with the alcohols at the ratios tested, reduce the trap catches obtained with the alcohols alone. Trap catch was reduced even when a relatively low ratio of acetates to alcohols (1:200) was used. Although it is not known whether the acetates are emitted by the females, these data indicate that they probably are not.

[5] Conducted August 3 to October 15, 1973, in Winchester, Virginia, using 5 mg of the 11–14:OH's in rubber septa (5 × 9 mm rubber stopper, sleeve-type, Arthur H. Thomas Co.) or polyethylene caps and 2 or 5 mg of the 11–14:Ac's. There were five replicates of each treatment.

TABLE 1. FIELD ATTRACTION OF MALE *Platynota flavedana* AND *P. idaeusalis* TO *E* AND *Z* MIXTURES OF 11-TETRADECENYL ALCOHOLS AND ACETATES[a]

Mixture[b] 5 mg 11–15:OH's *E*:*Z*		11–14:Ac's *E*:*Z* (mg)	x̄ Males/trap/sample[c] *P. flavedana*	*P. idaeusalis*
2:98			0.0 h	1.1 e
50:50			1.2 g	4.0 d
70:30			10.6 d	6.8 d
80:20			13.6 c	4.4 d
84:16			20.1 a	5.0 d
88:12			16.9 b	4.3 d
92:8			7.0 e	5.2 d
96:4			2.8 f	7.4 d
100:0			0.0 h	24.2 b
100:0	+	100:0 (5)	0.0 h	46.2 a
99:1	+	100:0 (5)	0.0 h	33.6 b
96:4	+	100:0 (5)	0.0 h	32.8 b
92:8	+	100:0 (5)	0.0 h	28.9 b
92:8	+	92:8 (0.025)	3.4 f	13.2 c
Unbaited			0.0 h	0.0 e

[a] Conducted May 30 to June 12, 1974, in Idaville, Pennsylvania. Adults of both species were present throughout this period.
[b] In polyethylene caps.
[c] For each column, means followed by the same letter are not significantly different at the 5% level according to Duncan's new multiple range test. Five replicates; rerandomized 5 times.

Platynota idaeusalis (Walker), known to use E11-14:OH and E11-14:Ac as pheromone components (Hill et al., 1974), also was trapped in the same test (Table 1) in Pennsylvania. Maximum catches were obtained with the known pheromone components, and trap catches diminished with addition of Z11-14:OH or Z11-14:Ac.

DISCUSSION

At present there are three *Platynota* species for which sex pheromone components have been identified: *P. stultana* (Walsingham) (Baker et al., 1975; Hill and Roelofs, 1975), *P. flavedana*, and *P. idaeusalis* (Hill et al., 1974). For *P. stultana*, which is not part of the leafroller complex on apples in the eastern United States, the primary pheromone components are the

11-tetradecenyl acetates in an *E*:*Z* ratio of about 90:10. Interestingly, *P. flavedana* uses approximately the same *E*:*Z* ratio of the corresponding alcohols. All four compounds have been found in the female tip extract of each of these two species. *P. stultana* appears to use small amounts (0.2–2%) of E11-14:OH as a secondary pheromone component along with the primary component acetates (Baker et al., 1975). In both cases, the corresponding functional group analogues of the primary sex pheromone components reduce trap catches when present in large proportions.

P. idaeusalis (tufted apple bud moth) is found sympatrically and synchronically with *P. flavedana* in the northeastern United States. It uses one of the primary sex pheromone components of *P. flavedana* as its primary sex pheromone component (E11-14:OH) and one of the primary sex pheromone components of *P. stultana* as a secondary sex pheromone component (E11-14:Ac). Although *P. flavedana* and *P. idaeusalis* co-occur spatially and temporally and have a primary sex pheromone component in common (E11-14:OH), a high degree of species specificity is achieved by the presence of other sex pheromone components. This is shown by the data in Table 1, where the best blend tested for *P. flavedana* (E11-14:OH/Z11-14:OH, 84:16) did not lure many *P. idaeusalis* males, and the best blend tested for *P. idaeusalis* (E11-14:OH/E11-14:Ac, 1:1) did not capture any *P. flavedana* males. It is evident that the presence of a different second component for each species is very effective in achieving attraction specificity. Similar situations have been documented before (Cardé et al., 1977, and references therein).

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HOST PREFERENCE OF *Pissodes strobi* PECK (COLEOPTERA : CURCULIONIDAE) REARED FROM THREE NATIVE HOSTS[1]

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Abstract—In forced feeding bioassays, *Pissodes strobi* reared from eastern white pine, Engelmann spruce, or Sitka spruce failed to discriminate between these three host species. However, results of choice feeding bioassays suggest that eastern white pine is the ancestral host of the two western *P. strobi* populations. Comparative feeding data obtained under conditions of choice support the hypothesis that *P. strobi* has dispersed westward across the North American continent, adapting its host selection behavior in turn to Engelmann spruce and then Sitka spruce. Whereas *P. strobi* reared from eastern white pine preferred to feed on its natural host rather than on the two western spruces, *P. strobi* reared from Engelmann spruce did not discriminate between eastern white pine and its natural host, Engelmann spruce, but fed significantly more on these two hosts than on Sitka spruce. *P. strobi* reared from Sitka spruce, on the other hand, fed equally on all three native host species. Our data support the contention of Smith and Sugden (1969) that the two western weevils are ecotypes of *P. strobi*. Comprehensive research on behavioral adaptations to new host species may allow the three *P. strobi* populations to be distinguished as legitimate ethospecies.

Key Words—*Pissodes strobi*, ecotype, phytophagy, speciation, host selection, feeding bioassay, weevil, dispersal, chemoreception, taxonomy.

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[2] Graduate student, Professor, and Post-Doctoral Fellow, respectively.

INTRODUCTION

The white pine weevil, *Pissodes strobi* (Peck), is a Nearctic species first described from the leading shoot of "Weymouth pine," *Pinus strobus* L. (Peck, 1817). Currently recognized as a major pest throughout the range of eastern white pine [(Fig. 1), Fowells, 1965] from the Atlantic Coast to

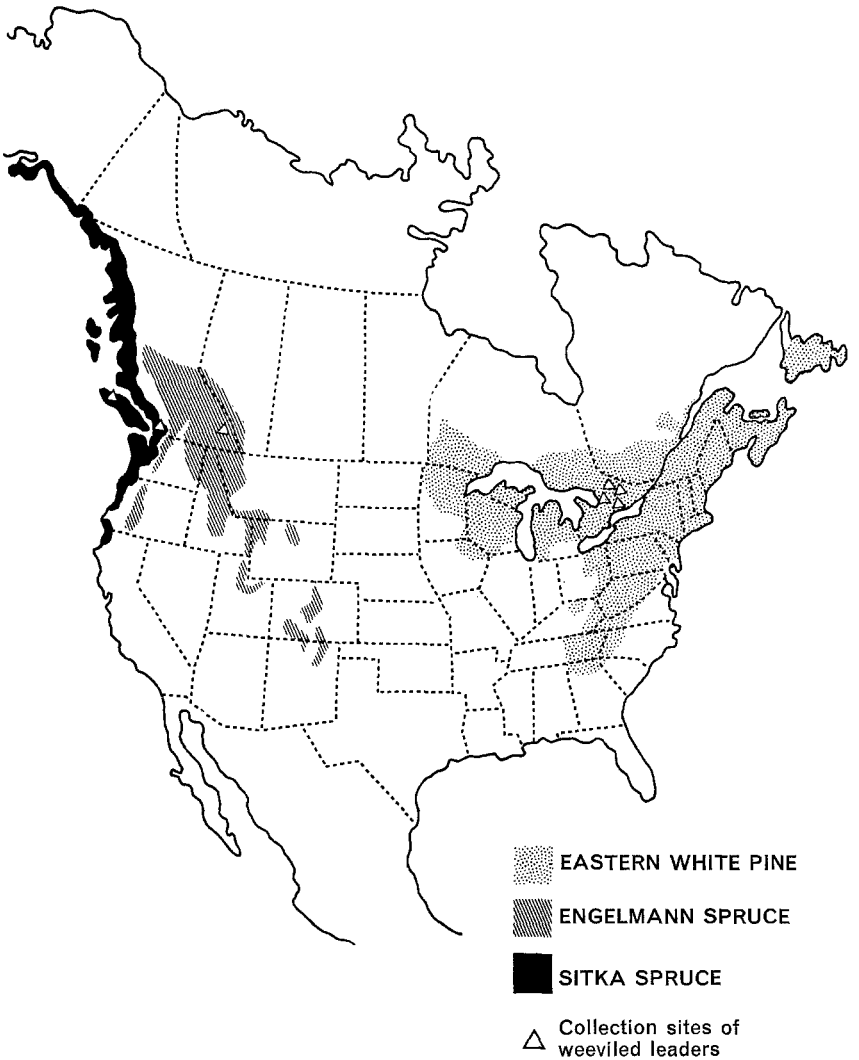


FIG. 1. Geographical distribution of three preferred host conifers of three *Pissodes strobi* populations. Collection sites of weeviled leaders of each of the three host species are indicated.

TABLE 1. NATIVE AND EXOTIC HOST SPECIES COMMONLY REPORTED FOR THE THREE POPULATIONS OF *P. strobi*

Host species	*P. strobi* (ex EWP)	*P. strobi* (ex ES)	*P. strobi* (ex SS)
Pinus strobus L.	x[a]		
P. sylvestris L.[b]	x		
P. banksiana Lamb.	x		
P. resinosa Ait.	x		
P. rigida Mill.	x		
P. pungens Michx.	x		
P. contorta Dougl.		x	
Picea abies (L.) Karst.[b]	x	x	x
P. glauca (Moench) Voss	x	x	x
P. mariana (Mill.) BSP	x	x	x
P. pungens Engelm.	x	x	x
P. rubens Sarg.	x		
P. sitchensis (Bong.) Carr			x[a]
P. engelmannii Parry		x[a]	x
P. glauca (Moench) Voss var. *albertiana* (S. Brown) Sarg.[c]		x	
Picea × *lutzii* Little[d]			x

[a] Preferred natural host species.
[b] Exotic species.
[c] Natural hybrid between *P. engelmannii* and *P. glauca*.
[d] Natural hybrid between *P. sitchensis* and *P. glauca*.

southeastern Manitoba, the weevil has been instrumental in the curtailment of reforestation programs (Belyea and Sullivan, 1956). Two western weevil populations that are damaging to *Picea engelmannii* Parry regeneration (Fig. 1) in the interior mountains (Stevenson, 1967), and to *Picea sitchensis* (Bong.) Carr. plantations (Fig. 1) on the Pacific Coast (Silver, 1968), were both accorded species status by Hopkins (1911) on the basis of his host-selection principle.

In studies on the taxonomic status of the genus *Pissodes* Germar, Manna and Smith (1959) and Smith (1962) were unable to distinguish Hopkins' (1911) species *engelmanni* and *sitchensis* from *P. strobi* on the basis of morphological or cytogenetic evidence. Accordingly, Smith and Sugden (1969) concluded that the two western weevil populations were in fact geographic races or ecotypes of *P. strobi*.[3]

Table 1 lists the host trees that are commonly reported for the three

[3] In this paper, the three weevil populations are identified by the host from which they emerged as follows: from eastern white pine (ex EWP), from Engelmann spruce (ex ES), from Sitka spruce (ex SS).

P. strobi populations (Stevenson, 1967; Mitchell, Johnson, and Wright, 1974; Belyea and Sullivan, 1956; Smith and Sugden, 1969). All host species possess vigorous erect terminals.

The behavioral ecology of the three populations of *P. strobi* on their respective hosts is essentially identical (Belyea and Sullivan, 1956; Stevenson, 1967; Silver, 1968). Females oviposit in spring in fresh feeding cavities excavated in the upper bark of vigorous 1-year-old leaders, preferring the more dominant trees in open grown, even-aged stands. *P. strobi* (ex ES), however, may oviposit in the upper portions of the leaders of the two preceding years, as well as in the 1-year-old leader (T.J.D. VanderSar, unpublished observation), or below the previous year's killed leader (L.H. McMullen,[4] personal communication). *P. strobi* (ex SS) may also oviposit in the 2-year-old leader when the needle growth is very dense on the 1-year-old leader of a suppressed tree (T.J.D. VanderSar, unpublished observation). After hatching, the larvae mine downward in the cambial zone, forming a "feeding ring" that results in the girdling and death of the leader. Following pupation beneath the thin outer bark, in the xylem or pith, the new adults emerge in August and September to feed on the living bark below the dead leader. Some flight dispersal to new hosts may occur in autumn. With the advent of unfavorable weather in November, the adults move to the base of the host trees and overwinter in the duff (Belyea and Sullivan, 1956; Stevenson, 1967), or on Sitka spruce, which is not subjected to a severe winter, remain on the lateral branches and upper bole (Gara et al., 1971; McMullen and Condrashoff, 1973).

Our objectives were: (1) to test the host specificity of the three weevil populations on each of the three preferred natural hosts using forced and choice feeding bioassays, and (2) to assess the revised taxonomic status of the former *P. engelmanni* and *P. sitchensis* on the basis of their feeding preferences relative to *P. strobi*.

METHODS AND MATERIALS

New-generation adult weevils were reared from naturally infested host material as described by VanderSar and Borden (1977). Infested Sitka spruce leaders were collected in mid-July, 1975, from three localities: the Kelsey Bay region of northern Vancouver Island; the University of British Columbia Research Forest, Maple Ridge, B.C.; and the District of Mission Municipal Forest, Mission, B.C. Weeviled Engelmann spruce leaders were collected in late August from natural regeneration in Kootenay National Park, B.C. Infested eastern white pine terminals were collected from plantations and

[4] Pacific Forest Research Centre, Environment Canada, Victoria, B.C.

natural regeneration in several southeastern Ontario localities. Collection sites are indicated in Fig. 1. Owing to the proximity of the Maple Ridge and Mission, B.C., sites, however, a single triangle denotes these two collection areas of weeviled Sitka spruce leaders.

For both the forced and choice feeding experiments, the methods and evaluation procedures described by VanderSar and Borden (1977) were utilized, except for modifications as noted. Current-year Sitka spruce and eastern white pine lateral branches were obtained from plantations in the University of British Columbia Research Forest, Maple Ridge, B.C. Engelmann spruce lateral branches were clipped from natural regeneration in Manning Provincial Park, B.C., and in the Merritt region of interior British Columbia. The branches were brought to the laboratory on the day of collection and stored at 0–4°C. All bioassays were conducted within 1–2 days of field collection of the lateral branches.

In the forced feeding bioassays, individual weevils were presented a single lateral branch section of one of the three preferred natural hosts. These experiments were conducted on 3 different days over a 3-week period in September–October, 1975. On each day, four replicates for each sex of each weevil population on each of the three host species, *Pinus strobus*, *Picea engelmannii*, and *P. sitchensis*, were completed. Each replicate tested a lateral branch section from a different tree, such that variability in the host–insect interaction was maximized. After 24 h, the feeding punctures initiated on each host lateral branch section were counted, and the weight of feces determined for each replicate.

In the choice feeding bioassays, each weevil was presented a choice of three 4-cm lateral branch sections, one each of eastern white pine, Engelmann spruce, and Sitka spruce. Each replicate consisted of a 448-cm^3 glass jar inverted over a filter paper floor on which rested the three branch sections. Thirty replicates were used for each sex of each weevil population. The choice bioassay was conducted in two parts: 18 replicates were completed on October 16, and an additional 12 replicates on December 8, 1975. At intervals during the choice bioassays, the position of each weevil in the apparatus was recorded. After 24 h, the number of feeding punctures on each host lateral branch section was counted.

In the forced feeding bioassays, analysis of variance (ANOVA) was performed on both the feeding puncture and feces weight data. Separate ANOVA on the number of feeding punctures initiated on each host species in the October 16 and December 8 choice bioassays indicated that differences between groups were not significant. Therefore, the feeding puncture data for all 30 replicates were combined in a single multivariate analysis of variance (MANOVA) using the numbers of feeding punctures produced on each of the three host species as variables. Hypothesis (H) and error (E) cross-product matrices were calculated using computer program BMD12V. Greatest

characteristic roots of each HE^{-1} matrix were determined using computer program SFU-EIGENP. Critical values were subsequently calculated and compared to Heck charts. After carrying out a profile analysis, multiple comparisons were made to determine significant differences between numbers of feeding punctures produced by each weevil population on each of the three host conifer species (Harris, 1975; Morrison, 1976).

To assess one population variable that may have influenced observed differences in feeding rates, 25 weevils of each sex of each weevil population were weighed on February 2, 1976. Prior to weighing, all weevils had been maintained at 2°C on lateral branch sections of their respective natural hosts. An analysis of variance was performed on the body weight data.

RESULTS

All three *P. strobi* populations initiated shallow and deep bark punctures on eastern white pine, in addition to feeding cavities in the needles, needle fascicles, and needle traces of this host. On both spruce species, however, nearly all feeding punctures were deeper bark cavities initiated on the sterigmatal ridges, and needle punctures were rarely observed. This between-host variability in the location and depth of feeding punctures may be related to the depth of inside and outside cortical resin canals (Stroh and Gerhold, 1966). Since eastern white pine resin is repellent to *P. strobi* (Anderson and Fisher, 1956), shallow punctures contacting the resin ducts may be abandoned. Such behavior has also been observed in *P. strobi* feeding on Sitka spruce (Overhulser, 1973).

Each feeding puncture is considered to represent the biting response that initiates feeding on a given host species (VanderSar and Borden, 1977). For this reason, an analysis of variance was performed on the total number of feeding punctures. However, the disparity in location and relative depth of feeding punctures both within and between host species precludes a simple correlation of numbers of punctures with the amount of food ingested. For this reason, we hypothesize that fecal weight is a more accurate and reliable measure of actual food intake. It was not feasible to measure this additional variable in the choice bioassays, however, as 19.4% of the weevils produced punctures on more than one host lateral branch section.

Forced Feeding Bioassay

In the forced feeding bioassay, analysis of variance indicated no significant differences in the number of feeding cavities initiated on each of the three host species by all three *P. strobi* populations combined, and no sig-

TABLE 2. FEEDING PUNCTURES PRODUCED BY PREWINTERING WEEVILS OF THREE *Pissodes strobi* POPULATIONS ON CURRENT-YEAR, 4-CM LATERAL BRANCH SECTIONS OF THREE PREFERRED HOSTS PRESENTED SINGLY DURING A 24-H PERIOD[a]

Weevil population	No. of feeding punctures initiated by males			No. of feeding punctures initiated by females			Total[b]
	Eastern white pine	Engelmann spruce	Sitka spruce	Eastern white pine	Engelmann spruce	Sitka spruce	
P. strobi (ex EWP)	106	80	64	119	105	121	595 a
P. strobi (ex ES)	93	51	57	72	56	83	412 b
P. strobi (ex SS)	68	46	83	75	63	78	413 b

[a] One weevil per replicate; 12 replicates for each sex of each *Pissodes* population on each host species.
[b] Totals followed by the same letter not significantly different (Newman–Keuls test, $P < 0.05$).

TABLE 3. WEIGHT OF FECES PRODUCED BY PREWINTERING WEEVILS OF THREE *Pissodes strobi* POPULATIONS FEEDING ON CURRENT-YEAR, 4-CM LATERAL BRANCH SECTIONS OF THREE PREFERRED HOSTS PRESENTED SINGLY FOR 24 H[a]

	Wt. of feces produced on eastern white pine (mg)			Wt. of feces produced on Engelmann spruce (mg)			Wt. of feces produced on Sitka spruce (mg)			
Sex	*P. strobi* (ex EWP)	*P. strobi* (ex ES)	*P. strobi* (ex SS)	*P. strobi* (ex EWP)	*P. strobi* (ex ES)	*P. strobi* (ex SS)	*P. strobi* (ex EWP)	*P. strobi* (ex ES)	*P. strobi* (ex SS)	Total[b]
♀♀	3.484	2.113	3.422	3.735	4.362	4.695	3.868	3.829	4.324	33.832
♂♂	2.520	3.303	2.834	2.956	3.254	3.114	2.082	2.106	3.505	25.674

[a] One weevil per replicate; 12 replicates for each sex of each *Pissodes* population on each host species.
[b] Totals significantly different, ANOVA, $P<0.01$.

nificant effect caused by weevil sex. However, *P. strobi* (ex EWP) initiated significantly more feeding cavities on the three host species combined than did either *P. strobi* (ex ES) or *P. strobi* (ex SS) (Table 2).

The feces weight ANOVA indicated there were no significant differences in the weight of feces produced on each of the three host species by all three weevil populations combined, and no significant effect caused by beetle population. Female weevils, however, produced significantly more feces feeding on the three hosts combined than did the males (Table 3).

Choice Feeding Bioassays

The results of the MANOVA indicated a significant weevil population effect ($P<0.01$), but no significant sex effect or beetle by sex interaction. In addition, the parallelism, flatness, and levels hypotheses were rejected ($P<0.01$), indicating significantly different feeding rates on the three host species by each beetle population, differential feeding on each of the three host species, and differential feeding by each beetle population, respectively. Multiple comparisons between the means of each weevil population feeding on the three host species showed that *P. strobi* (ex EWP) produced significantly more feeding punctures on eastern white pine than on Engelmann

TABLE 4. MEAN NUMBER OF FEEDING PUNCTURES PRODUCED ON EACH HOST BY PREWINTERING WEEVILS OF THREE *Pissodes strobi* POPULATIONS WHEN PRESENTED WITH A CHOICE OF THREE 4-CM HOST LATERAL BRANCH SECTIONS, ONE EACH OF EASTERN WHITE PINE, ENGELMANN SPRUCE, AND SITKA SPRUCE, FOR 24 H[a]

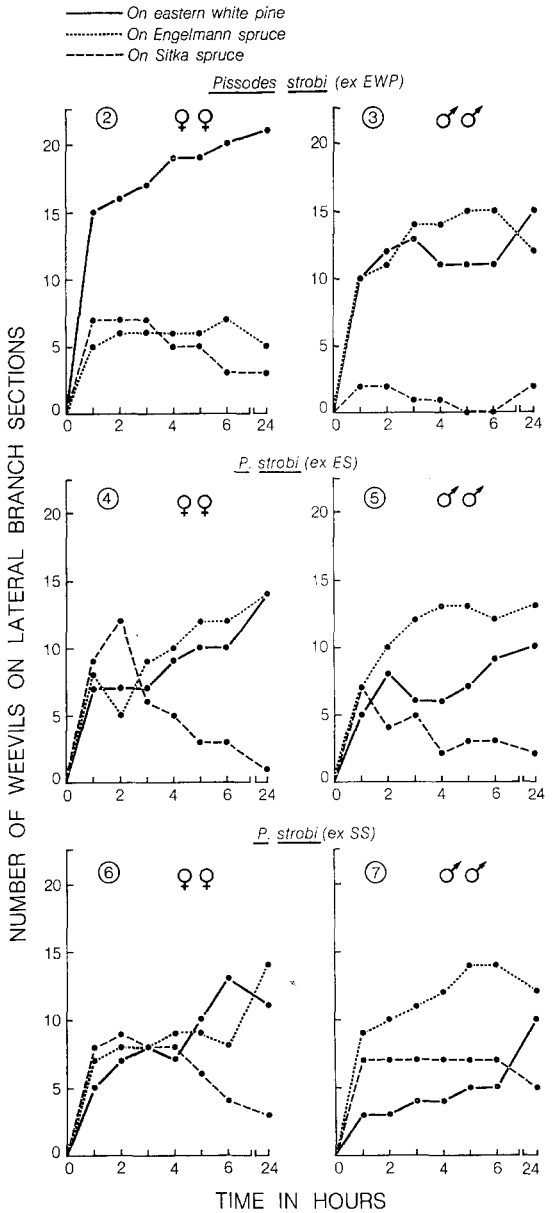
Host species	Weevil population[b]			Mean no. of feeding punctures produced on each host species[c]
	P. strobi (ex EWP)	*P. strobi* (ex ES)	*P. strobi* (ex SS)	
Eastern white pine	8.45 a	4.10 a	2.60 a	5.05
Engelmann spruce	3.60 b	2.43 a	1.88 a	2.64
Sitka spruce	0.43 c	0.42 b	1.13 a	0.66
Mean no. of feeding punctures produced per weevil in each population[d]	4.16	2.32	1.87	

[a] One weevil per replicate; 30 replicates for each sex of each population on the three-host combination.

[b] Differences between means in the same column followed by same letter not significant (MANOVA, $P<0.05$).

[c] Flatness hypothesis rejected (MANOVA, Profile Analysis, $P<0.01$).

[d] Levels hypothesis rejected (MANOVA, Profile Analysis, $P<0.01$).



FIGS. 2–7. Position of prewintering adults of three *Pissodes strobi* populations when presented a choice of three 4-cm host lateral branch sections, one each of eastern white pine, Engelmann spruce, and Sitka spruce, for 24 h. One weevil per replicate; 30 replicates for each sex of each weevil population on the three-host combination.

spruce and significantly more punctures on the latter host than on Sitka spruce (Table 4). While the difference between mean number of feeding punctures produced on eastern white pine and Engelmann spruce by *P. strobi* (ex ES) was not significant (Table 4), significantly fewer feeding cavities were produced on Sitka spruce than on either of the other two hosts. *P. strobi* (ex SS) did not show any preference for any of the three host species (Table 4).

When the mean numbers of feeding punctures produced on each host species were compared, *P. strobi* (ex EWP) produced significantly more punctures on its natural host, eastern white pine, than did the two western weevil populations combined ($P<0.05$). Although the differences between mean numbers of feeding punctures produced on Sitka spruce were not significant, *P. strobi* (ex SS) tended to produce more feeding cavities on its natural host, Sitka spruce, than did either *P. strobi* (ex EWP) or *P. strobi* (ex ES). In addition, the mean number of feeding punctures produced on Engelmann spruce by *P. strobi* (ex ES) was not significantly different from the mean number of feeding punctures produced on this host by either *P. strobi* (ex EWP) or *P. strobi* (ex SS). The mean number of feeding cavities produced on Engelmann spruce by *P. strobi* (ex EWP), was, however, significantly greater than the number of feeding punctures initiated on this host by *P. strobi* (ex SS).

Weevil positions during the 24-h test (Figs. 2–7) reflect the results shown in Table 4. All three host species were contacted by weevils of both sexes soon after the start of each bioassay. However, after 24 h, the majority of weevils of all three populations were found in contact with either eastern white pine or Engelmann spruce, rather than with Sitka spruce. The greatest incidence of weevils in contact with Sitka spruce after 24 h was 14.5% of *P. strobi* (ex SS).

The body weight of female weevils was significantly greater than that

TABLE 5. MEAN BODY WEIGHT FOR WEEVILS OF THREE *Pissodes strobi* POPULATIONS; $N = 25$ IN EACH CATEGORY

Weevil population	Mean body weight ± SD (mg)		
	Females	Males	Both sexes combined[a]
P. strobi (ex EWP)	9.266 ± 1.483	8.938 ± 1.745	9.102 ± 1.611 a
P. strobi (ex ES)	9.311 ± 1.707	7.852 ± 1.598	8.581 ± 1.795 a
P. strobi (ex SS)	10.087 ± 1.857	9.136 ± 1.916	9.611 ± 1.928 b
Three populations combined[b]	9.554 ± 1.709	8.642 ± 1.825	

[a] Figures followed by same letter not significantly different (Newman–Keuls, $P<0.01$).
[b] Females significantly heavier than males (ANOVA, $P<0.01$).

of males (Table 5). In addition, the weight of the *P. strobi* (ex SS) population was significantly greater than the body weight of either the *P. strobi* (ex EWP) or the *P. strobi* (ex ES) populations (Table 5).

DISCUSSION

Significantly more feeding by female weevils in the forced feeding bioassay (Table 3), is partially attributed to their significantly greater body weight (Table 5). It may also be related to the physiological requirements of ovary maturation prior to spring dispersal. The significantly greater number of feeding punctures initiated by *P. strobi* (ex EWP) on all three host species combined in the forced feeding bioassays may reflect our observation that this population did not feed extensively on its native host in holding jars and, therefore, were more strongly stimulated to feed. However, all weevils were conditioned equally by deprivation of food for 24 h prior to each experiment. Moreover, food ingestion (as measured by feces weight) by *P. strobi* (ex EWP) was approximately equal to that of the remaining two populations (Table 3), suggesting similar satiation levels.

The lack of discrimination between hosts in the forced feeding bioassay indicates that all three host species are acceptable to all three *P. strobi* populations. In choice feeding bioassays, however, the failure of the two western *P. strobi* populations to discriminate between their respective natural hosts (i.e., the conifer species from which they emerged) and eastern white pine (Table 4) suggests that the latter conifer is their ancestral host. Since *P. strobi* (ex EWP) prefers to feed on eastern white pine, *P. strobi* (ex ES) prefers to feed on both eastern white pine and Engelmann spruce, and *P. strobi* (ex SS) feeds on all three host species equally, these results suggest that *P. strobi* dispersed west across the North American continent, probably utilizing an alternate host species such as *P. glauca*. White spruce is sympatric with both Engelmann spruce and Sitka spruce in central and northwestern British Columbia, and hybridizes readily with both species (Roche, 1969). Engelmann and Sitka spruces, however, are allopatric species.

In retrospect, Hopkins (1911) was premature in according species status to the two western weevil populations on the basis of his host-selection principle. Our results support Smith and Sugden's (1969) contention that the three *P. strobi* populations are ecotypes or geographic races. Since the *P. strobi* populations are undergoing speciation that is reinforced by reproductive isolation on their respective preferred natural hosts whose ranges do not overlap [(Fig. 1), Fowells, 1965], behavioral adaptation leading to pronounced differences in host preferences may, with further analysis, allow the three populations to be distinguished as valid ethospecies.

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USING WOOD EXTRACTS TO DETERMINE THE FEEDING PREFERENCES OF THE WESTERN DRYWOOD TERMITE, *Incisitermes minor* (HAGEN)

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Abstract—Nymphs of the western drywood termite, *Incisitermes minor* (Hagen), were tested for their feeding preferences on wood and extracts of 11 tree species. The amount of wood consumed was inversely proportional to its specific gravity, but methanol extracts of least preferred woods were also least preferred when termites were confined to paper treated with extracts. In choice tests, only paper treated with extract of wood from which the colony developed and untreated and methanol-treated controls were significantly fed upon. Ponderosa pine extract applied to Douglas fir and sugar pine significantly decreased the amount of wood consumed. Resistance to termite feeding appeared to depend on the presence of repellent chemicals in the wood.

Key Words—feeding deterrents, feeding preferences, western drywood termite, *Incisitermes minor*, Kalotermitidae.

INTRODUCTION

The western drywood termite, *Incisitermes minor* (Hagen), has been found occurring naturally in eucalyptus, willow, sycamore, oak, alder, Monterey cypress, redwood, California laurel, buckeye, peach, pear, almond, walnut, and cherry (Light, 1934), but the frequency of these infestations is unknown. Drywood termites are also responsible for millions of dollars of damage to buildings and structural lumber each year. Nearly 80% of the termite inspection reports filed in California during 1965 indicated damage by drywood termites.

There have been several studies that indicate that drywood termites have feeding preferences. In laboratory tests, Williams (1934) showed that the heartwoods of ponderosa pine and redwood were more resistant to termite feeding than were their sapwoods and that the heartwoods contained more soluble extractives. He also found the following feeding preferences of *I. minor* on various heartwoods: western red cedar > Douglas fir > redwood > cypress > Port Orford cedar. Reierson (1966), in choice feeding tests with the same termite, reported the following preferences for sapwood: cedar > Douglas fir plywood > birch > mahogany > Douglas fir = oak = pine = redwood > walnut.

Several field observations also suggest that certain woods are preferred over others. Kofoid (1934) reported that, after decades of weathering and exposure, a natural degradation or removal of water-soluble components from redwood was the possible reason for termite attack.

This paper deals with the feeding preferences of *I. minor* toward several kinds of woods and the effect of chemical extracts from these woods on feeding preferences.

METHODS AND MATERIALS

Animal Maintenance

Nymphs of *I. minor* were removed from infested walnut logs by sawing the logs into 0.3-m sections and splitting the sections into smaller pieces with a metal wedge. The sections and pieces were lightly tapped with hammers, and the nymphs that fell out were collected in shallow porcelain or stainless steel trays. Termites were removed from the wood chips and debris by spreading paper towels over the chips and gently placing weight on the towels. A camel-hair brush was used every 15–20 min to brush nymphs adhering to the towels into covered 9-cm-diameter glass petri dishes provided with ~10 paper towel disks and several small walnut wood chips. Several hundred nymphs and 10–15 soldiers were placed into each dish. The termites were maintained at 22.7 ± 1.2°C and $60 \pm 5\%$ RH for at least 3 days prior to testing.

Determination of Specific Gravity and Moisture Content of Wood

Specific Gravity. The specific gravity (mg/cm^3) of each wood sample was calculated at room conditions, 22.7 ± 1.2°C and $60 \pm 5\%$ RH. Eleven types of wood were tested: Douglas fir, *Pseudotsuga menziesii*; koa, *Acacia koa*; ponderosa pine, *Pinus ponderosa*; red alder, *Alnus rubra*; red cedar, *Juniperus virginiana*; red oak, *Quercus* spp.; redwood, *Sequoia sempervirens*; sugar pine,

Pinus lambertiana; teak, *Tectona grandis*; walnut, *Juglans californica*; and western red cedar, *Thuja plicata* (nomenclature as reported by Harlow and Harrar, 1968). The woods, except walnut, were secured from a local lumberyard, and all the woods except walnut and ponderosa pine had been kiln dried. Three fragments of each wood sample ($\sim 1 \times 2 \times 2$ cm) were carefully weighed. The volume of each fragment was determined with a balance beam apparatus that measures water displacement (Brown *et al.*, 1952). This device was used to provide an assessment of the volume of the sample by determining the difference in weight (mg) of a vessel of water before and after the wood sample was submerged. The specific gravity was then calculated by dividing the initial weight of the sample by the weight of displaced water.

Moisture Content. Several pieces of wood placed into an oven were heated at 100°C until continued loss of weight of each sample was negligible. The reduction in weight due to heating divided by the initial weight represented the approximate percentage moisture content of the wood.

Feeding Tests on Wood

Pieces of lumber and logs were cut into ~0.1–0.3-m sections, and a flat surface was sanded smooth and clean. Since termites were unable easily to right themselves on glass surfaces and Reierson (1966) reported no feeding damage to masonite surfaces, control tests were conducted on 1.27-cm-thick masonite surfaces. A minimum of three replicates of 10 fourth through seventh instar nymphal termites was placed under inverted glass stendor dishes (3.0 cm ID) on the surface of each board. After 13 days, the termites were removed and the number of surviving termites recorded. The amount of feeding on each sample was determined by inserting modeling clay into all the grazing areas, cracks, pits, and tunnels formed by the feeding termites. The clay was then carefully removed and weighed (Reierson, 1966).

The volume of wood consumed by the termites was calculated by dividing the number of milligrams clay removed from the wood by the specific gravity of the clay (98.2 mg/cm^3). The weight (mg) of wood consumed was calculated by multiplying the consumed volume by the specific gravity of each wood. The amount of feeding and the specific gravity of each wood were analyzed using a Kendall's coefficient of rank correlation test (Conover, 1971) to determine whether any relationship existed between these two variables. The amount of wood consumed was analyzed using an analysis of variance, and the mean amounts were further analyzed using the Student–Newman–Kuels (SNK) test (Sokal and Rohlf, 1969).

Preparation of Wood Extracts and Feeding Tests

Approximately 70–80 g of wood chips, sawdust, and shavings obtained

from each wood were bathed for 4–5 h in 300–500 ml of methanol heated to 45°C. The methanol–wood extract was filtered and concentrated. All solutions were stored at −5°C.

Crown Contract® natural paper towels cut into 9-cm-diameter disks were evenly treated with 2.0 ml of methanol or 0.5–3.0 ml of each wood extract applied dropwise from a 1-ml pipette. Several treated disks were left uncut while others were cut into two or eight equal sectors. Treated sectors were joined to untreated sectors with a narrow piece of transparent adhesive tape. Random combinations of eight small sectors were joined in a similar fashion. The recombined test disks were fastened (with the taped junctions down) with a peripheral band of hot melted paraffin to the bottom of covered 9-cm-diameter petri dishes.

For each evaluation, 15 termites were placed into each petri dish. A minimum of five replicates of each particular combination were tested. Mortality was recorded every 2–3 days, and the termites were removed from the dishes on day 13.

Calculation of Feeding Preference

The recombined and intact disks were carefully removed from the petri dishes and the excess paraffin was scraped away with a razor blade. Each disk was photocopied on a black background so that areas where feeding had occurred appeared as dark spots. The outline of these areas was obtained on tracing paper, and the area was determined by carefully fitting the areas with as many appropriately sized circular dots as possible from a Paratipe® lettering sheet (55068) or a Staedther-Mars® general-purpose template 102 GP. The diameter of dots and circles ranged from 0.079 cm (0.00495 cm^2) to 1.111 cm (0.96987 cm^2), and the combined area of the dots used to fill each area was tabulated.

The choice feeding tests were analyzed by determining the total area consumed for the eight choices and calculating the percentage of feeding on each choice. A k-sample slippage test was performed on the data for each of the aged deposits (Conover, 1971).

RESULTS AND DISCUSSION

When drywood termites were confined to various wood blocks, their feeding propensity, as indicated by the mean amount of wood consumed, was as follows: Douglas fir > walnut > sugar pine = ponderosa pine = redwood = red oak = koa = red alder = western red cedar > red cedar = teak = masonite (Table 1). Significantly, more milligrams of Douglas fir were con-

TABLE 1. FEEDING AND MORTALITY OF DRYWOOD TERMITES CONFINED TO WOOD BLOCKS

Wood	Density (mg/cm³)	% Moisture	X̄ Consumption[a]			X̄ Live
			cm³ (×10⁻³)	Water (mg)	Wood (mg)[b]	
Douglas fir	594	8.2	111.9	5.45	66.48 c	9.0 bc
Koa	670	7.1	11.9	0.57	7.98 ab	7.3 abc
Ponderosa pine	493	9.2	20.4	0.92	10.04 ab	9.3 bc
Red alder	644	8.0	17.0	0.88	10.95 ab	6.7 abc
Red cedar	648	10.5	1.3	0.09	0.86 a	6.2 abc
Red oak	670	6.5	20.7	0.90	13.85 ab	7.7 abc
Redwood	544	9.0	45.4	2.22	24.70 ab	9.7 c
Sugar pine	350	7.2	69.2	1.75	24.24 ab	6.4 abc
Teak	626	7.2	2.5	0.11	1.59 a	4.3 ab
Walnut	489	7.6	65.1	2.42	31.82 b	10.0 c
Western red cedar	559	7.1	14.6	0.58	8.14 ab	9.7 c
Masonite	1149	4.8	1.0	0.06	1.17 a	3.7 a

[a] A minimum of three replicates each with 10 termites for 13 days; 30±1°C, 80±2% RH.
[b] Means followed by the same letter within columns are not significantly different at the 5% level of probability with the SNK test.

sumed than walnut cut from the logs in which the termites had been collected. Physical factors of wood such as density could affect the feeding of termites. There was a significant correlation between increasing specific gravity of the wood and decreasing amounts of wood consumed by the termites ($0.02 < P < 0.05$, $\tau = 0.4545$). At 13 days, the mortality was significantly higher in those groups of termites confined to masonite or teak than in those that fed on western red cedar, redwood or walnut. Termites exposed to red cedar consumed an average of only 0.86 mg but had significantly fewer mortalities than in trials with teak or masonite. It appeared, therefore, that a chemical factor in teak and masonite was an important factor influencing mortality.

Chemical extracts were prepared to eliminate possible effects of physical characteristics of each wood. In tests in which termites were continuously exposed to wood extracts, the following order of feeding propensity was found: untreated control = methanol-treated control = walnut > Douglas fir > sugar pine = redwood = red oak = western red cedar = red alder > koa = red cedar = teak = ponderosa pine (Table 2). Generally, there was a slight increase in mortality of those termites exposed to the least-preferred wood extracts, and there was a direct correlation ($\tau = 0.7818$, $P < 0.001$)

TABLE 2. EFFECT OF WOOD EXTRACTS ON THE FEEDING AND MORTALITY OF DRYWOOD TERMITES IN CHOICE TESTS WITH EXTRACT-TREATED PAPER

	Forced[a]			Choice[b]		
Extract	Rate ($\mu g/cm^2$)	Total % s.a. (consumed)	% (dead)	Total % s.a. (consumed)	% feeding (on extract)[c]	% (dead)
Douglas fir	580	2.77 b	6.7	4.00	0.0	13.3
Koa	630	0.39 a	22.2	1.07	2.2	24.4
Ponderosa pine	1160	0.24 a	2.2	5.13	0.0	13.3
Red alder	630	1.70 ab	17.8	1.44	26.0	22.2
Red cedar	630	0.31 a	17.8	1.30	2.4	33.3
Red oak	630	1.57 ab	24.4	1.48	8.6	13.3
Redwood	1160	1.63 ab	6.7	5.89	14.6	13.3
Sugar pine	640	2.17 ab	4.4	6.54	1.4	11.1
Teak	630	0.08 a	17.8	1.02	4.1	28.9
Walnut	1170	4.51 c	4.4	5.63	96.9	6.7
Western red cedar	580	1.49 ab	13.3	6.91	4.7	8.9
Methyl OH	—	5.14 c	0.0	4.72	64.6	11.1
Untreated	—	6.10 c	8.8	4.25	31.9	13.3

[a] Paper disks treated with extract. Surface area (s.a.) of each disk ~ 63.6 cm^2.
[b] Half of disk untreated, half of disk treated with extract of indicated wood.
[c] Of the total s.a. consumed, the percentage that occurred on extractive portion.

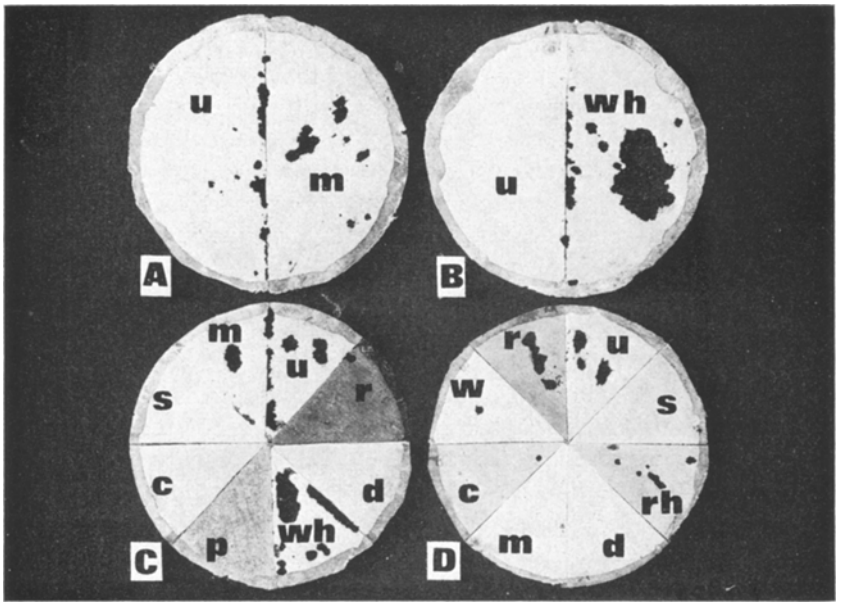

FIG. 1. Choice tests with paper treated with extracts of various woods showing areas consumed by 10 drywood termites. Choices shown include a check (A), trials with walnut homewood (B, C), and trials with redwood homewood (D). Extracts are untreated (u), methanol check (m), Douglas fir (d), suger pine (s), ponderosa pine (p), walnut (w), walnut homewood (wh), cedar (c), redwood (r), and redwood homewood (rh). Tests terminated at day 13.

between the amount of wood consumed and the amount of wood-extract-treated toweling consumed (Table 2).

In two-choice tests with untreated and methanol-treated disks as controls, an average of 48.25% (range 31.9–64.6%) of the feeding occurred on the portion of the disk designated as a control (Fig. 1A, B). Although a slightly higher mortality rate than that of the control was observed in several choice tests and there was increased consumption of paper treated with extracts of western red cedar, Douglas fir, sugar pine, and ponderosa pine, less than 5% of the total amount of feeding occurred on the extractive portions (Table 2). This indicated that, given a choice, the termites would readily avoid the extract of woods, except walnut, in favor of a more preferred substrate. In two-choice tests with walnut extract, nearly all feeding occurred on the extractive portion.

At day 13, highest mortality was attained in trials with red alder, koa, red cedar, and teak even when the termites had an untreated surface on which to feed. This indicated the possible presence of toxic and/or antifeedant chemicals in these woods. In tests with redwood or ponderosa pine, however,

a great increase in feeding activity without inordinate mortality indicated the absence of a very toxic fraction.

When the number of choices was increased to include eight equal, treated sectors (Fig. 1C, D), the results were similar to those obtained in the two-choice tests. Most (65.8%) of the feeding occurred on the walnut-extract paper, which constituted only 12% of the total paper available. Both controls totaled 22.12% of the total feeding, while paper with extracts of redwood (7.9%), ponderosa pine, western red cedar, Douglas fir, and sugar pine accounted for a total of only 12.08%.

Another group of termites removed from a 12-year-old redwood board was also given an eight-choice test with one sector containing an extract of their homewood. In this test, 70.4% of the feeding occurred on redwood and homewood extracts, and 23.63% on the control sectors. These results are similar to those found by McMahan (1966), where the powder post termite, *Cryptotermes brevis* (Walker), strongly preferred woods on which they had been accustomed to feeding. Unlike termites of other families, the nymphs of Kalotermitidae develop into reproductive alates. Therefore, feeding preferences developed during the nymphal state may be extremely important in host wood site selection of swarming queens. When Douglas fir and sugar pine boards were treated with ponderosa pine extract, there was a 55.5 and 39.3% reduction in the amount of feeding on the Douglas fir and sugar pine in six trials, respectively. The general feeding pattern changed considerably with the addition of the ponderosa pine extract. Instead of grazing shallow troughs in the springwood as in controls, termites exposed to the ponderosa pine extract excavated deeper tunnels through the treated sugar pine surface. Presently, the ponderosa pine, red cedar, and several exotic woods are being chemically characterized to identify potential feeding deterrents.

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THE DEFENSIVE GLAND OF OMALIINAE (COLEOPTERA : STAPHYLINIDAE)

I. Gross Morphology of the Gland and Identification of the Scent of *Eusphalerum longipenne* Erichson

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Abstract—A description is given of the abdominal defensive gland of *Eusphalerum longipenne* Erichson. Through the aid of GLC and TLC, the two major compounds of its secretion are identified as 3-methylbutyric acid and *trans*-hex-2-enal.

Key Words—*Eusphalerum longipenne*, Staphylinidae, abdominal defensive gland, gross morphology, 3-methylbutyric acid, *trans*-hex-2-enal.

INTRODUCTION

When disturbed, many staphylinid beetles are known to release a secretion from their posterior. The secretion is produced and stored in certain glands, which are usually situated dorsally near the tip of the beetle's abdomen. There are several different types of glands, which cannot be regarded as homologous (Dierckx, 1899, 1901; Berger, 1968; Jenkins, 1957; Happ and Happ, 1973; Araujo, 1973; Jordan, 1913; Pasteels 1968a, 1968b).

The glands contain quite a variety of compounds including quinones, lactones, amines, ketones, aldehydes, alkanes, and alkenes (Wheeler et al., 1972; Blum et al., 1971; Brand et al., 1973; Berger, 1968; Schildknecht et al.,

1976; Abou-Donia et al., 1971; Fish and Pattenden, 1975; Bellas et al., 1974).

Considering the few known details, we have started a comparative study of the scent organs of Staphylinidae. We report here the chemistry and morphology of a new type of gland in *Eusphalerum longipenne* Erichson (Coleoptera: Staphylinidae) which to our knowledge has not been reported before.

METHODS AND MATERIALS

The beetles can be found in great numbers in the blossoms of various plants, where they feed on pollen and nectar. Specimens were collected from blossoms of *Ranunculus* sp. (Ranunculaceae) around Frankfurt in early spring.

To study the glands in situ the beetles were dropped into a mixture of hydrogen peroxide and potassium hydroxide, following the method of Blackwelder (1936).

Scanning electron microscopy was done on specimens, whose eighth sternite was dissected to study the cranial margin. Observations were done on a Cambridge Stereoscan 600.[1] Whole beetles of both sexes were extracted with diethyl ether or with formic acid or a mixture of both (1:10).

The secretion was analyzed by GLC on a Perkin-Elmer 900 gas chromatograph isothermally with three different columns: (1) 4% Carbowax 1500 on Chromosorb G AW-DMCS 80–100 mesh; (2) 25% diethylhexyl sebacate and sebacic acid on silica gel 60–100 mesh; (3) 2.5% Carbowax 20 M on Chromosorb G AW-DMCS 80–100 mesh. For temperatures, see the Results section. All columns were metal, 180 cm × 2.7 mm. A nitrogen flow of 40–50 ml/min was employed.

TLC of 2,4-dinitrophenylhydrazones was carried out on silica gel. The plates were developed with petroleum ether (Urbach, 1963).

RESULTS

Gross Morphology

When handling the beetles, we always noticed an intense odor similar to fatty acids and aldehydes. The odorous secretion originates from a gland that occurs in both sexes, situated above the seventh and sixth sternite.

[1] We are grateful to Dr. Grashoff, Senckenbergische Naturforschende Gesellschaft, Frankfurt, for having given us the possibility of working with the scanning electron microscope.

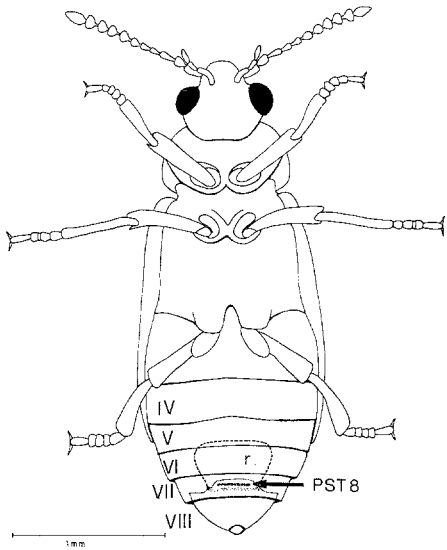


FIG. 1. Schematic ventral view of *Eusphalerum longipenne*. The gland is situated above the sixth and seventh sternite. At the opening of the gland between the seventh and eighth sternites, there is a characteristic projection (PST8) formed by the cranial margin of the eighth sternite.

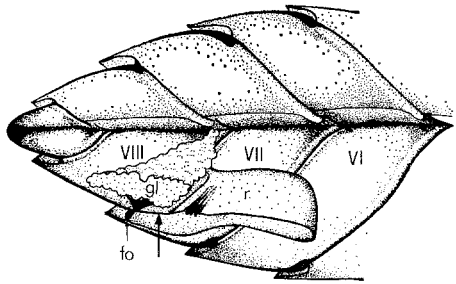


FIG. 2. Schematic longitudinal section through the abdomen of *Eusphalerum longipenne*. The arrow points at the projection of the eighth sternite (PST8). Lettering: r—reservoir; fo—fold, by which the opening of the reservoir is closed; gl—gland. The dorsal wall of the reservoir is wrinkled.

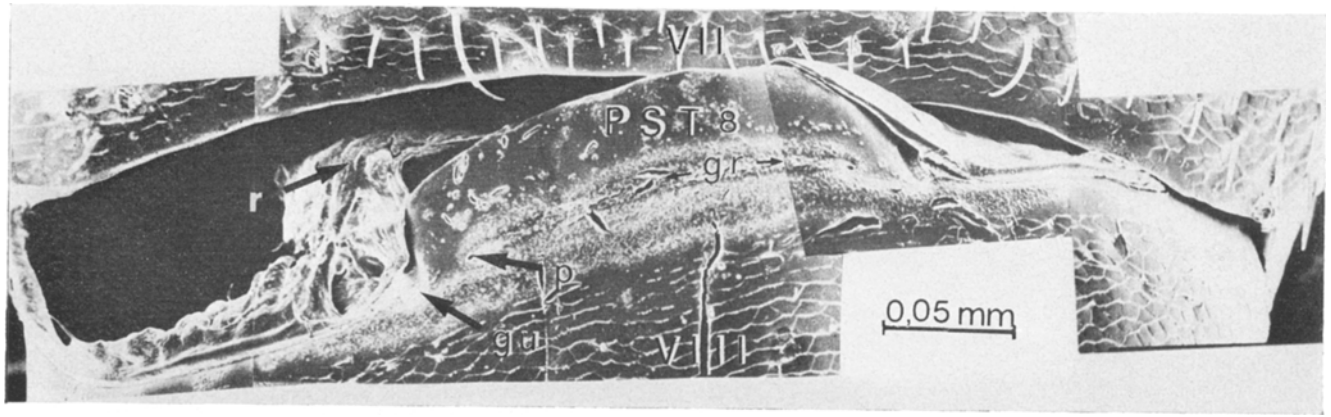
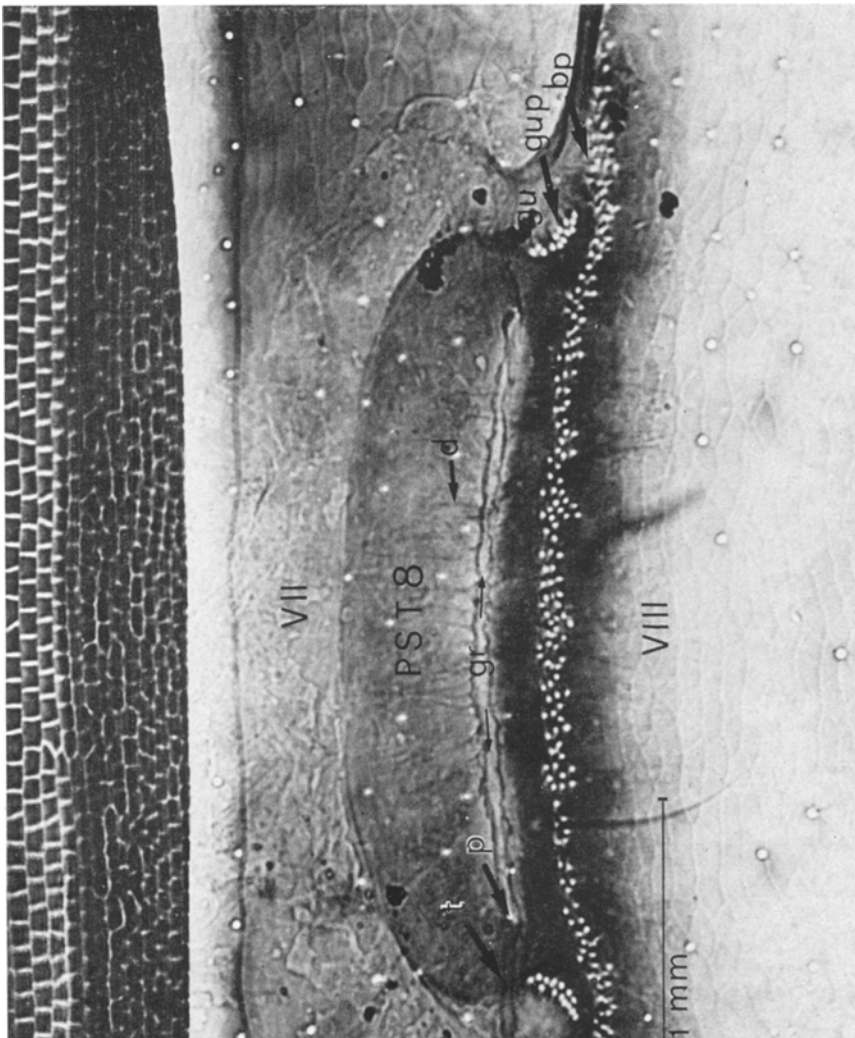


FIG. 3. Scanning electron microscope photograph (above) and light microscope photograph (facing page) of the cranial margin of the eighth sternite, which has a characteristic projection (PST8). PST8 is connected to the dorsal wall of the reservoir. The ductules (d) of the gland cells run into the groove (gr). The groove is extended by a tube (t). The pore (p) at both ends of the groove (gr) indicates the beginning of the tube. In analogy to Pasteels, the gutter pores (gup) and the basic pores (bp) are believed to be campaniform mechanoreceptors.


VII
PST8
gr
d
p
t
gu
gup
bp
VIII
0,1 mm

The organ consists of an unpaired sac like reservoir, opening between the seventh and eighth sternites (see Fig. 1) and a couple of cell units, which are situated above the eighth sternite near the opening of the reservoir. It is obvious that the reservoir is derived from a mediocranial invagination of the intersegmental membrane between sternite 7 and 8. The opening of the reservoir between the seventh and eighth sternites is closed by a fold formed by the intersegmental membrane at the posterior margin of the seventh sternite (see Fig. 2).

As particular gland muscles seem to be lacking, the regular intersegmental muscles between the seventh, eighth, and ninth segment are assumed to be involved in the process of opening and closing the reservoir.

Above the opening of the reservoir, the eighth sternite has a projection (called PST8 from now on) that is connected to the dorsal reservoir wall (see Fig. 3). Each side of PST8 is deepened to form a gutter (gu). A groove is cut into the surface of PST8 which runs parallel with the cranial brim of PST8. At both sides of PST8, which are deepened to form the mentioned gutter (gu), the groove (gr) is extended by a small tube (t) of $\sim 30\ \mu m$ in length.

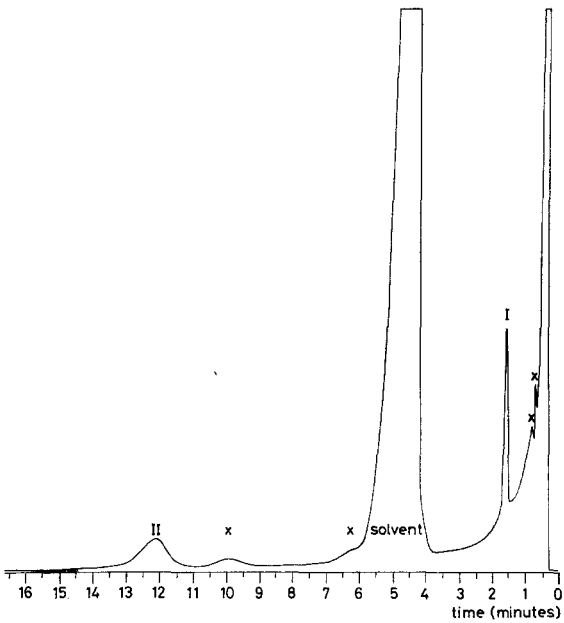


FIG. 4. Gas chromatogram of a formic acid extract of *Eusphalerum longipenne* (68 beetles of both sexes in 200 μl solvent). Column C, 2,5 μl, $\times 40$; peak I: *trans*-hex-2-enal; peak II; 3-methylbutyric acid. Further components ($\times$) are not yet identified.

Figure 3 shows the pore at both ends of the groove (gr) that indicates the beginning of the tube (t) (see Fig. 3).

In *Eusphalerum longipenne* the gland consists of about 600–800 single cell units, which are situated above PST8. Each of the cell units has a ductule of its own running into the groove (gr), into the tube (t), and into the surface of PST8 above both ends of the groove. The tiny pores perforating the groove (gr) are the endings of these ductules (d).

Somewhat larger pores (bp) can be seen at the beginning of the gutter and at the base of PST8 running parallel with the groove pores (gp). These basic pores (bp) and gutter pores (gup) remind us of similar pores in the tergal glands of diverse aleocharine beetles (Pasteels, 1968a). In analogy to Pasteels, we believe these pores to be campaniform mechanoreceptors.

Analysis of the Secretion

When checked with indicator paper, the secretion showed an acid reaction (pH ~ 4.2). GC analysis showed two major components in the beetles' secretion (see Fig. 4).

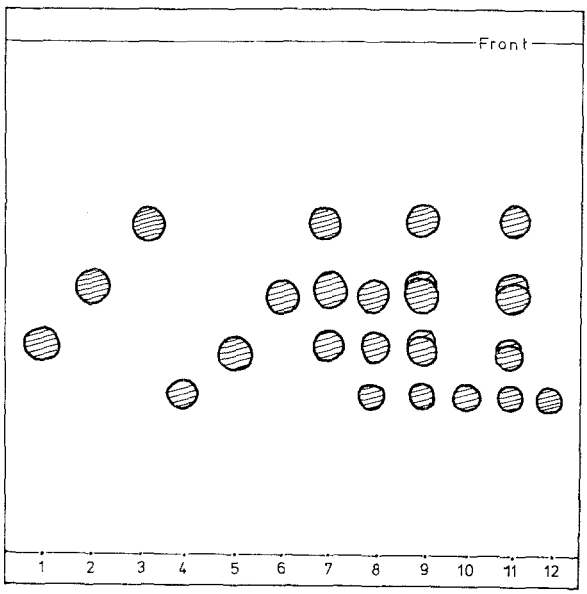


FIG. 5. Thin-layer chromatogram of DNP-aldehydes. (1) hexanal; (2) octanal; (3) decanal; (4) *trans*-hexenal; (5) *trans*-octenal; (6) *trans*-decenal; (7) substances 1, 2, and 3; (8) substances 4, 5, and 6; (9) and (11) substances 1–6; (10) and (12) DNP-aldehyde from the beetle's gland.

Component II had a retention time similar to that of 3-methylbutyric acid in all three columns used. Column A, 130°C: component II, 6 min 15 sec; 3-methylbutyric acid, 6 min 20 sec. Column B, 130°C: component II, 30 min 40 sec; 30 min 20 sec. Column C, 110°C, component II, 12 min 00 sec; 12 min 05 sec.

Component I is an aldehyde, which gave a characteristic precipitate with DNP. TLC showed that the R_F values were identical to those of *trans*-hex-2-enal (see Fig. 5). In addition, the retention time of component I was identical to that of *trans*-hex-2-enal in all three columns in GLC. Column A, 130°C: component I, 1 min 25 sec; 1 min 30 sec. Column B, 130°C: component I, 8 min 40 sec; 8 min 45 sec. Column C, 110°C: component I, 1 min 30 sec; 1 min 30 sec. Further components (x) are not yet identified.

Hexenal and 3-methylbutyric acid were also found in the secretion of *Eusphalerum anale*, *E. minutum*, and *E. abdominale*.

DISCUSSION

This is the first time that an abdominal gland is described in a species belonging to the rove beetle's subfamily Omaliinae. The subfamily Omaliinae includes species, the elytra of which sometimes cover the whole beetle's abdomen. The Omaliinae are closely related to the Proteinae, which resemble the Omaliinae in habit. Both Omaliinae and Proteinae possess notable primitive features with apparent affinities to the Oxytelinae (Crowson, 1950; Jeannel and Jarrige, 1949; Herman, 1970). All Omaliinae and Proteinae, that we have investigated (species belonging to the Omaliinae genera *Eusphalerum*, *Omalium*, and *Anthophagus*, and to the Proteinae genera *Megarthrus* and *Proteinus*) had a similar ventral gland organ; details, however, were different. The apparent homology of the glands in both subfamilies may reveal the stated relationship between these two subfamilies.

Considering the possible relationship between the subfamilies Omaliinae/Proteinae and Oxytelinae, it must be stated that the Oxytelinae form a huge subfamily, which is not sharply differentiated from other subfamilies. Some authors include several groups of staphylinid beetles that are not included by other authors (for details see Herman, 1970). The glands of *Bledius* (Araujo, 1973; Happ and Happ, 1973) and other Oxytelinae (Stein, 1847) are of a very peculiar type. It has been reported that the openings of this organ (and therewith the whole gland) are present in all Oxytelinae in the world (Herman, 1970). They are usually situated in the ninth segment, whereas the external opening of the omaliine gland has been found in the membrane between sternites 7 and 8. Therefore, these two organs cannot be regarded as homologous. Nevertheless, there are some interesting common

features in their morphology, which may help the understanding of the evolution of the highly developed glands in Oxytelinae. The gland cells of either side of the paired defensive gland of the Oxytelinae possess a common channel running into the basic part of the reservoir. In Omaliinae, some cell ductules run into paired small tubes (see Fig. 3).

This is the first time that *trans*-hex-2-enal has been reported to be a major component in the secretion of a beetle. Holometabolous insects usually produce secretions other than *trans*-hex-2-enal, except the ant *Crematogaster* (Blum et al., 1969). It is widespread as a defensive secretion among bugs and cockroaches (Calam and Youdeowei, 1968; Wallbank and Waterhouse, 1970). Additionally, in some bugs it is reported to act as an alerting pheromone.

Carboxylic acids are known to be produced in many insects. Among beetles, 3-methylbutyric acid occurs in carabids (Schildknecht et al., 1968; Moore and Wallbank, 1968), where it is supposed to be a repellent. It has also been found in *Zyras humeralis* (Staphylinidae), where it acts as a repellent against ants (Kolbe and Proske, 1973).

In *Eusphalerum longipenne*, the secretion is obviously a repellent against various predators, such as ants, spiders, and bugs. An alerting effect of the secretion similar to that in bugs has not been observed. We are carrying out a detailed study of the functions of this gland.

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RAT OLFACTORY RESPONSE TO ALIPHATIC ACETATES

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Abstract—Volatile compounds of the male rat preputial gland extracts were found to contain *n*-aliphatic acetates, by gas chromatography and mass spectroscopy. Female rats preferred the odor of various saturated and unsaturated *n*-aliphatic acetates while the male rats were repelled by or indifferent to, most of these compounds. A bioassay apparatus recorded the number of approaches and the investigation time for each animal offered the test compounds. The possibility of aliphatic acetates' involvement as sex attractants produced by the male rat is considered.

Key Words—sex attractants, pheromones, preputial gland, rodent behavior.

INTRODUCTION

Olfaction plays a major role in mammalian social behavior (Stoddart, 1974), regulating their territoriality (Mykytowycz, 1974; Thiessen, 1973; Thiessen et al., 1974), reproduction (Parkes and Bruce, 1961; Whitten, 1966; Bronson, 1974), and care of the young (Fleming and Rosenblatt, 1974). Rodents rely on olfactory signals in recognizing sexual partners and their respective hormonal state (Bronson, 1971; Carr et al., 1962, 1965, 1966, 1970; Curtis et al., 1971). In the white rat and mouse, it is the preputial gland that produces an attractant odor affecting the animals of the opposite sex and dependent on the hormonal state of the donor (Caroom and Bronson, 1971; Orsulak and Gawienowski, 1972)

Our previous attempts to analyze the rat preputial gland secretions

centered on volatile lipid substances (Gawienowski et al., 1975, 1976). Since the mouse preputial glands were found to contain volatile aliphatic acetates (Spener et al., 1969; Sansone-Bazzano et al., 1972), we decided to see if the rats were attracted to the aliphatic acetates. Male mouse preputial glands had relatively higher amounts of aliphatic acetates than did female glands and the acetate content could be increased in the female by testosterone injections.

Observations presented in this report demonstrate that many aliphatic acetates act as an attractant for female rats while being neutral or repulsive for male rats.

METHODS AND MATERIALS

Extraction and Analysis of Preputial Volatiles

Preputial glands, excised from mature male Sprague-Dawley rats, were homogenized in 0.9% saline and extracted 5 times with an equal volume of peroxide free ether. After drying over molecular sieves, the ether extract was subjected to concentration and then distilled under vacuum as reported previously (Gawienowski et al., 1975, 1976). The volatile male preputial lipids were analyzed by a combined GC-MS system. A Perkin-Elmer Model 900 gas chromatograph with a flame ionization detector was interfaced with a Hitachi–Perkin Elmer RMU-6L mass spectrometer; GLC columns (3.66 m × 3.2 mm) were packed with 15% Carbowax 1500 on 100–120 mesh Chromosorb W (HP). The results were processed and interpreted by an IBM 1800 computer system.

Animals

Sprague-Dawley rats of the same age (4–10 months old) were sexually experienced and housed in single cages away from the colony in a separate, well-ventilated room. Food (Purina laboratory chow) and water were available ad libitum, the temperature was stable (24–26°C), and the light cycle (12-h light, 12-h dark) reversed. The experiments with odorants were conducted for 5 days, followed by a 2-day rest period. During the 2 days of rest, the cages with animals of the opposite sex were placed in the experiment room to provide more natural social conditions. On experiment days, the animals did not have any olfactory exposure to the opposite sex.

Materials

All saturated and unsaturated aliphatic acetates were purchased from Nu-Check-Prep, Inc. (Elysian, Minnesota).

Testing Apparatus and Procedure

The testing apparatus was similar to that described previously (Gawienowski et al., 1975, 1976). The testing arena, however, was now circular, 61 cm in diameter, with 46-cm-high galvanized metal walls painted black. It was equipped with two tunnels, placed at a 150° angle rather than opposite each other. The rats could insert their heads in the 5×5 cm tunnels to investigate samples of odorants placed on cotton wads, with air flowing from the air tank (300 ml/min). A metal grid in each tunnel prevented the rats from touching and licking the samples. Each tunnel was equipped with a photocell, an elapsed time indicator, and an event counter, which recorded the cumulative investigating time and the number of approaches for each animal during a 5-min testing time. The indicators and event counters were connected to an interval clock that started and stopped them simultaneously.

Various *n*-aliphatic acetates (1–4 μl) were dissolved in ethanol, which also served as a control odor. The relative position of samples was changed every 30 min and new samples were placed in tunnels. One standard group of animals (20–30 rats) of the same sex was used, and the test compounds were presented randomly, one compound during each daily experimental session. Each animal, naive to this particular odor, was placed in the testing chamber for 5 min. The whole apparatus was washed with detergent every day, and the sawdust under the wire grid floor of the test chamber was changed simultaneously with switching of the sample position (every 30 min). In addition, the entrance to each tunnel and metal grid inside were cleaned with 75% ethanol after every rat.

Statistics

Cumulative investigating times and the number of approaches for the odor source were recorded for each animal, and the data were analyzed by a 2-way analysis of variance (Steel and Torrie, 1960).

RESULTS AND DISCUSSION

Preliminary analysis of the male preputial gland volatiles in a combined GC-MS system indicated the presence of ethyl, propyl, isopropyl, pentyl, and decyl acetates among other compounds. Since the vacuum distillation technique used limits the investigation to the most volatile compounds, the bioassays dealt also with longer chain aliphatic acetates previously found in the mouse preputial glands (Spener et al., 1969).

Tables 1 and 2 list the investigated compounds and indicate the animal

TABLE 1. RESPONSE OF MALE AND FEMALE RATS TO THE ODOR OF SATURATED ALIPHATIC ACETATES[a]

Alcohol from which acetate was derived		Time of investigation		Frequency	
		♂	♀	♂	♀
Ethyl	C-2				
Isopropyl	C-3	+			
Propyl	C-3		+++		+++
Butyl	C-4				
Pentyl	C-5		+++		++
Hexyl	C-6		+++		++
Heptyl	C-7		+++		++
Octyl	C-8		+++		
Nonyl	C-9		+++		++
Decyl	C-10		+++		
Undecyl	C-11	−−	+++		+++
Lauryl	C-12		++		+++
Tridecyl	C-13		+++		+++
Myristyl	C-14		+		
Pentadecyl	C-15				
Palmityl	C-16				
Heptadecyl	C-17	++			
Stearyl	C-18		+++		
Nonadecyl	C-19				
Arachidyl	C-20	−			

[a] The signs (+) and (−) mark attraction and avoidance, respectively. Lack of a sign indicates indifference toward the compound. Probabilities were determined using the F values obtained from the two-way analysis of variance:
+/− $P<.1$;
++/−− $P<.05$;
+++ $P<.01$.

aversion or attraction in terms of statistical significance of the data variation between the sample and the vehicle odor. Investigation time seems to be a better measure of the rat response to different odorants than the frequency of approach. This is not surprising in light of the well-known spontaneous alternating behavior of rats presented with two choices (Schultz and Tapp, 1973). Nevertheless, both measures often provide similar results.

Female rats were clearly more interested in acetates than were the male rats. Ten of the saturated, and four of the unsaturated, acetates elicited highly significant ($P<.01$, for the investigating time data) responses from the female rats, while none of the males displayed such interest in the acetates. The males appeared to avoid some of the acetates, even those that were attractive for

TABLE 2. RESPONSE OF MALE AND FEMALE RATS TO THE ODOR OF UNSATURATED ALIPHATIC ACETATES (SEE FOOTNOTE TO TABLE 1)[a]

Acetates of the following alcohols		Time of investigation		Frequency	
		♂	♀	♂	♀
10-Undecenyl	C-11:1,Δ^{10}[a]				
11-Dodecenyl	C-12:1,Δ^{11}	++			
12-Tridecenyl	C-13:1,Δ^{12}	−			
Myristoleyl	C-14:1,Δ^{9}				
Palmitoleyl	C-16:1,Δ^{9}				
Palmitelaidyl	C-16:1,Δ^{9}, *trans*	+	++		
Heptadecenyl	C-17:1,Δ^{10}				+
Oleyl	C-18:1,Δ^{9}	−			
Elaidyl	C-18:1,Δ^{9}, *trans*		+++		++
Petroselinyl	C-18:1,Δ^{6}			−−	
Ricinoleyl	C-18:1,Δ^{9},12-OH				
Vaccenyl	C-18:1,Δ^{11}				
Linoleyl	C-18:2,$\Delta^{9,12}$				++
Linolenyl	C-18:3,$\Delta^{9,12,15}$				
11-Eicosenyl	C-20:1,Δ^{11}		+++		++
11,14-Eicosadienyl	C-20:2,$\Delta^{11,14}$	++			
11,14,17-Eicosatrienyl	C-20:3,$\Delta^{11,14,17}$		+++		++
Homo-γ-linolenyl	C-20:3,$\Delta^{8,11,14}$		+	−	+
Arachidonyl	C-20:4,$\Delta^{5,8,11,14}$	++	+++		+++
Erucyl	C-22:1,Δ^{13}				
Brassidyl	C-22:1,Δ^{13}, *trans*				
Docosadienyl	C-22:2,$\Delta^{13,16}$	+			
Docosatrienyl	C-22:3,$\Delta^{13,16,19}$				
Nervonyl	C-24:1,Δ^{15}				

[a] Configuration *cis* unless otherwise stated.

females (i.e., undecyl acetate). Arachidonyl acetate was the most attractive odor for the females, but the males also showed a significant preference for this compound. Among the acetates that we found in the volatile fraction of the male preputial gland, propyl, pentyl, and decyl acetates were highly significant as attractant odors for female rats, while the male rats were indifferent to these odors.

It is possible that *n*-aliphatic acetates contribute to the sex-specific odor of rat preputial gland secretions. The differences in their relative content combined with the sex-dependent receptivity of the animals suggests that the acetates may act as sex pheromones for the female rat. Alternatively, the acetates could be part of a still more complex composition of odors, or serve to enhance the odor of another pheromonal substance.

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RHYTHMS OF MALE *Antheraea polyphemus*[1] Attraction and Female Attractiveness, and an Improved Pheromone Sythesis

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Abstract—Wild *Antheraea polyphemus* (Cramer) (Lepidoptera: Saturniidae) males were attracted to synthetic pheromone and to laboratory-reared females from 23:00 (EDT) until dawn. Males were attracted to the synthetic pheromone at a mean time of 01:33 and to the females at 03:23 (EDT). Synthetic traps accumulated the most males (61 compared to 19 in the female-baited traps), but there was no large difference in catch during the main interval of female attractiveness. Pheromone used in the study was prepared by an improved synthesis that utilizes *cis*-4-nonen-1-yl bromide as a key intermediate.

Key Words—*Antheraea polyphemus*, sex pheromone, rhythm of female attractiveness, male sex attractant rhythms, pheromone synthesis, *trans*-6-*cis*-11-hexadecadienyl acetate, *trans*-6-*cis*-11-hexadecadienal.

INTRODUCTION

In a continuation of our previous study (Kochansky et al., 1975) on the sex pheromone of *Antheraea polyphemus* (Cramer), we report the rhythms of male attraction to synthetic pheromone and virgin females. To obtain an

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adequate quantity of synthetic pheromone for these field studies, an improved synthesis with acceptable yields was necessary. The pheromone components are *trans*-6-*cis*-11-hexadecadienyl acetate (t6c11-16:Ac) and the corresponding aldehyde (t6c11-16:Ald).

METHODS AND MATERIALS

Traps used were as described previously (Kochansky et al., 1975). Virgin females (laboratory-reared on oak leaves, two per trap, 1–4 days old) were maintained indoors at 20°C on a photoperiod regime approximating the outdoor light–dark cycle (lights on at 1–2 h prior to sunrise, light off at 1–2 h after sunset) and were kept outdoors for 24 h prior to use to ensure their entrainment to the natural photoperiod. The synthetic attractant used was 1 mg of a 90:10 mixture of t6c11-16:Ac and t6c11-16:Ald (each >99% pure, collected from preparative GLC) on a silicone vial stopper (rubber stoppers, sleeve-type, 5 mm × 9 mm, Arthur H. Thomas Co.).

At dusk, traps were placed at ground level near the sides of rural roads around Geneva, New York, and were separated by at least 1000 m. Ten synthetic-baited and 10 female-baited traps were alternated. Traps were

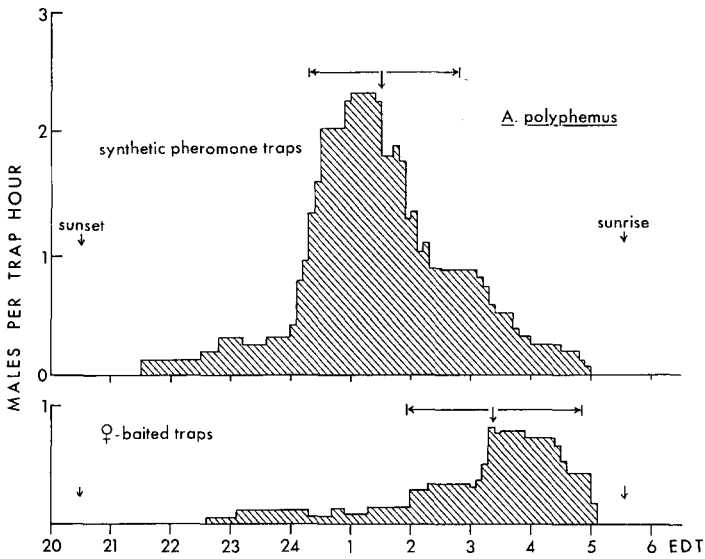

FIG. 1. *A. polyphemus* males trapped per hour in pheromone and in female-baited traps. Arrows above shaded areas represent mean time of catch ± standard deviation (EDT).

checked approximately every 1.5 h until full daylight. Captured males were removed from the traps. Time of capture was considered to be the midpoint of the trap-checking interval. Thus, if a trap that was empty at midnight contained three males 1.5 h later, the males were considered to have been caught at 00:45, and the trap caught two males/h for that interval. A few males found fluttering around the traps were caught by hand and the actual time of catch was recorded.

Trapping was done on the nights of July 1–2, July 3–4, and July 16–17, 1975.

RESULTS AND DISCUSSION

The earliest male caught was found in a synthetic-baited trap at 23:09 (EDT) while the earliest male in a female-baited trap was found at 00:19. Figure 1 shows the activity spectra for both traps. The mean time of catch to synthetic-baited traps was at $01:33 \pm 1$ h 16 min (standard deviation) ($n = 61$). For the female-baited traps, the mean was $03:23 \pm 1$ h 28 min ($n = 19$). These means were different by Student's t test (78 d.f., $t = 4.67$, $P < 0.001$). Means and summed catches per trap-h are shown Fig. 1.

Rau and Rau (1929) investigated the periodicities of male attraction to virgin females in a number of saturniid species in St. Louis, Missouri. They observed *A. polyphemus* males attracted to females during the following intervals (EST): 23:00–24:00, $n = 11$; 00:00–01:00, $n = 4$; 01:00–02:00, $n = 2$; 02:00–03:00, $n = 1$; 03:12–04:18, $n = 21$. Rau and Rau considered the rhythm to be bimodal. Our observations (Fig. 1) of male captures to synthetic pheromone indicate that males were attracted from 23:00 until dawn, with maximal attraction between 00:00 and 02:00. While females did lure males from 23:00 to dawn, maximal attractiveness occurred during the 2 h preceding sunrise, a finding very similar to the early observations of Rau and Rau.

In *A. polyphemus*, the comparatively broad periodicity of male attraction to synthetic pheromone, as contrasted with the more restricted interval during which females attract males, could aid in dispersal of males prior to mating. Their flight capability was demonstrated in one instance in which a marked *A. polyphemus* male flew 7.5 km in one evening before recapture in a pheromone-baited trap (Kochansky et al., 1975). Another example of relatively precocious male activity was found with *Holomelina aurantiaca* (Hübner) (Cardé, 1974).

The hourly differences in trap catches of synthetic vs. female lures illustrate the necessity of determining the rhythms of male attraction and female attractiveness for evaluation of the comparative efficacy of these lures (Cardé et al., 1974; Cardé, 1976). The use of summed nightly trap

catches as the only criterion suggests that the synthetic dispensers were several times more attractive than the females. However, comparison of catches during the interval of maximal female attractiveness reveals that both lures were of roughly equal potency. We have as yet been unable to measure the pheromone release rate from *A. polyphemus* females.

CHEMICAL SYNTHESES

Our previous synthesis (Kochansky et al., 1975) gave very poor overall yields ($\sim 1\%$), mainly because of the poor reaction of 6-heptyn-1-yl THP ether with *cis*-4-nonen-1-yl bromide. In our original reaction, this step proceeded in only $<20\%$ yield, presumably because of a side reaction of the bromide with the liquid ammonia solvent. Since this bromide is the product of a multistep synthesis, we could not use it in the desired excess to obtain optimum yields. Furthermore, on an attempt to repeat the synthesis, we were unable to obtain any of the desired product from this reaction.

Therefore, we designed an improved synthesis that utilizes readily accessible compounds in excess and that does not require purification of the intermediates (Fig. 2). It is based on successive elaboration of the molecule from the carbon terminal end, starting with 1-bromobutane and 4-pentyn-1-ol. This scheme also produces 1-bromo-*cis*-4-nonene, which was used in our previous preparation. This bromide was treated with lithium acetylide ethylenediamine complex (3 eq) in DMSO–liquid ammonia. GLC of the

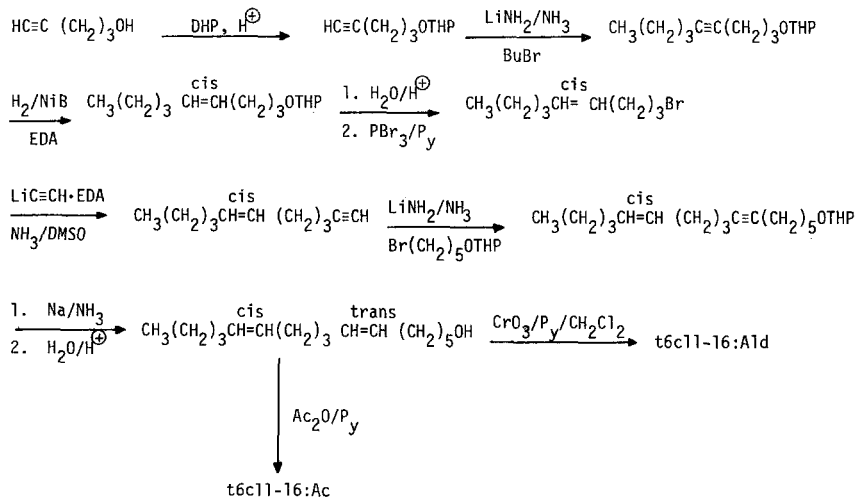

FIG. 2. Synthesis scheme for polyphemus pheromone components.

resulting terminal acetylene showed no impurities other than some residual solvent, and it was treated directly with 5-bromopentyl THP ether (3 eq) after formation of the anion with lithium amide in liquid ammonia. This step gave, after workup, a solution of 40% of the desired C_{16} enyne THP ether in the excess bromoether. This mixture was carried through successive steps as such, since removal of the bromide with Na–NH_3 and then hydrolysis of the THP ether gave a mixture of pentanol and *trans*-6-*cis*-11-hexadecadienol that were separated simply by distillation on a rotary evaporator, leaving the C_{16} alcohol 89% pure by GLC.

Retention times of the product were identical to those of the first preparation on polar and nonpolar GLC columns, and the IR spectra were superimposable. While we have no direct evidence about isomeric purity, our previous experience with the two reductions suggests that the product was >99% isomerically pure. The hydrogenation over nickel boride–EDA has consistently given us 99–99.5% *cis* hydrogenation, and we have never detected any *cis* from Na–NH_3 reduction. GLC showed no detectable (<1%) unreduced enyne or saturated material. The final proof of identity of the products of the two preparations is, of course, the activity of both in the field.

This synthetic scheme represents a great improvement in yield over the previous one. We obtained, in our first synthesis, less than 1% combined yields of acetate and aldehyde from the starting 4-pentyn-1-ol. In this case, we obtained 21% overall yield of *trans*-6-*cis*-11-hexadecadienol from 4-pentyn-1-yl THP ether.

5-*Bromo*-1-*Pentanol and Its THP Ether*

This compound was prepared by a slight modification of the method of Butenandt et al. (1962). A mixture of 54.7 g of 1,5-pentanediol, 350 ml of 48% HBr, and 50 ml of water was continuously extracted with Skellysolve B for 1 week at 25° and then for 1 week at 60°. A small amount of anhydrous K_2CO_3 was kept in the receiving flask for the Skellysolve to neutralize any traces of HBr that came over. After completion of the reaction, the extract was filtered to remove K_2CO_3 and evaporated on a rotary evaporator to give 45 g of crude product.

The crude product was treated with 75 ml of dihydropyran and 3 drops of sulfuric acid in the presence of a small handful of 4-A molecular sieves for an hour, with magnetic stirring. Neutralization of the acid with solid $NaHCO_3$ and extraction with ether gave, after washing with water and brine, drying with $MgSO_4$, and removal of solvent, 76 g of crude THP ether which was suitable for use without further purification.

4-Nonyn-1-yl THP Ether and the Corresponding Alcohol

To a solution of 44 g of $LiNH_2$ in 500 ml of liquid NH_3 was added a solution of 4-pentyn-1-yl THP ether (prepared by method given above, precursor alcohol from Chemical Samples Co., 30 g, 179 mmol) in ether (200 ml). After stirring for 1 h, 1-bromobutane (55 g, 401 mmol, 2.5 eq) was added. The mixture was stirred under reflux for an additional 4.5 h, then the NH_3 was allowed to evaporate overnight. The reaction mixture was hydrolyzed with ice and water, and the aqueous layer extracted 3 times with Skellysolve B. The organic layers were washed twice with brine, dried with $MgSO_4$, and evaporated to give 41.6 g (96%) of product; purity was not determined.

This product was dissolved in 400 ml of methanol; water was added until the solution turned cloudy (~150 ml was required), followed by ~2 ml of concentrated HCl. The mixture was heated under reflux for 2 h. The methanol was removed in vacuo, and the residue was dissolved in ether and washed with water (2×), saturated $NaHSO_3$ (2×), and saturated NaCl. The ether was evaporated to give 13.4 g of product (55%) that was ~95% pure by GLC on OV-1 at 170°.

cis-4-Nonen-1-ol

The acetylene was hydrogenated in ethanol using P2 nickel boride poisoned with ethylenediamine (Brown and Aruja, 1973). The catalyst was prepared from 40 ml of a 0.125 M solution of nickel acetate in 95% alcohol and 5 ml of a 1.0 M solution of $NaBH_4$, followed by the addition of 0.9 g of ethylenediamine. The alcohol (13.4 g) was added in 30 ml of ethanol, and the hydrogenation carried out for 6 h in an ice bath until hydrogen uptake ceased. The mixture was filtered to remove catalyst, the bulk of the ethanol was removed in vacuo, and the residue was diluted with water and extracted 3 times with ether. The extracts were washed with water (3 ×) and saturated NaCl (1×), and dried with $MgSO_4$. Solvent removal left 15 g of product.

cis-4-Nonen-1-yl Bromide

The reaction of the alcohol with PBr_3 was carried out in the presence of pyridine (Su and Mahany, 1974). A solution of the alcohol (15 g) and pyridine (18.6 g) was stirred under N_2 at 0° for 10 min. Phosphorus tribromide (22.2 g) in ether (70 ml) was added dropwise, and the mixture was allowed to stir overnight. The excess PBr_3 was hydrolyzed with ice and water, and the organic product extracted with Skellysolve B (3×). The organic layers were washed with water (1×), saturated $NaHCO_3$ (1×), and brine (2×), dried with $MgSO_4$, and the solvent was removed to yield 14.1 g of product

having an identical retention time to a previously prepared sample on OV-1, 175°. GLC showed the product to be ~95% pure. This represents a 72% yield from 4-nonyn-1-ol.

cis-6-*Undecen*-1-*yne*

To 250 ml of liquid ammonia in a 500-ml three-necked flask fitted with a dry ice condenser and a mechanical stirrer, were added 12.3 g (134 mmol) of lithium acetylide, ethylenediamine complex, then 9.0 g (44 mmol) of *cis*-4-nonen-1-yl bromide in 50 ml of ether, and then 50 ml of DMSO. The mixture was stirred for 4 h. Workup and GLC of a small aliquot showed that no bromide remained. Ammonium chloride (8 g) was added, and the NH_3 was allowed to evaporate as the flask was heated to 30°C with a water bath. After 2 h, 200 ml of water was added, and the product was extracted with 30–60° petroleum ether (150, 100, and 50 ml for the three extractions). The combined extracts were washed 3 times with water, once each with saturated $NaHCO_3$ and NaCl, and dried with $MgSO_4$ and a little activated charcoal. Removal of the solvent left 8.45 g of a pale yellow liquid. (According to GLC it was >99% pure except for about 10% residual solvent.)

The IR spectrum had bands at 3320 cm^{-1} (C≡CH) and 3030 cm^{-1} (—CH=CH– *cis*); there was no sign of a *trans* band at ~980 cm^{-1}.

cis-11-*Hexadecen*-6-*yn*-1-*yl Tetrahydropyranyl Ether*

Lithium amide was prepared from 1.5 g of lithium (1% Na) in 500 ml of liquid NH_3 in the above-described apparatus with the use of 50 mg of $Fe(NO_3)_3 \cdot 6\ H_2O$ as catalyst. After 20 min, the initial blue color had faded to gray. The enyne prepared above, in 75 ml of ether, was added over 30 min. After stirring for 15 min, 50 ml of DMSO was added, and the mixture was stirred for a further 15 min. 5-Bromopentyloxy THP (33 g, 3 eq) was added in 100 ml of ether. The ammonia was allowed to reflux for 5 h, and the stirring was continued as the ammonia evaporated overnight. The mixture was hydrolyzed with ice and 500 ml of H_2O, and the product was extracted with three portions of petroleum ether. The extracts were washed and dried as above, to give 16.4 g of an oil containing 40.5% of the desired $C_{16}OTHP$ by GLC (OV-1, 225°) along with the excess $Br(CH_2)_5OTHP$ as the major constituents.

trans-6-*cis*-11-*Hexadecadien*-1-*yl THP Ether*

To 500 ml of ammonia in a 1000-ml flask fitted out as above, was added 5.6 g of Na (250 mmol). When this had dissolved, 14.4 g of the crude enyne

THP ether was added in 125 ml of Et_2O. After 1-h stirring, most of the blue color had disappeared, so a further 4.8 g of Na was added, followed by 100 ml of ether, and the mixture was stirred under reflux for a further 6 h. Excess sodium was destroyed by addition of NH_4Cl (26 g), and the ammonia was allowed to evaporate overnight. Water (300 ml) was added, and the product was extracted with petroleum ether (2 ×) and ether (1 ×). The extracts were washed and dried as above, to give, after solvent removal, 12.9 g of a yellow oil.

The IR spectrum had bands at 980 cm^{-1} (—CH=CH— *trans*) and 3030 cm^{-1} (—CH=CH— *cis*).

GLC showed a large peak at 6.8 min on CHDMS at 220°, compared to hexadecyloxy THP at 6.0 min and the starting enyne THP at 9.1. No peak corresponding to the starting enyne was seen in the product.

trans-6-*cis*-11-*Hexadecadien*-1-*ol* (*t6c*11-16:*OH*)

The crude THP ether from the Na–NH_3 reduction was hydrolyzed by heating under reflux for 3 h in a mixture of 300 ml of EtOH, 75 ml of H_2O, and 1 ml of conc. HCl. After completion of the reaction (as shown by GLC), the EtOH was removed by rotary evaporation; the residue was then diluted with 300 ml H_2O and extracted 3 times with ether. The combined ether extracts were washed with 100-ml portions of water (2 ×), saturated $NaHSO_3$ (2 ×, to remove hydroxyvaleraldehyde from the THP group), water (2 ×), saturated $NaHCO_3$ (1 ×), and saturated NaCl (1 ×). After drying with $MgSO_4$ and solvent removal, 5.64 g of a yellow oil remained (89.6% pure by GLC on CHDMS, 195°). This represents 5.05 g of pure alcohol or a 54% yield overall from *cis*-4-nonenyl bromide, or 21% from 4-pentyn-1-yl THP ether.

The IR spectrum had bands at 3030 cm^{-1} (—CH=CH— *cis*), 980 cm^{-1} (—CH=CH— *trans*), and 3400 cm^{-1} (OH).

trans-6-*cis*-11-*Hexadecadien*-1-*yl Acetate* (*t6c*11–16:*Ac*)

A mixture of the crude t6c11-16:OH (4.50 g), acetic anhydride (10 ml), and pyridine (10 ml) was heated on a steam bath for 1 h. The mixture was dissolved in 100 ml of ether and washed with equal volumes of water (2 ×), 5% HCl (cold, 2 ×), water (2 ×), saturated $NaHCO_3$ (1 ×), and saturated NaCl (1 ×). It was then dried with $MgSO_4$ and a little activated charcoal to remove color. Removal of the solvent left 4.65 g of a pale yellow oil that was 85.7% pure by GLC (OV-1, 195°). The IR spectrum contained bands at 3030, 980, and 1750 cm^{-1} (C=O). Pure material was obtained for use in the field by preparative GLC on a 5% SE-30 column (2.5 m × 10 mm, glass, 225°C).

trans-6-*cis*-11-*Hexadecadienal* (*t6c*11-16:*Ald*)

Oxidation was carried out by the method of Ratcliffe and Rodehorst (1970). A mixture of CrO_3 (6.0 g), pyridine (9.5 g), and CH_2Cl_2 (150 ml) was stirred for 15 min. A solution of the alcohol (1.60 g) in 20 ml of CH_2Cl_2 was added in one portion, and stirring was continued for a further 15 min. The solution was poured into a separatory funnel, and the tarry residue in the reaction flask was rinsed with enough ether to make the total extract lighter than water. The extract was washed with water (2×), cold dilute HCl (2×), water (2×), saturated $NaHCO_3$ (1×), and brine (1×), and dried with $MgSO_4$ and charcoal. Solvent removal left 1.77 g of an almost colorless oil smelling strongly of long-chain aldehyde. The crude product was 84.7% pure by GLC (OV-1, 195°). Its IR spectrum contained bands at 3030, 980, and 2730 cm^{-1} (RCHO), and at 1736 cm^{-1} (C=O). Purification was by preparative GLC as described above.

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AGGREGATION ATTRACTANT OF THE EUROPEAN ELM BARK BEETLE, *Scolytus multistriatus*[1] Production of Individual Components and Related Aggregation Behavior

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Abstract—The three components of the *Scolytus multistriatus* aggregation attractant—4-methyl-3-heptanol (1), α-multistriatin (2), and α-cubebene (3)—were collected from beetles and elm wood by aeration, solvent extraction of host tissue, or solvent extraction of beetles and beetle parts. Bioassays and analysis of extracts demonstrated that (a) compounds 1 and 2 are produced by virgin females and 3 is host-produced, (b) cessation of the production of 1 is coincident with diminished attractiveness of females after they have been joined by males, (c) the release of 3 from elm wood is augmented by attacking beetles, and (d) the level of 3 is related to the condition of the wood, and fungal growth (particularly *Ceratocystis ulmi*) may lead to increased levels of 3. Compound 1 was concentrated in the upper abdominal area and 2 in the abdomen tips, but neither component was in the hindgut. A gland opening through the vaginal palpi (a pair of sclerotized conical structures) was circumstantially associated with the release of 2. Compounds 1, 2, and 3 are regulatory agents that con-

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tribute to the initiation and maintenance of the beetle–elm–microorganism biosystem.

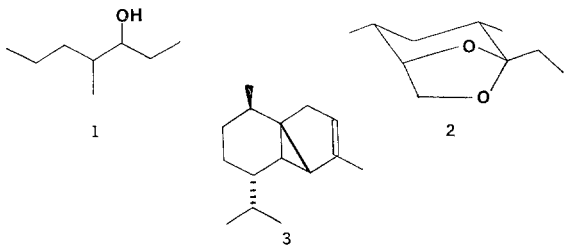
Key Words—*Scolytus multistriatus*, European elm bark beetle, Scolytidae, Dutch elm disease, aggregation pheromone, kairomone, aggregation attractant.

INTRODUCTION

European elm bark beetles, *Scolytus multistriatus* (Marsham), aggregate on declining elms, where the females bore into the inner bark to construct egg galleries and the males join the females to mate. Beetles are attracted to diseased or degenerating elms (Martin, 1935), and the rate at which beetles arrive at the breeding site is greatly enhanced by the boring activity of pioneer beetles (Meyer and Norris, 1967). Virgin females boring in elm bark stimulate the aggregation of large numbers of male and female beetles (Peacock et al., 1971). Attraction decreases rapidly after males join tunneling females (Elliott et al., 1975). Elm wood (Peacock et al., 1971) or frass (Peacock et al., 1973) from logs containing only males is only slightly attractive.

Some beetles initiate new breeding attacks directly after dispersal from brood trees;[6] others, unable to locate host trees weakened by disease or other factors, may feed in twig crotches of healthy elms. During twig crotch feeding, sticky spores of the disease-causing fungus, *Ceratocystis ulmi* (Buisman) C. Moreau, may be transferred to healthy elms.

Aeration of elm logs infested with virgin female *S. multistriatus* yielded an attractive extract (Peacock et al., 1975), from which the principal pheromone components (−)-4-methyl-3-heptanol (1), (−)-α-multistriatin (2), and (−)-α-cubebene (3) were isolated by monitoring individual and recombined fractions with laboratory bioassays (Pearce et al., 1975; Gore et al., 1975). Subsequent laboratory and field assays (Lanier et al., 1977) and extensive field trials with synthetic 1 and 2, and 3 from cubeb oil (Cuthbert and Peacock, 1977; Lanier et al., 1976) verified that the aggregation attractant is a synergistic mixture of 1, 2, and 3.



1 2 3

[6] Gut content content analysis of a sample of 292 beetles arriving at an attractant source revealed that 65% had not fed (Lanier, unpublished data).

The production of aggregation pheromones by Coleoptera with respect to sex, age, virginity, and host components has been reviewed (Jacobson, 1972; Carle, 1974; Borden, 1974; Lanier and Burkholder, 1974). In three bark beetles—*Trypodendron lineatum* (Olivier) (Schneider and Rudinsky, 1969; Borden and Slater, 1969), *Ips confusus* (LeConte)[7] (Pitman et al., 1965), and *Dendroctonus pseudotsugae* Hopkins (Zethner-Moller and Rudinsky, 1967)—the hindgut, or some portion of it, was implicated by bioassays as the site of pheromone production. In contrast, dermestid species of the genus *Trogoderma* produce pheromone in glandular cells lining the inner surface of the seventh abdominal sternite and release it from pore-like structures scattered over the surface of the sternite (Hammack et al., 1973).

The objectives of this study were to identify the sources of each of the three attractant components within the tree–beetle–microorganism association, to investigate their roles in the aggregation behavior of the beetles, and to investigate the structures that we believe to be associated with pheromone production and release.

METHODS AND MATERIALS

Collection of Host- and Beetle-Produced Components

Attractant components were collected by three methods: aeration of logs, logs infested with virgin females, and logs infested with both males and females; solvent extraction of both healthy and diseased elm tissue; and solvent extraction of whole beetles and beetle parts.

Beetles used in the aeration studies were reared according to the method described by Peacock et al. (1973). In studies involving the extraction of beetles and beetle parts, newly emerged beetles were obtained directly from the logs as they emerged; virgin females and mated males and females that had bored into elm tissue were obtained from the elm bolts 4–5 days after the initial infestation. All beetles or beetle parts were stored at $-10°C$ prior to extraction.

The Porapak Q aeration system (Peacock et al., 1975) was used for the collection of volatile components. In studies comparing the volatile substances produced by virgin females with those produced by mated beetles, two separate chambers were aerated simultaneously; one chamber contained 4000 virgin females on logs, while the other chamber contained mated beetles (4000 ♂♂+4000♀♀) on logs. The airflow through each chamber was 9–11 liters/min. Wood from healthy American elm, *Ulmus americana* L., was

[7] Now *Ips paraconfusus* Lanier.

infested 1 week after cutting. Six to eight logs with a surface area of approximately 11,000 cm^2 were used in each chamber.

Each collection column contained Porapak Q (50–80 mesh, Waters Associates) that had been conditioned by pentane extraction (Byrne et al., 1975). Fresh Porapak collection columns were placed on each chamber at 72-h intervals starting with the time of beetle introduction; thus, three separate aeration periods for days 1–3, 4–6, and 7–9 were monitored. After the given aeration period, the Porapak Q was extracted with pentane and the extracts were concentrated to approximately 2 ml as described by Byrne et al. (1975). As an estimate of beetle activity, the frass produced in each chamber was also collected during each aeration.

For the collection of volatile components from elm, logs (surface area 11,000 cm^2) uninfested with beetles and uninfected with Dutch elm disease (DED) were slashed to expose phloem tissue. These logs were aerated for 7 days, and the volatile components were collected and concentrated under conditions equivalent to those used for aeration of infested logs. A 250-μl aliquot of the concentrate (2.1 ml total) was analyzed by GLC.

Host production of pheromone components was further investigated by solvent extraction and analysis of the volatiles from healthy and diseased (DED) elm tissue. Healthy tissue originated from an elm tree (*U. americana*, Syracuse, New York) that was not infested by *S. multistriatus* and had not contracted DED. The samples consisted of 40 g of phloem and 40 g of outer xylem, both of which had been ground in a Wiley mill. The phloem and xylem tissues were extracted separately in a Waring Blendor with 2×300 ml of *n*-hexane. The phloem and xylem extracts were then combined and concentrated by distillation at reduced pressure through a Vigreux column; final concentration to 670 μl was accomplished with a gentle stream of dry N_2, and an aliquot equivalent to 30 g of tissue was analyzed by GLC.

Diseased elm tissue was from a tree with DED (probably inoculated by root graft) but with no *S. multistriatus* infestation. Phloem (43 g) and xylem (44 g) tissues were extracted as described above. A 130-μl aliquot of the extract concentrate (equivalent to 30 g of tissue) was analyzed by GLC.

Frass collected from virgin females boring in elm was extracted with hexane (Peacock et al., 1975). As with the elm tissue extracts, the hexane solution was concentrated and analyzed by GLC.

Whole beetles and beetle sections were extracted and analyzed for the presence of 1, 2, and 3. In the extraction of whole beetles, 1400 boring virgin females were removed from bark and extracted three times in a Waring Blendor with hexane. The extracts were combined and concentrated by fractional distillation. In a similar manner, extracts were prepared of 1400 virgin males that had been tunneling in elm bark, 2000 emergent males, 2000 emergent females, 1300 boring mated males, and 1300 boring mated females.

Beetles and beetle parts were extracted by crushing in 50 μl of twice-distilled hexane, freezing the mixture, thawing, and removing the supernatant liquid with a syringe. The extraction was repeated, and the combined extracts were injected into the gas chromatograph. The following preparations were made: 125 live virgin female beetles were divided into head, prothorax, and mesothorax-abdomen sections; a second group of 125 whole beetles taken from the same infestation were retained as a control; an additional group of 125 beetles were laterally bisected to remove the last three abdominal segments (tip) from the remainder of the beetle; hindguts were pulled from a group of 1000 boring virgin females; the accessory glands (Figs. 1–3) were removed from 175 virgin females; fluid from accessory glands was removed from 175 virgin females.

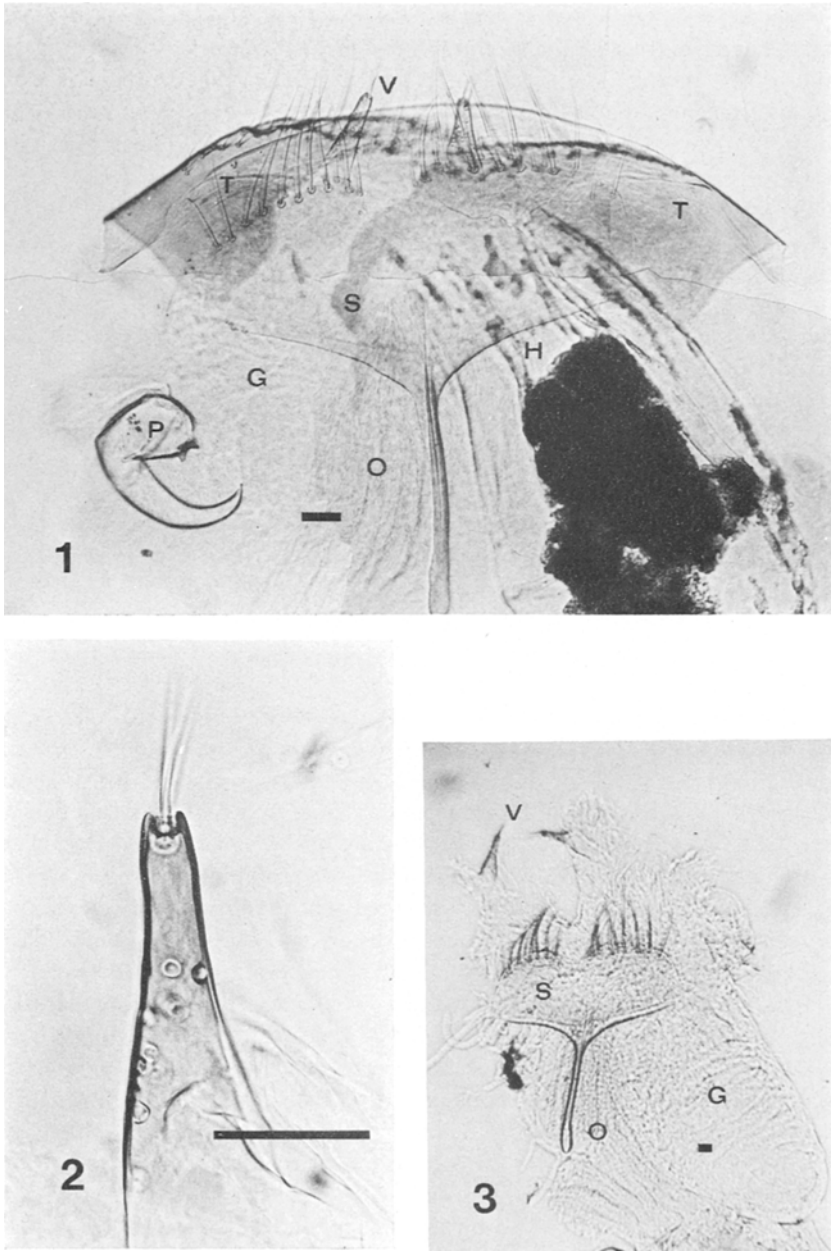
The accessory gland was removed by squeezing the abdomen with forceps to evert the gland, which was then removed under physiological saline. Glandular fluid was sampled by inserting the tip of a drawn 5-μl glass capillary into the exposed glandular tissue. About 0.1 μl of solution was taken into the capillary and subsequently expelled into a hexane solution (25 μl). In these experiments, the beetles without glands and those without gland fluid were retained and extracted as described above.

Analysis of Pheromone Components

The crude extracts from aeration, beetles or beetle parts, or elm wood were fractionated by preparative GLC with an SE-30 column, and the fractions were collected at the retention-time intervals marked by authentic samples of 1, 2, and 3. The target fractions were then chromatographed on analytical GLC columns. If the SE-30 fractions were complex, they were fractionated on a preparative Carbowax 20M column, and each of the target Carbowax fractions was then analyzed. The identity of each compound was confirmed in each of the crucial extracts of beetles and beetle parts, elm tissue, and aeration extracts by GLC coinjection and by spectrometry.

Quantitative analyses were obtained, where possible, from preparative runs, but quantitative determinations for complex fractions were made from analytical chromatograms. Tetradecane was used as the quantitative standard, and peak areas of 1, 2, and 3 were adjusted by factors of 0.82, 0.78, and 0.76, respectively, to correct for differences in the flame-ionization-detector response. These factors were calculated on the basis of the appropriate model compounds.

Similar GLC conditions were used throughout the analytical procedure, and specific conditions given here for the analysis of whole beetles are representative of the analytical procedure. Extracts of whole beetles were fractionated by GLC (on-column injection) on a 5% SE-30 preparatory



FIGS. 1–3. Photomicrographs from slide-mounted terminalia of *Scolytus multistriatus* females. 1: The terminal segment and attached organs (V—vaginal palpi, G—accessory gland, H—hindgut, O—oviduct, P—spermathecal pump, S—sternite, T—tergite). 2: Vaginal palpus. 3: The eighth sternite with the vaginal palpi freed from their membranous attachment; note gland. The horizontal bar in each figure denotes 50 μm.

column (60–80 DMCS AW Chromasorb G, silylated glass, 6 mm × 3.7 m, He at 60 ml/min, 100° at 6°/min to 160°). Four fractions corresponding to target compounds were collected: fraction 1, 7.8–10.8 min; fraction 2, 12.6–16.2 min; fraction 3, 19.2–23.4 min; fraction 4, 34.8–37.2 min. Fractions 1, 2, 3, and 4 corresponded to compounds 1, 4 (a postulated precursor of 2), 2, and 3, respectively. The SE-30 fractions were analyzed on a 5% Carbowax 20M analytical column (100–120 DMCS AW Chromasorb G, stainless steel, 3 mm × 6.2 m, He at 25 ml/min), and on a 5% Apiezon L column (100–120 DMCS AW Chromasorb G, glass, 3 mm × 6.2 m, He at 25 ml/min). The extracts of glands and the beetles without glands, the gland fluid, and the beetles without gland fluid were analyzed directly on the Carbowax 20M column. Retention times of unknowns and standards were compared by coinjection, and a positive identification with one column was confirmed by coinjection on the second column. In the case of the whole virgin females, the presence of 1, 2, and 3 in the SE-30 fractions was confirmed by GLC and mass spectra.

The analysis of beetles for 3,5-dimethyl-1,2-epoxyoctan-6-one (5) was obtained indirectly from the level of 2 and by direct analysis. A standard solution of 5 (mixed isomers) was quantitatively converted to 2 when chromatographed on the SE-30 column, with the observed peaks having the same retention time as injected samples of 2. Therefore, the absence of 2 in SE-30 fraction 3 indicated an absence of 5 in the extract. When 2 was present in the SE-30 fraction 3, a sample of the crude extract was chromatographed on the Carbowax 20M analytical column on which 5 was stable.

Preparation and Staining of Accessory Glands

When pressure was applied to the abdomen of a live virgin female, the glands were everted at the posterior of the beetle. The glands, a small portion of the oviduct, and the last sternite were removed from the beetles, stained, and examined by light microscope. The staining was accomplished by soaking the glands in Chlorazil Black (Carnoy solution) for 30–60 min. When the tissue was rinsed with ethanol and mounted, the glands and surrounding tissue acquired a pale green color. When the ethanol rinse was replaced with soaking in physiological saline for about 10 min, the glands acquired a red color and most of the surrounding tissue remained green.

RESULTS AND DISCUSSION

Aeration of Infested and Uninfested Logs

Aeration experiments provided a chemical basis for observed temporal

changes in the attractiveness of tunneling females, and for the role of the host in the aggregation response. Peacock et al. (1971) demonstrated that in-flight beetles responded preferentially to elm logs infested with virgin females rather than to elm logs with either males or both sexes. Subsequent studies (Peacock et al., 1975) demonstrated that a pheromone could be collected by aerating logs and absorbing the attractant in a porous polymer. In a model compound study, Byrne et al. (1975) had established that this technique was a reliable method for qualitative and quantitative determinations of volatile compounds released over extended time periods. We applied this technique to the monitoring of the release rates of 1, 2, and 3 from slashed elm logs and from virgin females and mixed males and females boring in elm logs.

Field studies indicated that the attractiveness of virgin females reached a maximum on the fourth day after boring commenced (Peacock et al., 1975; Elliott et al., 1975). This change in activity was reflected in the release rates of 1, 2, and 3, which were relatively low for the first three days, increased by day 4, and remained level through day 9.

A comparison of the quantities of 1, 2, and 3 released by virgin females

TABLE 1. PHEROMONE COMPONENTS COLLECTED BY AERATION OF ELM LOGS AND BEETLE-INFESTED ELM LOGS, AS DETERMINED BY GLC AND MASS SPECTROMETRY

Infestation	Days of collection[a]	Release rates of pheromone components[b]			Frass produced (g)
		1	2	3	
Virgin females[c]	1–3	0.79	0.70	0.69	9.67
Mated beetles[d]	1–3	0.86	1.9	1.2	15.43
Virgin females[c]	4–6	5.0	3.8	10.5	7.96
Mated beetles[d]	4–6	0.23	2.1	9.2	16.46
Virgin females[c]	7–9	6.7	5.6	12.2	9.53
Mated beetles[d]	7–9	0.16	1.8	9.2	15.85
Virgin females[e]	4–10	6.9	12.0	113.0	
Healthy elm, uninfested		0	0	0.0773	

[a] Day 1 beginning at time of infestation.

[b] Release rates $\times 10^4$ μg/bh for beetles and as μg/h for elm wood; 1 beetle — hour (bh) represents the boring activity of one beetle for 1 h.

$$\text{Beetle equivalence} = \text{initial infestation} - \frac{\text{dead beetles at termination.}}{2}$$

[c] Infestation of 4000 virgin females, Ohio collection

[d] Infestation of 4000 virgin females and 4000 virgin males, Ohio collection.

[e] Infestation of approximately 5000 virgin females, Syracuse collection.

and by mixed sexes revealed that the production of 1 was significantly reduced by the inclusion of males (Table 1). The release rates of the three components were initially low in both groups, but the quantities released by the virgin females increased sharply and maintained a near constant level for days 4–9. In contrast, the release rate of 1 for the mated beetles was 4.6% of that for the virgin females for days 4–6 and diminished further over days 7–9. The release rate of 2 by mated beetles was about 50% of its value in the virgin females.

The termination of aggregation on a given host following the arrival of sufficient numbers of each sex of the attacking beetles is a well-documented phase of scolytid host-selection behavior (Borden, 1974, and references therein). Although pheromone "masks" or "response inhibitors" have been implicated for several scolytid species [e.g., 3-methyl-2-cyclohexen-1-one for *Dendroctonus pseudotsugae* (Rudinsky et al., 1973) and verbenone for *D. frontalis* Zimmerman (Renwick and Vité, 1969) and *D. brevicomis* LeConte (Wood, 1972)], the cessation of attractant release is also a common mechanism for attack termination. *Ips paraconfusus* Lanier (Borden, 1967), *I. calligraphus* (Germar) (Vité et al., 1972), and *I. pini* (Say) (Swaby and Rudinsky, 1976) reduce pheromone production after the sexes intermingle. Whether cessation of pheromone production in *Ips* results from mating per se, acoustical communication, or some other mechanism is unknown.

Approximately 80% of the biological activity of the aeration extracts of mated elm bark beetles was restored with the addition of 1 (Lanier et al., 1977). This result, in combination with the field studies of Elliott et al. (1975), established that mated beetles do not produce an antiaggregation pheromone. Since the field bioassays showed that attractiveness of females tunneling in elm logs decreased rapidly following the introduction of males (Peacock et al., 1971; Elliott et al., 1975), we conclude that the reduced production of 1 is at least the initial mechanism by which the attraction to females is diminished after the arrival of males.

The ratio of the compounds 1, 2, and 3 varied from 1:1:2 to 1:1:10 in the aeration of different batches of infested logs, whereas slashed elm logs cut from healthy trees yielded small amounts of compound 3 but no measurable quantities of 1 and 2. These results indicated that 3 was host-derived and that the release rate of 3 was related to the condition of the logs.

The extremely small quantities of 3 obtained from the aeration of uninfested logs was an unexpected finding. A 7-day aeration of virgin female-infested logs yielded 0.5–2 mg, far more than the 13 μg collected in the aeration of a similar quantity of uninfested logs. This result, in combination with the observations on beetle-infested logs over time, clearly suggested that the large release of 3 from beetle-infested logs is associated with the presence of the beetles even though, as our results will show, the beetles

themselves do not produce 3. We also observed that the amounts of 3 collected from aerations of mated and virgin beetles on logs were correlated with greater frass production by mated beetles during the first 3 days, but thereafter they were similar even though the boring activity of the mated beetles was nearly double that of the virgin females.

Solvent Extraction of Elm Wood

The large variation in the amount of 3 in infested logs led to an investigation of the origins of this component. Samples of elm tissue (phloem and xylem) were taken from cross-sectional disks cut from a healthy elm tree and from a tree that had DED but no beetle infestation. Compound 3 was found in both samples, but 1 and 2 were not detected. Furthermore, the level of 3 in the diseased sample (0.17 ppm) was approximately 15 times that in healthy tissue (0.0027 ppm).

This result accounts for the considerable variation in levels of 3 observed in the aeration experiments. The extent of beetle boring, the number of beetles per unit of log surface, and the condition of the logs varied; hence, growth of microorganisms and the consequent release of 3 also varied. The increased levels of 3 in wood infested with *C. ulmi* indicated either that 3 is biosynthesized by both the tree and *C. ulmi*, or that the rate of biosynthesis of 3 in elm tissue increases in response to the fungus.

TABLE 2. OCCURRENCE OF PHEROMONE COMPONENTS OF *Scolytus multistriatus* IN SOLVENT EXTRACTS OF WHOLE BEETLES

Beetle source[a]	No. of beetles in sample	Analyzed compounds (ng/beetle)[b]				
		1	2	3	4	5
Virgin females,						
Batch 1	1400	2.6[c]	2.6[c]	2.5[c]	0	0
Batch 2	125	1.5	0.5	0	na	na
Mated females	1300	0	0.7	2.6	na	na
Emergent females	2000	0	0	0	0	0
Emergent males	2000	0	0	0	na	na
Virgin males	1400	0	0	0	na	na
Mated males	1300	0	0	0	na	na

[a] Virgin and mated beetles were removed from elm logs during days 4–5 of the infestation. Emergent beetles were collected 1–6 days after leaving brood logs.
[b] 0 means compound was not detected, lower limit of detection was 0.1 ng/beetle as determined by the GLC detector response to standard samples of 1, 2, and 3; na means not analyzed.
[c] Identity confirmed by GLC and mass spectrometry.

Extraction of Beetles

The results of these experiments (Table 2) are consistent with the aeration and field bioassay studies in that boring virgin female beetles contained 1 and 2 in approximately equal quantities (1.5–2.6 ng/beetle), while 3 was present in more variable quantities (<0.1–2.5 ng/beetle). In mated females, amounts of 2 and 3 were equivalent to the quantities in virgin females, but the quantity of 1 was diminished (<0.1 ng/beetle). The variable levels of 3 present in elm wood were apparently reflected in the amount of 3 ingested by, and hence extracted from, boring females.

The quantities of 1 and 2 extracted from virgin females were small (2.6 ng/beetle) relative to the total amounts of 1 and 2 that a virgin female produced in 6 days (72 ng). With 2.6 ng of 1 or 2, a beetle could sustain a typical release rate of 0.5 ng/h for only 5.2 h. Although quantities of 1 and 2 obtained by aeration and extraction exhibited some variation from batch to batch, the presence of small amounts of 1 and 2 was continuous throughout the pheromone production period. In the event that pheromone biosynthesis was terminated, as was apparently the case for 1 soon after the female had mated, the release of pheromone soon ceased.

Production of Pheromone Components and Biological Responses

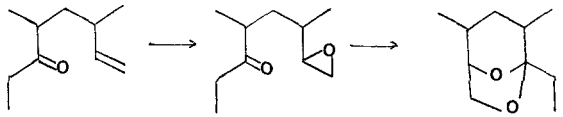
The release rates of the individual pheromone components are of particular importance when the biological responses to synthetic 1, 2, and 3 are considered. In previously reported laboratory bioassays (Lanier et al., 1977), individual components and binary combinations of components produced very weak responses, whereas the mixture of 1, 2, and 3 gave responses equal to those of the crude attractant. Similar results were observed in field bioassays, with the exception that the mixture of 1 and 2 was more attractive than the other binary mixtures, although significantly less attractive than the combination of 1, 2, and 3 (Lanier et al., 1977). Therefore, the diminished release rate of 1 in the mated female would result in a substantial reduction in the pheromone activity. Furthermore, the increased levels of 3 in infested wood could result in an increase in the aggregation response toward trees with DED.

Peacock et al. (1973) have demonstrated that walking beetles are attracted to frass and frass extracts in the laboratory olfactometer but that these materials are weakly attractive to in-flight beetles in the field. In this study, frass was extracted and analyzed to determine whether the beetles' response to frass was related to the production of 1, 2, and 3. Compounds 1 (0.018 ppm), 2 (0.280 ppm), and 3 (260 ppm) were found. The quantity of 3 present in frass corresponded to the quantity of 3 present in diseased elm wood. The

very low level of 1 in frass supports the hypothesis that, although frass contains sufficient active components to stimulate beetles at close range in the laboratory olfactometer, it does not contain the necessary concentration of all the components to elicit the aggregation response in the field.

Recently Hendry et al. (1975) and Hendry (1976) proposed that pheromone components for the oak leaf roller were present in the leaves of the host tree at very low concentrations, and that during the life cycle of the insect sufficient leaf tissue was ingested to account for the quantities of released pheromone.[8] That some bark beetles utilize plant-produced compounds as components of attractants had been demonstrated (Bedard et al., 1969), and, in the case of *S. multistriatus*, we have shown that 3 is a host-produced component. The possibility that 1 and 2 existed in elm tissue at levels below the limits of detection in the gas chromatograph, and that these components were concentrated in the insect, was tested. A conservative estimate of the lower limit of detection of 1 and 2 is 1 ng/g (0.001 ppm). As previously discussed, a virgin female beetle could release at least 72 ng of 1 or 2; to sequester this amount, a beetle would have to ingest a minimum of 720 mg of elm tissue. Since a beetle produces an average of 6 mg of frass in 6 days (some of which is not ingested by the beetle), we conclude that the observed levels of 1 and 2 cannot be sequestered from ingested host material.

The in vitro synthesis of 2 via the epoxidation of 4 to give 5, and the acid-catalyzed cyclization of 5 have been reported (Pearce et al., 1975). Since the epoxidation and acid-catalyzed ring closure appear to be reasonable biosynthetic transformations, compounds 4 and 5 were regarded as potential biosynthetic intermediates in the in vivo formation of 2. However, com-



4 5 2

pounds 4 and 5 were not detected in extracts of emergent or boring females within the limits of the analytical system (0.1 ng/beetle). These compounds cannot be disregarded as intermediates in the biosynthesis of 2, but the possibility that the beetles maintain a large pool of these compounds is eliminated.

Site of Pheromone Production

The third phase of this study was a combined morphological and chemical investigation to determine the site of pheromone production within the virgin

[8] For rebuttals, see Miller et al. (1976) and Hindenlang and Wichmann (1977).

S. multistriatus female. Beetles and various beetle parts were analyzed by GLC to determine the areas of the beetle where the individual components of the pheromone were localized. The beetles were partitioned in three ways: (1) Major body segments were separated by clipping between the head and the prothorax and between the prothorax and the mesothorax; (2) the last three abdominal segments (tip) were removed from whole beetles; (3) the hindguts were pulled from whole beetles. Whole beetles and body parts were extracted, fractionated, and analyzed for 1, 2, and 3.

Compounds 1 and 2 were found in the mesothorax-abdomen, but not in the head or the prothorax (Table 3). When the abdominal section was divided, compound 2 appeared in the tip, while 1 was concentrated in the anterior section. Hindguts removed from whole beetles contained only trace amounts of 1 and 2, but substantial quantities of 1 and 2 were found in the beetles from which the hindguts had been removed. Thus, 1 and 2 appeared to be concentrated at different sites within the abdomen; but, contrary to reports on the site of pheromone storage in other bark beetles, neither 1 nor 2 was found in the hindgut.

No unique features were observed for the portion of the females that

TABLE 3. OCCURRENCE OF THE COMPONENTS OF THE AGGREGATION ATTRACTANT OF *Scolytus multistriatus* IN SOLVENT EXTRACTS OF BEETLE PARTS

	Analyzed compounds[b] (ng/beetle)		
Source (virgin females)[a]	1	2	3
Whole boring virgin females	1.5	0.5	0
Heads	0	0	0
Prothorax	0	0	0
Mesothorax-abdomen	1.3	0.3	0
Beetle tips	0	0.7	0
Beetles without tips	0.9	0	0
Hindguts	0	0	0
Beetles without hindguts	1.5	1.5	0
Accessory glands	0	0	0
Beetles without accessory glands	1.8	0	0
Glandular fluid	0	0	0
Beetles without glandular fluid	1.0	0	0

[a] Virgin females were removed from elm logs during days 4–5 of the infestation.

[b] 0 = compound not detected, lower limit of detection = 0.1 ng/beetle.

was associated with 1, although numerous pores are present on the thorax and abdomen of male and female beetles. An examination of the abdominal tip revealed that the general features of the digestive and reproductive tracts of *S. multistriatus* are similar to those reported for *Dendroctonus pseudotsugae* (Zethner-Moller and Rudinsky, 1967) with one outstanding exception. On the female, two sclerotized conical tubules (Figs. 1–3) extended in a posterior direction from the accessory glands between the anal and the genital openings to the exterior of the beetle. These structures, termed "vaginal palpi", appear in Fisher's (1937) illustration of the reproductive tract of the female of *Scolytus scolytus* F. Nusslin (1911–1912), according to Fisher (*op. cit.*), believed that this pair of organs was the vestige of the ninth sternite (we agree) and was used during oviposition.

The glandular region is exposed (Fig. 4) when the abdomen is gently squeezed with forceps. The glands were dissected from the virgin female beetles and examined with a light microscope. The vaginal palpi were clearly visible; however, examination of the unstained glandular tissue was difficult. Glands stained with Chlorazil Black in Carnoy solution and rinsed with ethanol acquired a pale green color. When the ethanol rinse was replaced with a physiological saline treatment, the glandular tissue became red, and the surrounding tissue (i.e., the oviduct) green. These differentially stained tissue preparations clearly showed that the vaginal palpi were continuous with the accessory glands. Unfortunately, the color differences were not helpful in discriminating these tissues in the black and white photomicrographs (Figs. 1 and 3).

The accessory glands were dissected from the beetle in saline solution, and the remainder of the beetle and the glands was extracted and analyzed by GLC for 1, 2, and 3. The beetles from which the glands had been removed contained 1 but not 2; however, neither 2 nor any other compound was

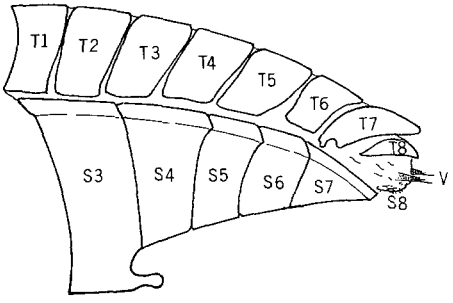


FIG. 4. The abdomen of a *Scolytus multistriatus* female with the terminal portion distended to show vaginal palpi.

found in the gland extracts. The loss of 2 was probably caused by rupture of the glands during removal from the beetle and dispersal of 2 into the physiological saline solution.

In the second attempt to obtain 2 directly from the beetle, the tip of a micropipette was inserted into the glandular area, and about 0.1 μl of fluid was removed. The fluid and the beetles from which the fluid had been removed were analyzed. As shown in Table 2, 1 was present in the beetles from which fluid was removed, but 2 could not be found in the beetle or the fluid. The loss of 2 from the fluid and from the beetle reflects some of the difficulties encountered in this type of experiment. The process of obtaining the gland fluid may cause the release of the small amount of 2 (2.5 ng) that was stored in the beetle. The release could result from a muscular response of the beetle or from disruption of tissue with the pepette tip.

The failure to obtain 2 directly from the gland area does not preclude the possibility that these organs are involved in pheromone production. However, the loss of 2 from whole virgin females with disruption of tissue at the posterior section of the reproductive tract is consistent with the finding that 2 is present in the beetle tip and virtually absent in the hindgut. On the basis of the chemical and morphological evidence, we believe that the accessory gland and attached vaginal palpi of the *S. multistriatus* female are probably the organs for production, storage, and release of 2.

SUMMARY AND CONCLUSION

This study is the first chemically monitored mapping of the production sites of individual pheromone and kairomone components of a complex insect–host–fungus association. Virgin *S. multistriatus* females produce two of the components, 1 and 2, and a third component, 3, is a kairomone, the production of which can be stimulated by DED fungus in the host. Components 1 and 2 are concentrated at two separate sites in the virgin female, with 2 occurring in the posterior tip and 1 anterior to the tip in the mesothorax-abdomen. A uniquely modified accessory gland, observed in the abdomen tip, could be the organ for the production and release of 2.

The release of the individual attractant components is related to the behavioral patterns of *S. multistriatus*. Female beetles reduce their production of 1 after mating, apparently as a means of discontinuing the attractant response. This effect could be the mechanism by which the beetle shifts the aggregation response from an established infestation area where extensive mating has occurred to a new breeding site that has been recently attacked.

The presence of 3 in the elm wood is at least one factor directing the beetle aggregation to elm trees. The relationship between *S. multistriatus*

and the DED pathogen, *C. ulmi*, is well known in that the beetle is the vector for the fungus, and the fungus produces additional breeding sites for the beetle. The finding that the level of 3 is significantly greater in diseased elm than in healthy elm explains why a diseased tree is more attractive to *S. multistriatus* than an uninfected tree. Thus, the aggregation attractant of *S. multistriatus* is a complex pheromone–kairomone combination that serves to synchronize the relationship not only between male and female beetles, but also between the beetles, the host, and the associated microorganism.

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IOWA EUROPEAN CORN BORER SEX PHEROMONE
Isolation and Identification of Four C_{14} Esters[1,2]

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Abstract—Four C_{14} straight-chain acetate esters—(*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, (*E*)-9-tetradecenyl acetate, and tetradecyl acetate—were isolated and identified from the diethyl ether extracts of the abdominal tips of adult female European corn borers, *Ostrinia nubilalis* (Hübner). The compounds were isolated using a combination of high-resolution gel-permeation, adsorption high-pressure liquid, and micropreparative gas chromatography. Identifications were made by gas chromatography, a combination of gas chromatography and mass spectrometry, and microdegradative chemical methods.

Key Words—sex pheromones, European corn borer, *Ostrinia nubilalis*, acetate esters, gas chromatography, mass spectrometry, gel-permeation chromatography, liquid chromatography, (*Z*)-11-tetradecenyl acetate, (*E*)-11-tetradecenyl acetate, (*E*)-9-tetradecenyl acetate, tetradecyl acetate.

[1] Mention of a commercial or proprietary product in this paper does not constitute a recommendation or an endorsement of that product by the U.S. Department of Agriculture or the Energy Research and Development Administration.

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INTRODUCTION

The functional importance of 11-tetradecenyl acetate (11-tda) geometrical isomers in the sex pheromone communications of the European corn borer, *Ostrinia nubilalis* (Hübner), has been established (Klun et al., 1973; Kochansky et al., 1975). Borers throughout much of North America and central Europe have been characterized as *Z*-type populations; that is, they respond to a blend of isomers that is predominately *Z*-11-tda, and borers of northern Italy and New York are known to respond to a blend of isomers that is predominately *E* (Klun and cooperators, 1975; Kochansky et al., 1975). The basis of this intraspecific pheromonal variation and other possible pheromonal variabilities of the species is not yet fully elucidated. A more complete understanding of the borer pheromone system can be expected, however, as chromatographic technology and chemical identification capabilities become more refined and additional chemical-biological studies are conducted. We report techniques used to isolate and identify some pheromonally active components from the female borer. Moreover, these techniques lend themselves to the efficient isolation and identification of trace compounds from the chemically complex mixtures frequently encountered in pheromone studies. We also report physical-chemical data that confirm the identity of pheromonal components of the Iowa borer previously described by Klun and Brindley (1970) and furnish evidence of the occurrence of a heretofore undetected acetate ester in ether extracts of the female European corn borer (ECB).

METHODS AND MATERIALS

Extraction and Bioassay

All ECB moths used in the study were reared (Lewis and Lynch, 1969) at the Corn Insects Research Unit, Ankeny, Iowa. The adult moths were allowed to emerge in a large walk-in screened cage held at conditions of constant light, 27°C, and 80% RH. The females were collected daily, placed in 56 × 28 × 45.8 cm screened cages, sprayed with water daily, and conditioned for 2 days by exposure to a photophase–scotophase cycle of 16 h at 27°C and 8 h at 20°C. The females were then chilled to 6°C and their abdominal tips were excised into diethyl ether. Routinely, about 3000 tips were placed in 10 ml of ether. These ether preparations were stored at 0°C while the tips of 24,000 females were being accumulated over a 1-month period. When sufficient tips were on hand, they were homogenized by hand at 20°C by using a 30-ml Teflon® pestle tissue grinder. Normally, a set of 3000 tips was ground 4 times with 30 ml of diethyl ether. The combined ether extracts

from 24,000 tips were filtered through Pyrex Wool®, dried over anhydrous Na_2SO_4, and the solvent was removed by rotary distillation (35°C) at atmospheric pressure to yield about 3 ml of yellow oil. All subsequent steps in purification and identification were regularly monitored for pheromonal activity by exposing caged adult male ECB moths (preconditioned for at least 72 h at 27°C and 80% RH in constant light and then held in a 300 ft/min airflow at 20–22°C from an air conditioner) to the odor stimulus on the tip of a glass capillary tube held 4–5 cm upwind. The males signaled the presence of pheromone by rapid wing vibration, extension of genitalia, and clasper responses.

Isolation

Four 30 cm × 7 mm (ID) stainless steel columns packed with 100-Å μ Styragel® (Waters Associates) were connected in series to a Waters Associates Model ALC/GPC-202/R401 liquid chromatograph equipped with a refractive index detector. A mixture of esters and primary alcohols was used to calibrate these gel-permeation chromatography (GPC) columns for molecular size separations. The eluant was $CHCl_3$ pumped through the columns at a rate of 1.5 ml/min and a head pressure of 1000 lb/in^2.

GPC data on the standard chemicals are plotted in Fig. 1. Tripalmitin fell outside the range of selective permeability and was eluted at the void volume. Molecules with apparent molecular sizes smaller than tripalmitin selectively permeated the column matrix and were eluted in order of their decreasing molecular sizes.

The components in the crude extracts of ECB females were separated on the μ Styragel columns by repetitive injections of 100-μl aliquots. Only the elution volume (V_e) of 22.6–24.2 ml contained pheromonal activity as measured by the bioassay. From the data in Fig. 1, this fraction should have contained C_{14} acetate esters and compounds of similar molecular size because tetradecyl acetate has a V_e of 23.4 ml in GPC. The combined pheromone-active fraction collected from successive injections on GPC was reduced from 48 ml to 0.3 ml by partial vacuum rotatory distillation at 40°C. The concentrate was then rechromatographed twice on the GPC column to ensure that the sample would contain only components having a molecular weight range of 235–275. The GPC-purified material was then chromatographed on a 30 cm × 4 mm (ID) stainless steel column packed with μ Porasil® (Waters Associates). The mobile phase was hexane–diethyl ether (80:1, v/v) pumped through the column at a rate of 1.5 ml/min. The pheromone-active material was found in the 16.5–18.6-ml fraction. This V_e interval coincided with the elution volumes of 11-tetradecenyl acetate (11-tda) and tetradecyl acetate (tda) standards.

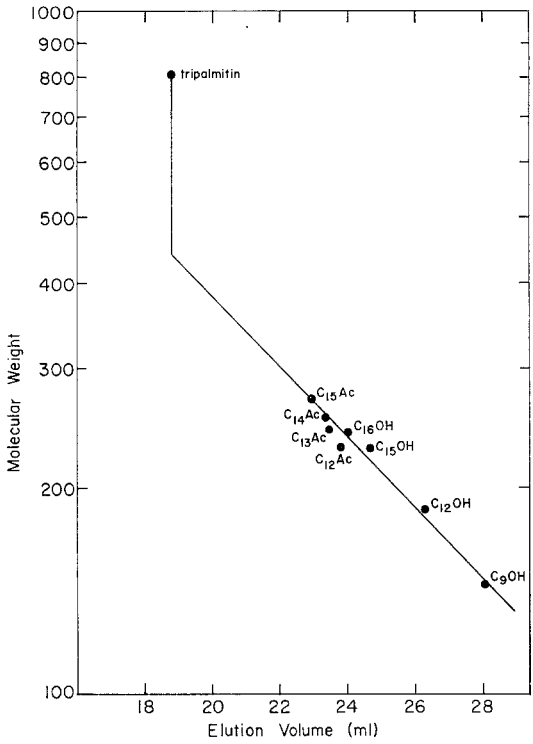

FIG. 1. Molecular-weight separations on μ Styragel columns eluted with 1·5 ml $CHCl_3$/min. Abbreviations: $C_{15}Ac$—pentadecyl acetate; $C_{14}Ac$—tetradecyl acetate; $C_{13}Ac$—tridecyl acetate; $C_{16}OH$—1-hexadecanol; $C_{12}Ac$—dodecyl acetate; $C_{12}OH$—1-dodecanol; C_9OH—1-nonanol.

The pheromone-active fraction from the μ Porasil column was purified further by micropreparative GLC after most of the hexane–diethyl ether had been removed by partial vacuum and rotatory distillation at 50°C. The micropreparative GLC separations were made on a 2.54 m × 4 mm (ID) glass column packed with 5% SE-30® on DMCS-treated 60–80 mesh Chromosorb G.® The separated components were detected in the flame ionization unit of a Beckman GC-4 chromatograph. The column was operated at 190°C with a He flow of 66 cm^3/min, and the column effluent was split so that 10% entered the flame ionization detector and the remaining effluent was routed to a fraction collection device. The fraction collector was a heated (150°C) stainless steel line that extended 3 cm beyond the detector compartment (250°C). The end of the heated line was fitted with a 1/16-in.

Swagelok® union, a stainless steel nut, and a Teflon® ferrule into which the tip of a disposable glass Pasteur pipette was inserted to collect chromatographic fractions.

Identification

Micropreparative GLC on the SE-30 column showed that the μ Porasil-collected material contained seven components. Each component was fraction-collected in a pipette and recovered by flushing the pipette with CS_2. Only one of the chromatographic peaks was pheromone-active, and its retention time (T_r) coincided with the retention time (15.2 min) of standard tda and monounsaturated C_{14} acetate esters. Previous research (Klun and Brindley, 1970; Kochansky et al., 1975) has shown that silicone liquid phases (SE-30 and OV-1®) separate acetate esters according to length of the carbon chain, but that they will not resolve saturated, monounsaturated positional, and geometrical isomers of compounds having the same overall

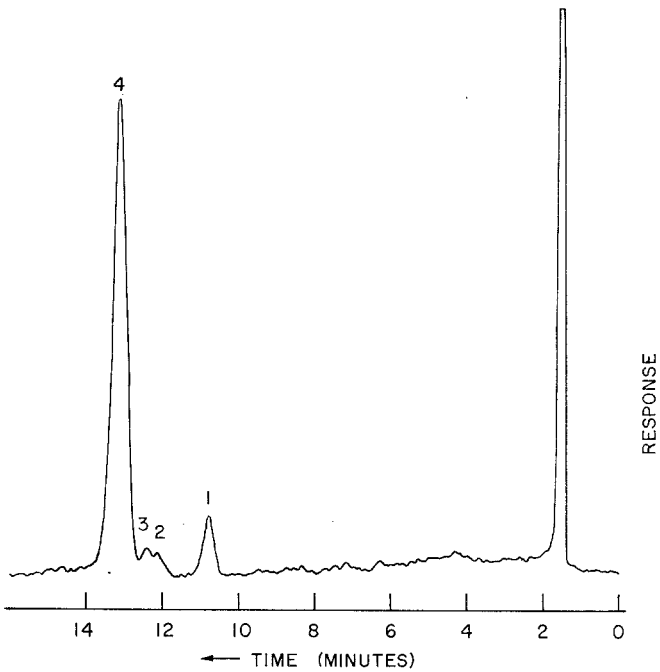

FIG. 2. PDEAS column chromatogram of active extract collected from SE-30 column chromatography: 1) tetradecyl acetate; 2) (*E*)-9-tetradecenyl acetate; 3) (*E*)-11-tetradecenyl acetate; 4) (*Z*)-11-tetradecenyl acetate.

chain length. Thus, the pheromone-active material collected from the SE-30 column probably represented a mixture of C_{14} saturated and monounsaturated acetate esters.

The pheromone-active fraction from the SE-30 column was chromatographed on a 6 m × 2 mm (ID) glass column packed with 3% PDEAS on 100–120-mesh Gas-Chrom Q (Applied Science Labs., Inc.) at 16 cm^3 He/min and 175°C. The chromatography (Fig. 2) showed that the SE-30 collected material was a mixture of at least four compounds and that the T_r values of the compounds on the PDEAS column fell within a range characteristic of the monounsaturated C_{14} acetates. The 13.2-min T_r of the major component in the mixture was identical to that of standard (*Z*)-11-tda. Two unresolved minor components (2 and 3) were observed with peak maxima at 12.2 and 12.5 min, respectively. The T_r of component 3 coincided with the T_r of standard (*E*)-11-tda, and GLC retention-time analyses with tetradecenyl acetate standards indicated that component 2 probably was (*E*)-9-tda. The 10.8-min T_r of component 1 coincided with the T_r of authentic tetradecyl acetate. Chemical reduction of an aliquot sample of the pheromone-active material with H_2 over Pd on carbon destroyed the pheromonal activity of the preparation. The GLC analysis of the reduced material on the PDEAS column showed a single chromatographic peak that had a T_r identical to that of tetradecyl acetate. The SE-30 collected material was therefore a mixture of tda and three monounsaturated tetradecenyl acetates.

Because the efficiency of our 6-m PDEAS packed column [5500 theoretical plates calculated for (*Z*)-11-tda] was insufficient to completely resolve all the components in the pheromone-active mixture, we tested eight 15 m × 0.5 mm (ID) stainless steel support coated open tubular (SCOT) chromatographic columns (Perkin-Elmer Corp.) with a standard blend of tda, (*Z*)- and (*E*)-11-tda, and (*E*)-9-tda. All the columns tested (Apiezon-L, Dexsil 410, OV-17, OV-225, XE-60, Carbowax 20M, butanediol succinate, and DEGS) had efficiencies greater than that of the 6-m packed PDEAS column, but only the DEGS column, with ~25,000 theoretical plates for (*Z*)-11-tda, provided complete separation of the standard mixture. We then chromatographed the pheromone-active mixture on the DEGS (SCOT) column operated at 135°C and 5 cm^3 He/min and found four resolved peaks (Fig. 3) whose T_r values were identical to those of the tda, (*E*)-9-tda, (*E*)-11-tda, and (*Z*)-11-tda standards, respectively.

The pheromone-active mixture was also chromatographed on a 50 m × 0.27 mm (ID) glass, open tubular column (GOTC) coated with Carbowax 20M. This column had an efficiency of 80,000 theoretical plates for (*Z*)-11-tda at 1.1 cm^3 He/min and 155°C. Results obtained with the GOTC confirmed the chromatographic identifications made on the DEGS (SCOT) column, and peak area determinations showed that the pheromone-active

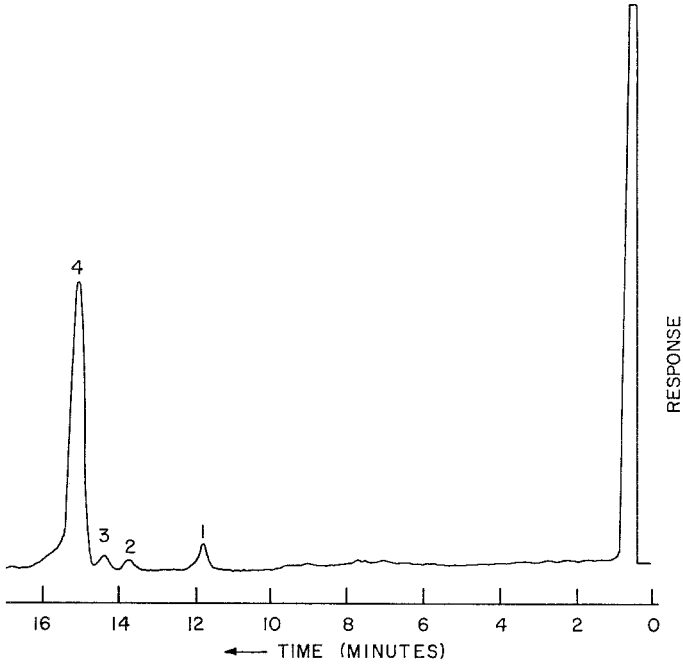

FIG. 3. DEGS high-resolution SCOT column chromatogram of active extract collected from SE-30 column chromatography: 1) tetradecyl acetate; 2) (*E*)-9-tetradecenyl acetate; 3) (*E*)-11-tetradecenyl acetate; 4) (*Z*)-11-tetradecenyl acetate.

mixture contained 84% (*Z*)-11-tda, 4.8% (*E*)-11-tda, 3.2% (*E*)-9-tda, and 8% tda.

Additional physical data for identification of the pheromone-active mixture collected from the micropreparative SE-30 column were obtained by combination gas chromatography–mass spectrometry (GC-MS), micro-ozonolysis of the olefins, and GC-MS of the ozonolysis products.

All mass spectra were obtained with a DuPont 21–490–1 GC-MS instrument. The chromatographic separations were made on a modified Varian Model 1400 gas chromatograph interfaced to the mass spectrometer with a metal–jet separator. The GC-MS was operated at the following conditions: on-column injection; 225°C injector; 250°C transfer line; 250°C separator; 270°C ion source; and He flow at 10 cm^3/min. The chromatograph was fitted with either a 1.83 m × 1.5 mm (ID) stainless steel column packed with 3% Dexsil on AW-DMCS treated 80–100-mesh Chromosorb W or a 4.57 m × 1.5 mm (ID) stainless steel column packed with 3% PDEAS on 100–120-mesh Gas-Chrom Q.

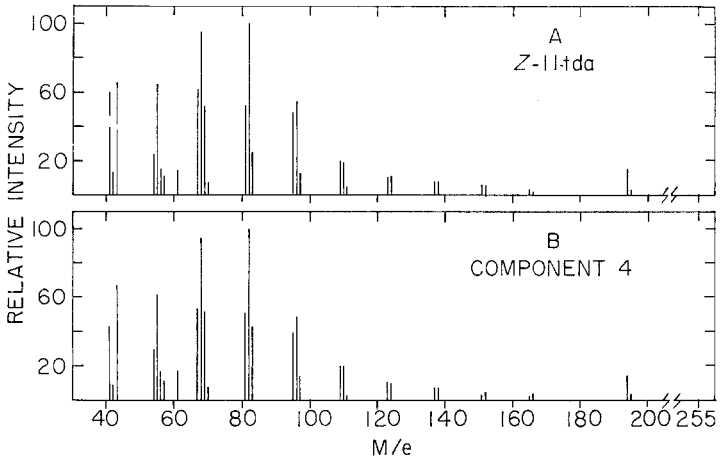

FIG. 4. Evidence for (*Z*)-11-tetradecenyl acetate from GC-MS analysis. A) Authentic sample; B) mass spectrum recorded at center of a resolved GC peak.

GC-MS analysis with PDEAS separation of the pheromone-active fraction from the SE-30 chromatography showed that the T_r and the mass spectrum of component 4 were identical to those of (*Z*)-11-tda. The mass spectrum shown in Fig. 4 was recorded at the center of the GC peak. Other mass spectra recorded over the profile of the GC peak were all constant and indicative of a single component.

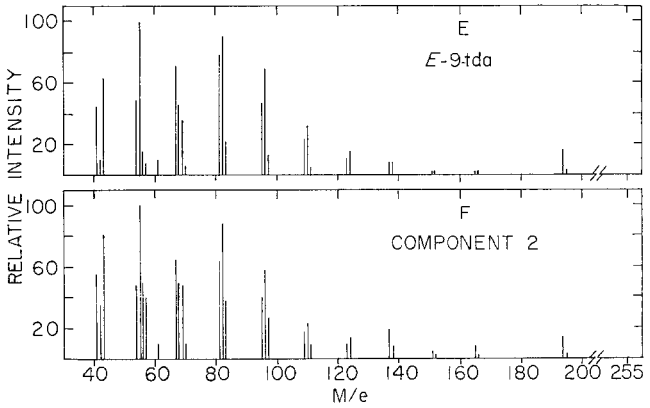

FIG. 5. Evidence for (*E*)-9-tetradecenyl acetate from GC-MS analysis. E) Authentic sample; *F*) mass spectrum recorded on the front side of an unresolved GC peak (see text and peak nos. 2 and 3 in Fig. 2).

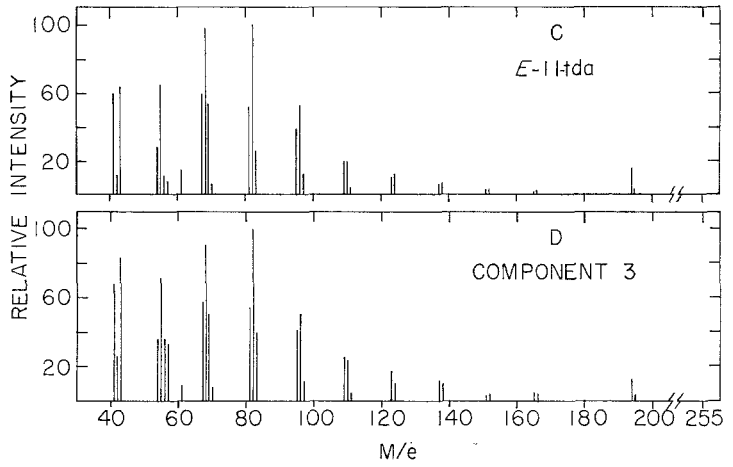

FIG. 6. Evidence for (*E*)-11-tetradecenyl acetate from GC-MS analysis. C) Authentic sample: D) mass spectrum recorded on the back side of an unresolved GC peak (see text and peak nos. 2 and 3 in Fig. 2).

Components 2 and 3 were not even partially resolved on the 4.57-m PDEAS column used in the GC-MS system. For this reason, mass spectra were recorded every 4 sec over the unresolved GC peak. Gradual changes were observed in successive spectra. Interpretation of these mass spectral scans showed that the mass spectrum obtained at the front side of the peak was nearly identical to the mass spectrum of standard (*E*)-9-tda (Fig. 5). The mass spectrum recorded at the backside of the GC peak was consistent with the mass spectrum of standard (*E*)-11-tda (Fig. 6).

Component 1 was fully resolved on the PDEAS column, and the T_r and the mass spectrum of this component were identical to those of authentic tda (Fig. 7).

Microozonolysis of the pheromone mixture was carried out by using procedures based on the work of Beroza and Bierl (1966, 1967). A microflask for ozonolysis was prepared by flaming a 35 mm × 12 mm Kimble screw-cap vial into a pear-shaped configuration. This was accomplished by rotating the base of the vial in a glassblower torch. The treatment produced a microflask with an internal volume of $\sim$0.5 cm^3 and a sharply conical interior that made it possible to quantitatively recover 2–3 μl of residual solution. Furthermore, the microflask was suitable for conducting ozonolysis reactions in as little as 30 μl of solvent.

Ozonolysis of the pheromone-active mixture was conducted as follows. A 50-μl CS_2 solution of the mixture containing $\sim$5 μg of the compounds

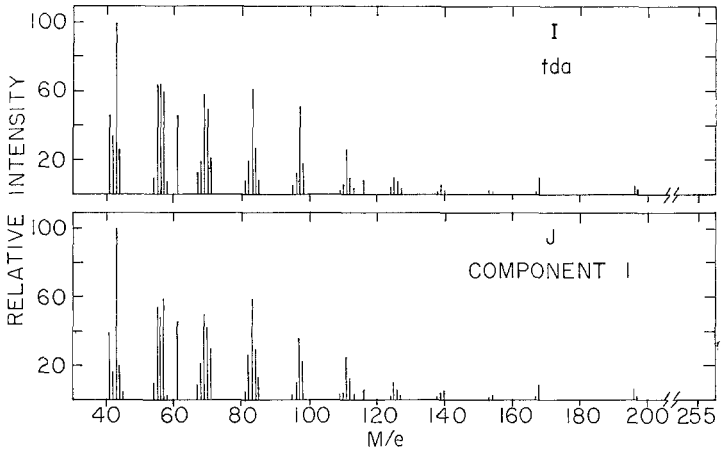

FIG. 7. Evidence for tetradecyl acetate from GC-MS analysis. I) Authentic sample; J) mass spectrum recorded at the center of a resolved GC peak.

was placed in the microflask. The flask was then attached to an O_3 generator in the manner described by Beroza and Bierl (1967) and immersed in an ice–isopropanol bath at $-10°C$ for 5 min. Next we generated and bubbled O_3 through the CS_2 solution at 1–2 cm^3/min for 45 sec. The gas flow was then shut off, and droplets of solvent that had splashed onto the side of the

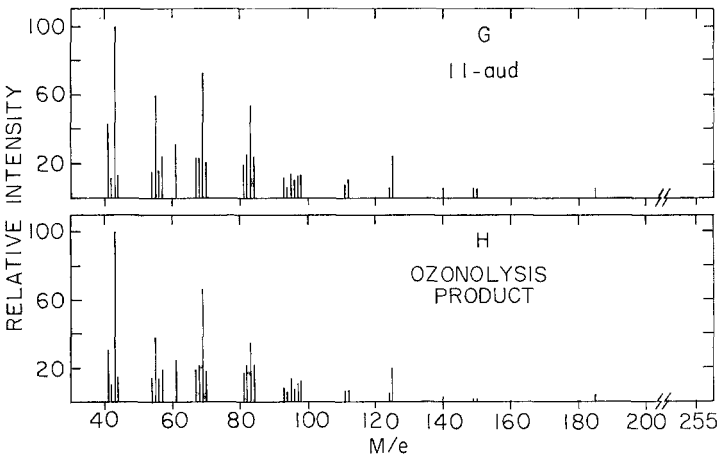

FIG. 8. Evidence for 11-acetoxyundecanal from GC-MS analysis of the ozonolysis products. G) Authentic sample; H) mass spectrum recorded at the center of a resolved GC peak.

flask were allowed to flow back into the cone of the microflask. About 0.5 mg triphenylphosphine was then added to reduce the ozonides.

When the pheromone-active mixture was subjected to the described ozonolysis reaction, the pheromonal activity was destroyed. GLC of the ozonolysis products on the 6-m PDEAS column showed that it contained three compounds whose T_r values were identical to those of standard tda (10.8 min), 9-acetoxynonanal (17.35 min), and 11-acetoxyundecanal (33.73 min). GC-MS analysis with a Dexsil chromatographic column confirmed the presence of tda and 11-acetoxyundecanal. The T_r values of these compounds and their mass spectra (Figs. 7 and 8) were identical to those of authentic tda and 11-acetoxyundecanal, respectively. There was not enough 9-acetoxynonanal in the sample to obtain a complete mass spectral confirmation of this component, but single-ion monitoring of *m/e* 97 (characteristic of 9-acetoxynonanal) showed a mass fragmentogram with a T_r that coincided with that of authentic 9-acetoxynonanal.

DISCUSSION

The data we have presented, in combination with previous chemical and behavioral research (Klun and Brindley, 1970; Klun et al., 1973), provide conclusive evidence that the geometrical isomers of 11-tda are an integral part of the pheromonal system of the Iowa ECB. It is noteworthy that the *Z*:*E* isomer composition of 11-tda found in the extracts of 24,000 Iowa ECB female tips was 95:5. This isomer proportion closely approximates the most favorable *Z*:*E* combination for attraction of Iowa ECB males in the field (Klun et al., 1973; Klun and cooperators, 1975). The data also show that the extracts of the female abdomen tip contain (*E*)-9-tda and tda. However, the ethological function of (*E*)-9-tda and tda in the pheromonal repertoire remains to be determined. Behavioral studies with various combinations of (*Z*)- and (*E*)-11-tda, (*E*)-9-tda, and tda are in progress and will be reported in a forthcoming paper.

The results of our analysis of the components of the extract of abdominal tips of the Iowa ECB compare favorably with the results published by Kochansky et al. (1975) for the London, Ontario, ECB. Thus, borers at London and Iowa are pheromonally similar. Indeed, comparative field-trapping studies showed that male populations at the two locations are also similar inasmuch as they were attracted to the same isomer blends of 11-tda (Klun and cooperators, 1975). Kochansky et al. (1975) ostensibly did not detect (*E*)-9-tda in the tip extracts of the London borer. Therefore, the females of the Iowa and London strains may differ with reference to (*E*)-9-tda. Alternatively,

a reinvestigation of the London borer might reveal the occurrence of (*E*)-9-tda.

Of the chromatographic methods we used for isolation of the C_{14} acetate esters, high-resolution GPC proved to be indispensable. This form of chromatography greatly simplified the isolation of the pheromonal chemicals from the complex mixture of chemicals encountered in the crude extracts of the female. The data plotted in Fig. 1 show that the μ Styragel columns could be used effectively to isolate the pheromones (C_{12}–C_{18} alcohols and acetates) of most Lepidoptera (moths) from the crude extracts of the insects. The high-resolution GPC columns resolved alcohols and acetate esters of the same carbon chain length. Therefore, this form of high-resolution GPC would be useful in preliminary studies of multicomponent sex pheromone systems that might involve, for example, alcohol–acetate ester or C_{12}–C_{18} chain length combinations of compounds. High-resolution GPC could also prove useful in isolation of lower-molecular-weight (<100) entities that often defy resolution from crude extracts by traditional isolation methods.

Another highly useful technique that we employed in the present study was high-resolution GC with support-coated, open tubular and wall-coated, open tubular glass columns. High-resolution open tubular GC greatly enhances the probable detection of small amounts of heretofore unresolved components in a pheromone mixture. These minor components may well play a vital direct and/or synergistic role in pheromonal communications.

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CHEMICAL RECOGNITION OF THE MOTHER BY THE YOUNG OF THE CICHLID FISH, *Cichlasoma citrinellum*

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Abstract—When placed in a *Y*-maze, the 1–10-day-old free-swimming fry of the Midas cichlid can chemically distinguish between their mother and plain water and between another mother and plain water. They do not distinguish between the two mothers offered together or between their father and plain water. Predation pressures make these responses adaptive.

Key Words—communication, fish, cichlid, *Cichlasoma citrinellum*, pheromone, maternal, development, predation, chemoreception, fry.

INTRODUCTION

Cichlid fish demonstrate efficient, persistent, parental care toward their brood, which may consist of 5 to 10,000 fry, depending on the species (Brichard, 1975; Fryer and Iles, 1972). Vision is one of the primary senses by which parents (Noble and Curtis, 1939; Myrberg, 1975) and fry (Kuenzer, 1968; Hay, 1976; Russock and Schein, 1974) recognize each other, but it is not the only one. Using chemical cues, parents can recognize their fry (Kühme, 1963; Myrberg, 1975; McKaye and Barlow, 1976) and each other (Caprona, 1974), and fry can recognize other fry (Kühme, 1963). However, efforts to show that fry can chemically recognize their parents have, until now, been unsuccessful (Cole and Ward, 1970). This paper presents the first evidence that cichlid fry can use chemical cues alone to respond to one of their parents.

METHODS AND MATERIALS

Normally colored Midas cichlids (*Cichlasoma citrinellum*) were obtained from stocks bred from adults collected in Lake Masaya, Nicaragua. Seven males were paired with slightly smaller females (standard length: males 146–171 mm, females 134–165 mm, all 3–5 years old) and bred in 240 liter aquaria (27–28°C, 12-h photoperiod). The eggs of each pair produced about 1500 nonswimming wrigglers (prolarvae) which, in about 3 days, became free-swimming fry (postlarvae).

The 5-mm-long fry were tested during the daylight portion of their light cycle in an opaque Plexiglas *Y*-maze (9 cm high, 6 cm wide, and 13 cm from start gate to choice point). Filtered, aerated water flowed from a reservoir through silicon tubing into two 15-gal (56.8 liter) stimulus tanks; each tank then fed one arm of the maze. Tests with dye showed that the water from both arms traveled down the alley in laminar flow, without mixing. The water emptied into a drain. The rate of flow (22.5 ml/sec) and the temperature (27–28°C) of the water entering each arm of the maze were about equal.

Before each test, both parents were removed from their home aquarium; one was placed in a stimulus tank, the other held in a separate aquarium. This procedure ensured that, on any day of testing, all fry had been exposed to both sexes for equal times. Five minutes after an adult was put in the stimulus tank, two fry were transferred by net to a start box. (If only one fry were used, it would remain motionless, but two fry swim up the maze readily). They remained there for 15 sec, then a gate was opened by raising a screen. After the first fry made a choice by entering an arm of the maze, or if no choice was made within 3 min, they were retrieved with a small net and discarded; thus, no fry was tested more than once.

Two more fry were put in the start gate and the process was repeated following Cole's (1962) modification of a sequential sampling design. This design determines the preference of the brood as a group by sequentially sampling the individual preferences of the fry. Fry were tested until the brood showed either a significant ($P<0.05$) preference for one side or no preference at all.

Broods that showed a significant preference for one side were retested by reversing the hoses leading into the maze and repeating the test for brood preference using additional untested fry. Only if a significant preference were now shown for the other side, ruling out a bias to swim consistently to one side, would the brood preference be considered valid.

The number of individual fry to swim toward each stimulus was also recorded, whether they came from a brood that showed a preference or from one that did not. Broods were tested on 8 of the first 10 days in which they were free-swimming.

RESULTS

The fry in a brood can distinguish between water from their own mother and plain water and between water from another mother and plain water.

TABLE 1. RESPONSES OF FRY TO WATER FROM PARENTAL ADULTS AND TO PLAIN WATER[a]

Stimulus	No. of times brood showed		Number of fry	χ^2 value	P
	A preference	No preference			
All days (1–10)					
Mother	6	2	113	56.12	0.001
Plain water	0		25		
Mother	1	3	42	1.50	ns
Other mother	1		54		
Other mother	3	2	74	32.52	0.001
Plain water	0		19		
Father	0	8	83	1.71	ns
Plain water	0		67		
Day 1					
Mother	4	0	60	54.26	0.001
Plain water	0		2		
Father	0	4	47	0.95	ns
Plain water	0		38		
Day 10					
Mother	2	2	53	11.84	0.001
Plain water	0		23		
Father	0	4	36	0.75	ns
Plain water	0		29		

[a] Brood preference ($P < .05$) was determined by sequentially sampling the preferences of individual fry in the brood. This yielded three data for each brood: the number of fry to choose one stimulus, the number of fry to choose the other stimulus, and the overall brood preference. Even broods showing no preference contained fry that chose one stimulus or the other. The number of fry making each choice was summed across all broods tested, both those that showed a preference and those that did not. The χ^2 value was calculated for number of fry only.

They do not distinguish between the two mothers offered together, or between their father and plain water (Table 1). Both mothers had fry of about the same age.

On the first day of free-swimming, 97% of the tested fry preferred their own mother over plain water. This percentage dropped significantly ($\chi^2 = 16.83$, $P<0.001$) to 70% on the tenth day. The number of broods preferring the mother also dropped.

During every run, both the water temperature and the rate of flow were recorded in each arm. No correlation could be found between slight fluctuations in these values in one arm of the maze and a preference for or avoidance of that side.

DISCUSSION

The impact of predators on the brood in nature suggests a function for the fry's differential abilities to chemically recognize their parents. Mated pairs of Midas cichlids defend territories up to about 1 m in diameter in front of rocky outcrops along the shores of freshwater lakes. Territories often abut one another, and each usually contains a crevice or cave into which the parents put the brood at night. During the day, parents chase away potential predators such as *Cichlasoma managuense*, *C. dovii*, *C. nigrofasciatum*, *Neetroplus nematopus*, *Gobiomorus dormitor*, and juvenile Midas cichlids. Without such parental defense, the brood would be consumed in minutes (Barlow, 1976; McKaye and Hallacher, 1973).

Although separation from the parents is fatal, a fry may become lost or may be displaced by the disturbance from an intense, territorial, agonistic encounter between its parents and another mated pair. In clear water both cichlid parents and their fry can find each other by using visual cues, but if the water is murky, as it often is in the Midas cichlid's natural habitat (Barlow, 1976), then parent and fry may orient by chemical cues. Since a brood contains up to 5000 fry, the parents presumably cannot retrieve every errant offspring, thus placing greater pressure on the fry's ability to find its parents. Chemical orientation could also occur at night, since predatory, nocturnal catfish (*Rhamdia nicaraguense*) sometimes scatter the brood from the cranny in which the parents have bedded it down (McKaye, unpublished, Yale University).

Because the parents have different roles in defending the brood, the fry respond to them differently. The male parent spends more of his time defending the territory—swimming out to threaten encroachers—than does the female, who, conversely, spends more time with the brood (McKaye, unpublished, Yale University). If a fry is displaced from its parents, an

approach to the male has a greater probability of placing it away from the brood and at the periphery of the territory, where predation is most intense. Thus, the fry's failure to swim toward the father in the maze may reflect an adaptive strategy rather than a lack of motivation.

This negative response to the father does not apply to all behavioral interactions. Noakes and Barlow (1973) have observed in the laboratory that, by day 25, fry contact and ingest dermal mucus from the father in preference to the mother; before day 25, contacts are equally distributed. The sensory basis for this choice has not been experimentally determined.

The fry's inability to distinguish between its own and another mother may also be adaptive. Because breeding sites are separated from one another by about a meter, the closest female to a separated fry is likely to be its mother. Thus, merely approaching the nearest female will usually return the fry to its brood. However, if a fry is displaced relatively far from its home territory, it can gain protection by approaching another female's brood. Parents have been shown to accept foreign fry into their brood if the foreign fry are the same age or younger than their own (Noakes and Barlow, 1973). and McKaye and McKaye (1977) have mathematically demonstrated that it is advantageous for them to do so. I am suggesting, therefore, that fry can survive by detecting "femaleness." I am conducting tests to determine whether the fry can discriminate maternal females from nonmaternal ones.

Myrberg (1966) suggested that cichlid parents recognize wriggling (nonswimming) young primarily by chemoreception' and then shift mainly to vision as the fry become free-swimming. It is possible that the dominant modality for recognition of the parent by the fry also changes from chemoreception to vision with increasing age. I am currently testing this hypothesis because the increase in the proportion of fry failing to recognize their mother on day 10 indicates a decline in sensitivity to maternal chemical cues.

Little (1975), working with crayfish larvae, has obtained parallel results. His studies, and those on Cichlidae, suggest that a chemically polluted environment can interfere with the success of parental care.

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SEX PHEROMONE COMMUNICATION IN THE NEMATODE, *Rhabditis pellio*

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Abstract—Both males and females of *Rhabditis pellio* release pheromones that attract the opposite sex prior to copulation. A quantitative bioassay for the female-produced pheromone was designed, based on male movement toward a pheromone source placed at one end of a 10-mm strip of bacterial material maintained on nutrient agar in a petri plate. Females produced pheromone from the age at which they attained the adult stage (~3 days following hatching from the egg) and maintained a relatively constant production level until at least the ninth day of life. Similarly, males became responsive to the female pheromone by the third day and remained responsive through the ninth day, although the time required for the males to migrate toward a female pheromone source increased with increasing age. No daily rhythm of pheromone responsiveness by males or pheromone production by females was observed when the nematodes were conditioned to a 12:12 h light–dark cycle.

Key Words—nematode, *Rhabditis pellio*, pheromone, quantitative bioassay, daily rhythms, age effects.

INTRODUCTION

The study of sex pheromones in nematodes is in its infancy, being mainly at the level of descriptions of which sexes and species employ premating chemical communication. In the first report of chemical attraction between nematodes, Greet (1964) found that, when males and females of *Panagrolaimus rigidus* were placed on opposite sides of a cellophane barrier in an agar trough, each sex accumulated at the barrier. Since that time, the existence

of pheromones that are released from one or both sexes and that attract nematodes of the opposite sex has been documented for a variety of plant-parasitic, animal-parasitic, and free-living nematode species (Anya, 1976).

The nematode, *Rhabditis pellio* (Schneider), is a facultative parasite of the earthworm, *Aporrectodea trapezoides* Duges (Poinar and Thomas, 1975). The nematodes encyst when in the third larval stage in the nephridia or coelom of the earthworms and remain there until the death of their hosts. Thereupon, the nematodes become activated and feed on the bacteria that grow on the decomposing earthworms. They mature, mate, and lay eggs, and several generations may ensue on the carcass during a few weeks. The primary bacterium which was isolated from *A. trapezoides* earthworms and which supported abundant growth of *R. pellio* was *Flavobacterium* sp. Using this bacterium growing on nutrient agar as a substrate for nematode development and behavior, we have recently characterized a number of the factors involved in the reproductive biology of *R. pellio* (Somers et al., 1977). This paper describes the role of male and female sex pheromones in the aggregation of the sexes prior to copulation, the influence of several biological variables in controlling pheromone communication, and the development of a rapid quantitative bioassay for the female-secreted pheromone.

METHODS AND MATERIALS

R. pellio was reared and isolated by sexes in the fourth larval stage, as described by Somers et al. (1977). The nematode colony was maintained at 22°C and a 12:12 h light–dark photoperiod.

Generally, pheromone was obtained by placing 55 adult virgin males or females on individual 35×10 mm nutrient agar plates previously inoculated with bacteria. After a specified time, usually 24 h, the nematodes were removed from the plates and the bacterial material containing the pheromone was collected with a camel-hair brush and placed in 1 dr glass vials. Control bacterial material was prepared similarly, from plates that were inoculated at the same time with bacteria but that had not contained nematodes.

Following a number of preliminary experiments, a bioassay technique was designed that was based on directed movement of test nematodes in a 2×10 mm strip of uncontaminated bacterial material painted onto the surface of an agar plate. Immediately before the assay period, 10 mg of freshly collected pheromone-containing and control bacterial material were diluted to 125 μl in distilled water. Approximately 3 μl of the diluted pheromone-containing material (representing ~ 1.3 nematode equivalents) and of diluted control material were placed on opposite ends of the bioassay strip. After 15 min, 10 assay nematodes were placed in the center of the strip

and, except when noted otherwise, their positions were recorded 15, 20, and 25 min thereafter. Percentage response to pheromone was determined by the formula

$$\frac{(T-C)\ 100}{T+C}$$

where T and C are the mean numbers (averaged over the three observation times) of nematodes located in the half of the strip containing the pheromone-treated and the control bacterial material, respectively. Each experiment was replicated 14–20 times, with no more than five replicates per day. A fresh bacterial strip and new responding nematodes were used in each replicate. Except when indicated, all females and males used in the various experiments were 5 and 4 days old, respectively. Ages were determined from the date of egg or larval deposition.

RESULTS

Comparison of Male and Female Responses

Both males and females (separately) were used in the preparation of pheromone-containing bacterial material. Also, both sexes were tested as responders to the pheromones of either sex. The mean percentages of nematode response to pheromones were:

(a) females responding to male pheromone, 44.6 ± 7.7 (SE);
(b) males responding to female pheromone, 26.3 ± 7.7;
(c) males responding to male pheromone, -13.8 ± 8.0;
(d) females responding to female pheromone, -7.9 ± 5.9.

Negative numbers indicate that the majority of responders were on the side away from the pheromone. Based on Duncan's Multiple Range Test at the 5% confidence level, categories (a) and (b) were significantly different from (c) and (d), although (a) was not different from (b), and (c) was not different from (d). Although the attraction of either sex to pheromones produced by the opposite sex [categories (a) and (b)] is significantly different from zero response, the tendency toward a repulsion of either sex when exposed to pheromones produced by the same sex [categories (c) and (d)] is not supported by statistical significance.

Duration of Female Pheromone Preparation

When females were left on bacterial plates for 2, 6, or 24 h before the pheromone-containing bacterial material was collected and used in bioassays,

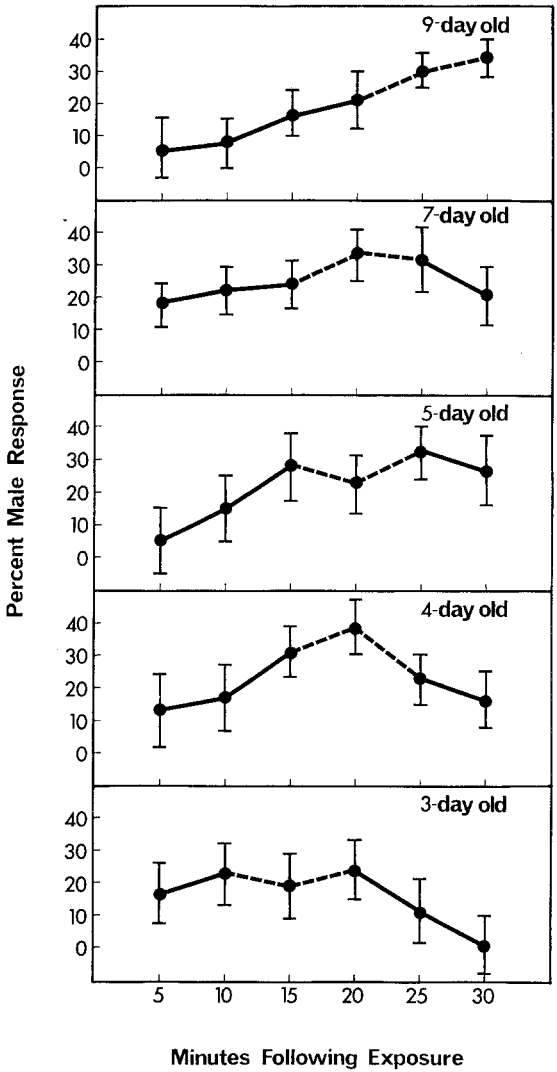

FIG. 1. Responses by *R. pellio* males of different ages at various times following exposure to pheromone produced by 5-day-old females. Broken lines connect the three consecutive means at times when the response was greatest. Vertical lines indicate standard errors.

male percentage responses of 35.8±6.5 (SE), 41.7±8.4, and 37.5±4.3, respectively, resulted. In each case, the pheromone-producing plates had been inoculated with bacteria 24 h prior to the assay, although the females were not placed on the plates until the indicated times before assay.

Age of Pheromone-Producing Females

Pheromone collected from virgin females of various ages elicited the following mean percentage responses by 3-day-old assay males (determined by averaging 10, 15, and 20-min observations of the assays): 3-day-old, 37.3±6.6 (SE); 4-day-old, 28.6±8.7; 5-day-old, 39.3±10.8; 7-day-old, 38.3±7.4; and 9-day-old, 17.4±10.4. None of the percentage responses differ significantly (Duncan's Multiple Range Test, 5% confidence level), although the response to pheromone produced by 9-day-old females tended to be the lowest.

Age of Responding Males

The responses of males isolated from females until the ages of 3, 4, 5, 7, 9 days and then used as responders to female-produced pheromone are shown in Fig. 1. The dashed-line portions of the curves showing the assay results at 5-min intervals indicate the time of the three highest responses for each age of male. Thus, 3-day-old males responded more rapidly, reaching their maximum response level at 10 to 20 min after initiation of the assay. On the other hand, 9-day-old males, which visually appeared less reactive, did not reach their maximum response until 20–30 min after assay initiation.

Daily Rhythms

Following conditioning of nematodes for several generations in a 12:12 h light–dark cycle, female pheromone was collected from plates four times at 6-h intervals during several separate assay days. In each case, the pheromone-producing plates had been set up 24 h prior to assay, and females had been placed on the plates either 6 or 12 h prior to assay. Thus, assay males at 6-h intervals beginning at the onset of the light period were exposed to pheromones that had been collected from females for 6 or 12 h prior to each assay. The male responses were:

0 h of light period, 22.5±6.6 (SE);
6 h of light period, 23.8±7.6;
0 h of dark period, 29.4±8.6;
6 h of dark period, 38.8±6.4.

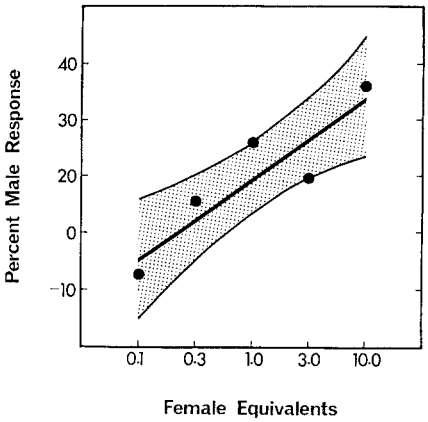

FIG. 2. Responses of *R. pellio* males to various concentrations of female pheromone ($Y = 11.91 + 14.37X$, where Y = percentage male response and X = female equivalents transformed to a log scale). The shaded area indicates a 95% confidence limit on each side of the regression line.

None of these responses differs significantly from the others, although a slight tendency is seen, indicating possible higher female pheromone activity (or higher male responsiveness) in the middle of the dark period.

Concentration–Response Curve for Female-Produced Pheromone

Different concentrations of pheromone were obtained by maintaining differing numbers of females for 24 h on bacteria-coated plates. After dilution with distilled water and dispensing of subsamples onto the assay strips, plates that had contained 3, 10, 30, 100, or 300 females provided 0.1, 0.3, 1.0, 3.0, or 10.0 female equivalents for bioassay. Percentage male responses to this array of pheromone concentrations are shown in Fig. 2. The linear regression between male response and pheromone concentration is statistically significant at the 5% confidence level ($F = 11.92$).

DISCUSSION

This research has resulted in a relatively simple and rapid bioassay for a nematode pheromone. The fairly low level of reaction to the pheromone, averaging ~30% response for males exposed to 1–10 female equivalents,

may be caused by a number of factors. First, the typical communication distance in nature may be very short, even shorter than the 5 mm on our assay strip between the initial position of the responders and the pheromone source. Second, habituation or sensory adaptation may have caused some of the nematodes to cease responding to the pheromone and to leave the pheromone source after initially being attracted there (Croll, 1970); such factors may have caused the apparent reduction in responsiveness of 3-day-old males after they had been in the bioassay for longer than 20 min. Third, the initiation of the assay only 15 min after the pheromone was inoculated at one end of the bacterial strip may not have allowed time for formation of a pheromone concentration gradient adequate for directional nematode orientation; however, preliminary experiments with longer lag periods between pheromone inoculation and initiation of the assay did not result in higher nematode responses. Fourth, much pheromone may have been lost through its diffusion into the relatively large agar mass underlying the bacterial material, both during the time the nematodes were releasing pheromone into the material and after the material was inoculated into the bioassay strip. Such a possibility is suggested by the finding that bacterial material which had held females for 2 h was no less biologically active than that which had held females for 24 h. Fifth, the pheromone may be highly labile, being transformed rapidly to an inactive state in the bacterial material. Sixth, males and females might produce secretions repellent to others of the same sex (see later discussion). If this is the case, then 10 assay responders might cause ambiguity, with their mutual attraction to a pheromone source interacting with their mutual repellency. Obviously, much fertile research remains to be conducted on these problems.

The mutual attraction of males for females and females for males appears to be typical for several zooparasitic nematodes, including *Trichinella spiralis* (Bonner and Etges, 1967), *Aspicularis tetraptera* (Anya, 1976), and *Nippostrongylus brasiliensis* (Bone et al., 1977). Also, Bonner and Etges suggested that males of *T. spiralis* exerted a repellent effect on other males, an effect that is also suggested by our data. Such a repellent reaction in *R. pellio* might be advantageous in maintaining an optimum distance between competitive members of the same sex for increased probability of mating.

Both males and females of *R. pellio* are capable of mating many times following their attaining of the adult stage at 3 days old and continuing throughout their lives (Somers et al., 1977). This continued reproductive activity is correlated with sustained pheromone secretion by females and pheromone responsiveness by males, at least through the age of 9 days. On the other hand, the *speed* of responsiveness decreased with increasing male age (Fig. 1). The 9-day-old males on the assay strips were observed to be very slow moving, corresponding with prior observations that the metabolic

activities and the locomotion rate of nematodes decrease with increasing age (Van Gundy, 1965; Zuckerman, 1974).

The reproductive potential of *R. pellio* males and females shows a much more discrete peak than does their responsiveness to or production of female pheromone (Somers et al., 1976). At 4 days of age, both sexes, when allowed unlimited mating, produce more offspring than do older or younger nematodes.

It seemed reasonable to suspect the presence of a diurnal rhythm of pheromone production or pheromone responsiveness in *R. pellio*. Circadian rhythms have been demonstrated in other nematode species. For example, *Nippostrongylus brasiliensis* was demonstrated to have an increased migration rate at night as compared to day (McCue and Thorson, 1965). However, except for a tendency (not statistically significant) for *R. pellio* males tested in the middle of the dark period to be most responsive to female pheromone collected during the previous 6 h, no rhythm was evident in this species. Perhaps rhythmic behavior in nematodes such as *R. pellio*, which live in the ground, might be entrained by cues other than light or dark. One such cue might be changes in temperature.

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THREE HIGHLY ODOROUS METABOLITES FROM AN ACTINOMYCETE: 2-ISOPROPYL-3-METHOXY-PYRAZINE, METHYLISOBORNEOL, AND GEOSMIN

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Abstract—The highly odorous metabolites isopropylmethoxypyrazine, methylisoborneol, and geosmin were identified from a *Streptomyces* sp. Their role in the odor pollution of water is discussed. 2-Isopropyl-3-methoxypyrazine has not been reported previously from actinomycetes.

Key Words—Actinomycete, *Streptomyces*, 2-isopropyl-3-methoxypyrazine, methylisoborneol, geosmin, actinomycete odor, metabolite odor.

INTRODUCTION

In 1974 some actinomycetes were received from M. Taylor of Apopke, Florida. The odor emanating from a single culture tube of one of them was sufficient to be noticed by most persons within a moderately large laboratory. Like that of geosmin (I) (Gerber and Lechevalier, 1965) the odor tended to linger and cling to apparatus and people. Many reported the odor as familiar and described it, according to their degree of chemical sophistication, as "musty," "like roots," "like pyridine or lutidine." However, none of the several experienced microbiologists here had ever before noticed this odor produced by a microorganism. Furthermore, the odor was distinctly different from that of geosmin (I) or methylisoborneol (II), which are previously known odorous metabolites of actinomycetes (Gerber, 1968, 1969; Medsker et al., 1969) and which have been isolated from odor-polluted waters (Piet et al.,

1972; Rosen et al., 1970), and their inhabitants (Kikuchi et al., 1973). We were able to produce in liquid cultures and isolate in pure form enough of the new odorant to identify it as 2-isopropyl-3-methoxypyrazine (III).

METHODS AND MATERIALS

The NMR, IR, and UV spectra were obtained with the Varian T-60, Perkin-Elmer Infracord, and Carey Model 14 instruments, respectively. Eastman Chromagram 13179 Silica Gel (No. 6061) cut into 4×10 cm strips was used for TLC. GC was carried out on an F & M model 700 dual-column instrument with a thermal conductivity detector and 1.83 m $\times$ 3.2 mm copper columns packed with 10% SE-30 on Diatoport S, 60–80 mesh. The alumina for column chromatography, Baker aluminum oxide powder, had been previously stirred with distilled water for several hours and air dried. Applied Science "Hi-Flosil" silica 60/200 mesh was used for column chromatography without further treatment. Cultures were grown at 28°. Shakers used were the New Brunswick Scientific Co. model V rotary action at 220 rpm or the model 5713 reciprocal action at 68 strokes/min. The Bennett's medium contained 1 g Difco yeast extract, 1 g Difco beef extract, 2 g Sheffield Farms N-Z amine-A (a casein hydrolysate), and 10 g Cerelose (a commercial grade of glucose) made up to 1 liter with tap water, and the pH was adjusted to 7.3 before autoclaving. The "OPG" medium contained 2 g Difco yeast extract, 2 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4 \cdot 7H_2O$, and 10 g Cerelose per liter of distilled water; no pH adjustment was made, but 0.5 g $CaCO_3$ was added to each 2-liter flask before autoclaving.

Preliminary Experiments

The odorous actinomycete was identified as a *Streptomyces* sp. by M.P. Lechevalier and designated A-10. It was grown in submerged culture, and steam-volatile products were isolated as has been described (Gerber and Lechevalier, 1965). The nature and yields of volatile products as measured by GC were extremely dependent on the growth medium used. When a soybean–peptone–NaCl–glucose medium was used, there were only traces of volatile substances. With "OPG" medium, mainly geosmin, I, and methylisoborneol, II, were obtained. With Bennett's medium, however, the major volatile product had the characteristic odor of the culture and during GC it was eluted well before II, which was also observed. Geosmin and methylisoborneol were identified by comparison with authentic samples. The comparison pairs were identical in odor, retention time, and, in the case of II, by the facile dehydration to a mixture with a characteristic GC pattern of three peaks.

TABLE 1. RESULTS OF SOME PRELIMINARY EXPERIMENTS IN THE PRODUCTION OF VOLATILE SUBSTANCES BY *Streptomyces* SP. A-10 AND TWO OF ITS VARIANTS

Strain	Aeration[a]	Result[b]
A-10-1	higher	no volatiles
	lower	II and III, both 0.5 mg[c]
A-10-9	higher	II, 0.2 mg; III, 2 mg
	lower	II, 0.5 mg
A-10	higher, lower	II, 0.2–0.3 mg

[a] For higher aeration, use less liquid medium in each flask and/or more rapid shaking; for lower aeration, the reverse.
[b] All harvested after 6 or 7 days of shaking. All prepared from 48-h seed flasks.
[c] Yields, obtained by GC analysis, are given in milligrams per liter of whole broth and are ±20%.

The retention times of III, II, and I were 11, 14, and 20 min at 80°, programmed at 5°/min. In subsequent runs, however, the yield of volatile products declined. Therefore, variants of A-10, designated A-10-1 and A-10-9, were tested on Bennett's medium under conditions of higher and lower aeration. The results are shown in Table 1. Subsequent experiments using A-10-9 verified the requirement for the higher aeration and for the 48-h seed flasks. Yields of the new volatile substance, III, were higher after 8 days of shaking than after 6 or 4 days.

Production and Isolation of III

Streptomyces sp. A-10-9 was maintained in sterile soil, incubated at 28° until well grown, then stored in the refrigerator. For production, a small amount of the soil was inoculated into several flasks of Bennett's broth, with 50 ml of broth per 250-ml Erlenmeyer flask. After 48-h shaking at 220 rpm, the whole broth (cells and beer) from each flask was divided among five flasks of Bennett's broth, 200 ml per 2 liter flask. After 8 days of shaking at 68 strokes/min, the combined whole broth was distilled until one tenth of its volume had been collected as distillate. The distillate was extracted twice with methylene chloride and the extracts concentrated. Yields, 2–5 mg/liter of whole broth, were measured by GC, but preparative GC was not satisfactory. Thus, purification was accomplished by column

chromatography. Eluate fractions were monitored by GC and by TLC with benzene–chloroform 4:1 as the solvent system. As soon as the solvent had evaporated, III could be well visualized by iodine vapor or alkaline permanganate spray; $R_f = 0.7$. Compound III could also be measured by its UV absorption at 275–290 nm. A methylene chloride extract of a distillate, which contained about 50 mg of III by GC assay, was purified first on 20 g of alumina, then on 25 g of silica by eluting with CH_2Cl_2 in both cases. The chromatography column used with alumina had an inner diameter of 15 mm; for silica the inner diameter was 19 mm. The volumes of the eluate fractions, 20 ml and 25 ml, respectively, were equivalent to the weight of absorbent used. From alumina, II and III eluted together in fractions 1–3; from silica, II was found in fraction 5 and III in 7–9.

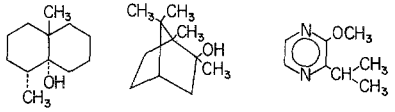
Identification of III

The UV absorption spectra of III suggested that it was an aromatic, very weak base. The maxima were found at 275 (290) nm in neutral methanol, (275) 295 nm in slightly acidic methanol, and 305 with about double intensity in methanol and concentrated HCl. By means of a spectrophotometric assay of upper and lower layers, it was determined that III was only slightly extracted (5%) by 10% HCl from chloroform but was well extracted (>75%) by concentrated HCl. On the nonpolar GC column, III eluted between *p*-cymene and naphthalene, with a log R_t which suggested a boiling point of about 190°. [I and II have reported boiling points of $252 \pm 2°$ and $208 \pm 2°$ (Piet et al., 1972) and 254° and 210° (Rosen et al., 1970).] The NMR spectra showed methyl bands at 1.2 and 1.3 δ, methoxy at 4.0 δ, and two aromatic protons at 7.9 and 8.05 δ. Thus, III was presumptively identified as an isopropyl methoxypyrazine. The confirmation of III as 2-isopropyl-3-methoxypyrazine followed from its IR absorption spectrum, which matched the published maxima (Seifert et al., 1970) and from the agreement of the NMR spectra with published values (Bramwell et al., 1972) as well as from a comparison of the mass spectrum with published values (Seifert et al., 1970; Murray et al., 1970). In a direct comparison with an authentic sample, the retention times were also identical: 10.8 min at 80°, programmed at 5°/min.

DISCUSSION

It has been known for a long time that some actinomycetes produce earthy-musty odors. The role played by actinomycetes in giving water and fish undesirable odors and tastes has been investigated; see the references given by Gerber and Lechevalier (1965). However, not until geosmin and

methylisoborneol, I and II, had been identified as earthy-smelling and camphor-like metabolites of actinomycetes (Gerber, 1968, 1969; Medsker et al., 1969) and reference samples were available for comparison, were I and II isolated and characterized from odor-polluted waters (Piet et al., 1972; Rosen et al., 1970; Kikuchi et al., 1973).



geosmin, I. methylisborneol, II. 2-isopropyl-3-methoxypyrazine, III.

2-Isopropyl-3-methoxypyrazine (III) is known to be found in galbanum oil and peas (Murray et al., 1970) as well as in potatoes (Buttery and Ling, 1973; Meigh et al., 1973). Compound III has also been isolated from *Pseudomonas perolens* grown on sterile fish muscle (Miller et al., 1973) and has been chemically synthesized (Seifert et al., 1970; Bramwell et al., 1972). Compound III is considered to be "likely of major significance in the flavour of peas" (Murray et al., 1970), the major contributor to the earthy aroma of potatoes (Buttery and Ling, 1973), responsible for the musty aroma produced in sterile milk by *Ps. taetrolens* (Morgan et al., 1972), and responsible for the musty, potato-like odor of some spoiled, chilled fish (Miller et al., 1973).

The threshold odor concentration of III has been reported to be 0.002 ppb (Seifert et al., 1970; Murray et al., 1970). Since the threshold odor concentration of I and II are 0.2 and 0.1 ppb, respectively (Rosen et al., 1970), it is obvious that III is likely to be isolated as an odor pollutant of rivers, lakes, and their inhabitants. In order to obtain sufficient I and II from water to identify them by mass spectrometry, Rosen et al. (1970) passed 3000 liters of odorous water through carbon filters. Piet et al. (1972) utilized 50-liter water samples, but identified I and II by odor and GC retention time only. Kikuchi et al. (1973) obtained 15 μg of geosmin from 1.5 liters of packed blue-green algal cell mass taken from shallow water.

Furthermore, because of its greater volatility, III would be lost during some solvent concentration steps that were satisfactory for I. A 50% loss was noted when 30 ml of a CH_2Cl_2 solution containing 20 mg of III was evaporated to 1 ml. Therefore, to isolate III from natural water, one should probably use special freezing-out techniques (Silvey et al., 1968) or cold-trapping of volatiles (Henley et al., 1969) or further concentration by back-flushing (Piet et al., 1972) or absorption onto a porous polymer as has been used for the volatiles from urine (Liebich and Al-Babbili, 1975).

The GC tracing of a reservoir water concentrate published by Piet et al. (1972) contains a small peak labeled "potatoes." This tracing also shows

peaks for I and II; II is labeled "camphoric." The relative retention times shown are in full accord with the "potato" peak being III. It should also be noted that a "very small basic fraction with an extremely powerful smell resembling that of cyclic bases such as pyridine and quinoline" was obtained from the steam distillate of previously heat-sterilized silicate rocks that had been exposed for some time to a warm, dry atmosphere (Bear and Thomas, 1964). These experiments successfully duplicated the well known "argillaceous odour" or "smell of rain." Thus, III or similar substances may be detected when such experiments can be repeated using GC and mass spectrometry.

Once exposed to the characteristic odors of I, II, and III, most people can readily distinguish among them. The difficulty is that most people do not possess the precise vocabulary needed to describe these differences. Clearly, II is "like camphor," by our custom, I is called "earthy," and III "musty." However, before our chemical characterization of III, no one suggested it smelled like potatoes and some still disagree with any description of III as smelling like potatoes.

A musty agent (mucidone) reported from a streptomycete (Dougherty et al., 1966) which had been implicated in musty taste and odor problems in the Cedar River was later identified as 3-isobutyl-6-ethyl-2-pyrone (Sipma et al., 1972). Since the synthetic pyrone had a weak, fruity odor, the intense mustiness of the natural material, mucidone, was felt to have been caused by an extremely odorous trace contaminant, possibly I. The GC retention times of mucidone and I were very nearly identical (Committee Report, 1970). From another *Streptomyces* sp., I was coproduced and it did cochromatograph with an 11-carbon lactone of somewhat different structure (Gerber, 1973). However, again considering that the threshold odor concentration of III is 1/100 that of I, there remains the possibility that the mustiness of mucidone was caused by traces of III, since the published GC tracing (Dougherty et al., 1966) did show minor peaks with significantly lower retention times, any one of which may be III. In addition, potassium permanganate was used successfully to treat the Cedar River taste and odor problem (Cherry, 1962) although potassium permanganate is usually considered to be of little or no value in the removal of odors from water (Gauntlett and Packham, 1973). This belief is reinforced by the knowledge that I and II are saturated tertiary alcohols which are not affected by permanganate. However, III is rapidly destroyed by permanganate, which further suggests that the Cedar River musty episode may have involved III.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS

V. Moth Scales as a Stimulus for Predation of *Heliothis zea* (Boddie)[1] Eggs by *Chrysopa carnea* Stephens Larvae[2–4]

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Abstract—Data are presented that demonstrate the responsiveness of *Chrysopa carnea* Stephens larvae to kairomones in the moth scales of *Heliothis zea* (Boddie). Rates of predation by *C. carnea* on eggs of *H. zea* were increased when *H. zea* moth scales or a hexane extract of the scales was applied to the search area under laboratory and greenhouse conditions.

Key Words—*Chrysopa carnea*, *Heliothis zea*, kairomones, predators, biological control.

INTRODUCTION

General predators have potential as biological control agents of pest insects infesting annual plant crops because they can maintain their populations by feeding on alternate prey during periods of low pest density (Ehler and

[1] Lepidoptera: Noctuidae.
[2] Neuroptera: Chrysopidae.
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[4] Mention of a commercial or proprietary product in this paper does not constitute endorsement of this product by the USDA.
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van den Bosch, 1974). *Chrysopa carnea* Stephens is such a predator (van den Bosch and Hagen, 1966), and it has been reported as a valuable control agent against some insect pests (Quaintance and Brues, 1905; Whitcomb and Bell, 1964; Reed, 1965; van den Bosch and Hagen, 1966; Ridgway and Jones, 1968; Lingren et al., 1968; Ridgway, 1969; Ehler and van den Bosch, 1974). The potential for *Chrysopa* spp. in the biological control of various pest species was discussed by Doutt and Hagen (1949), Lingren et al. (1968), Ridgway and Jones (1968, 1969), Scopes (1969), and others.

Laing (1937) and Lewis et al. (1971, 1972, 1975a, 1975b) reported that kairomones for the egg parasite *Trichogramma* spp. are left by ovipositing moths. Lewis et al. (1972) demonstrated that moth scales are at least one source of these chemical mediators. Jones et al. (1973) identified the hydrocarbon tricosane as the primary kairomone in *Heliothis zea* (Boddie) moth scales for *T. evanescens* Westwood. The use of kairomones by predaceous insects has also been demonstrated (Wood et al., 1968; Vité and Williamson, 1970; Wilbert, 1974; Hagen et al., 1976).

The studies reported herein determined that the presence of moth scales increased predation by *C. carnea* and that the cue(s) is (are) an extractable kairomone(s) with potential value in pest control procedures.

METHODS AND MATERIALS

The *C. carnea* used in these studies were purchased from Rincon Insectary, Inc., Oak View, California. The larvae were fed *H. zea* eggs that had been processed according to the procedure described by Burton (1969), and were held with food for at least 2 days at ~26°C and 70% RH prior to their use in the bioassays.

Heliothis zea eggs were used to monitor the feeding activities of the *C. carnea* in the experiments. These eggs were also processed according to Burton (1969), were irradiated with 25 krad (CO^{60}) when they were 8–36 h old to prevent eclosion, and were stored at ~10°C.

The laboratory experiments with natural *H. zea* moth scales and a hexane extract of moth scales were conducted in 150 mm × 15 mm petri dishes containing six *H. zea* eggs and two *Chrysopa* larvae per dish. The host eggs were placed directly on the glass with a small camel's hair brush, by using saliva as an adhesive, and were equally distributed throughout the dish. After exposure, the number of eggs fed on was determined with a dissecting microscope.

The greenhouse experiment with a hexane extract of *H. zea* moth scales was conducted on pea seedlings grown in pie pans 23 cm in diameter. Ten *Chrysopa* larvae and 15 *H. zea* eggs were placed on plants in each pan and,

after a 3-h exposure, the number of eggs fed on was determined as before.

The paired t test was used to determine whether differences between treatments were statistically significant. Arcsin transformations were conducted on percentages prior to analysis.

PROCEDURES AND RESULTS

Experiment 1

Experiment 1 was conducted to determine whether *H. zea* moth scales would stimulate increased predation by *C. carnea* larvae. First the inside bottom surface of a series of petri dishes was lightly brushed with a small amount of *H. zea* scales, using a camel's hair brush. Then *C. carnea* were introduced, and predation was determined in 10 paired replications of four dishes each for treatment and control during a 4-h exposure period. The mean percentage of eggs fed on in the dishes brushed with moth scales of *H. zea* was 66.1%, significantly ($P = .02$) more than the 48.5% fed on in the control dishes. These results demonstrate that moth scales of *H. zea* possess cues that stimulate increased predation by larvae of *C. carnea*.

Experiment 2

To determine whether the cues were chemical mediators that could be extracted in a similar manner as those for egg parasitoids, *Trichogramma* spp., a hexane extract (Jones et al., 1973) of the *H. zea* scales (diluted 1/1000) was evaluated as a stimulus. The eggs used in this experiment were removed from the paper linings of oviposition containers with a brush and were not processed with a sodium hypochlorite solution as were the eggs used in the other experiments.

The treated dishes were sprayed with the extract at a rate of $\sim$1 ml/dish with an aerosol spray device (Lewis et al., 1972); control dishes received no spray. The eggs were exposed to the *C. carnea* larvae for 2 h. The results were evaluated on the basis of results from 16 replications consisting of 20 dishes/treatment per replication.

The mean percentage of eggs fed on in the treated dishes (28.2%) was significantly higher ($P = .05$) than the feeding observed for the control dishes (25.5%). These results demonstrate that there are extractable kairomone(s) in *H. zea* moth scales that stimulate increased predation.

Experiment 3

The greenhouse experiment was conducted to determine whether the extract could be used to increase predation by *C. carnea* on plant substrates.

The treated plants were sprayed with the 1/1000 dilution of the hexane extract of *H. zea* scales at the rate of 1 ml/pan. The experiment consisted of five replications with 15 treated and 15 control pans/replication.

The mean percentage of eggs destroyed on the treated seedlings (40.6%) was significantly higher ($P = .05$) than that on the control seedlings (33.4%). These results demonstrate that the kairomone(s) does (do) influence the rate of predation by *C. carnea* on plant substrates and indicate that it could be used to increase predation under field conditions.

DISCUSSION

The results demonstrate that scales left by ovipositing *H. zea* moths stimulate predation by larvae of *C. carnea*. A hexane extract of the scales also caused a slight increase in predation, indicating that the activity was the result of one or more kairomones, perhaps hydrocarbons similar in nature to those identified for certain parasitoids, which may offer potential for manipulation procedures in pest-control programs. The level of response was not as great or as consistent as that reported for *Trichogramma* spp. (Lewis et al., 1972, 1975a). This may be because *H. zea* is not a preferred prey, or perhaps *C. carnea* is not as well adapted for this mechanism of prey finding.

Honeydew of aphids, mealybugs, and other Homoptera possesses kairomones that attract and induce oviposition by adults of *C. carnea* (Hagen et al., 1970). The amino acid tryptophan has been identified specifically as the source of an attractant for the adults of *C. carnea* (Hagen et al., 1976). Perhaps trails of aphids and other preferred prey provide a stronger prey-seeking stimulus for the larval stage of this predator.

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PHENOLICS IN BLACK OAK BARK AND LEAVES

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Abstract—Catechin, quercitrin, robinin, quercetin 3-methyl ether, scopoletin, cholorogenic acid, several leucoanthocyanins, and condensed and hydrolyzable tannins were identified in bark and leaves of *Quercus velutina* Lamarck. The concentrations of most phenolics in leaves increased as the growing season progressed, whereas those of most phenolics in bark remained essentially unchanged. Qualitative differences in bark and leaf phenolics among different trees were negligible.

Key Words—*Quercus velutina*, *Fagaceae*, phenolics, flavonoids, coumarins, tannins, leucoanthocyanins, fungal inhibitors.

INTRODUCTION

The presence of certain phenolics in higher plants seems to inhibit the feeding of phytophagous and bark-boring insects (Feeny, 1970; Berryman, 1972). Phenolics also affect invasion by parasitic fungi (Kuć, 1963; Hare, 1966). Some of our preliminary work indicates that the concentrations of phenolics in oak twig bark tend to decline after the trees have been defoliated by such insects as the gypsy moth, a change that could help to explain why trees become more susceptible to secondary attacks by fungi and insects after they have been defoliated.

Black oak (*Quercus velutina* Lamarck) is often attacked by fungi and bark beetles after it has been defoliated by phytophagous insects (Staley, 1965; Nichols, 1968), but little is known about phenolics in black oak. The present work was undertaken as a preliminary study to identify the principal phenolics in the leaves and bark of black oak, as well as to detect seasonal variations and variations among trees.

METHODS AND MATERIALS

Twelve trees, about 10 m tall, from one area were sampled each month from May through November 1974; another 9, of similar height, from a different area were sampled in the same months of 1975; both areas are in North Madison, Connecticut. Branches about a meter long were severed with a pole pruner about 4 m above ground and brought to the laboratory within half an hour. Usually, only one branch per tree was removed, but in some cases, several branches were taken for within-tree comparisons. Leaves were removed and bark was excised down to the cambium at about the fourth internode. A 5-g sample of fresh tissue from each tree was homogenized in a Waring blender[1] with 100 ml of 80% ethanol; the homogenate was filtered under suction and rinsed with another 50 ml of 80% ethanol. The resulting liquid filtrate was evaporated to 15 ml and stored at −30°C until analyzed.

Paper chromatography (PC)

n-Butanol:water:acetic acid (BWA) (4:5:1), 5% acetic acid (HAc), and isopropyl alcohol:water (IPW) (1:4) were used as solvents for descending development for one-dimensional (1-D) PC. For two-dimensional (2-D) PC, first HAc, then BWA, was used as the solvent (Parker, 1974). Phenolics were detected with Pauly's reagent (Linksens, 1959), 2% $FeCl_3$ in water (Hänsel, 1959), silver nitrate-NH_3 (Stange, 1959), or vanillin reagent (Stange, 1959). Fluorescent compounds were initially detected with long-wave UV (Ultra-Violet Products Inc., model UVSL-58).

High-Pressure Liquid Chromatography (HPLC)

HPLC was performed with a Waters model M-6000 chromatography pump with differential refractometer, model R-401. The particular fraction to be tested was dried under an airstream and taken up in about 3 ml of 60% methanol. This solution was filtered under pressure through a 50-nm filter before injection. A 10-μl sample was injected and eluted with 60% methanol carrier solution flowing at 0.9 ml/min through a Waters micro-Bondapak C_{18} column. The pump operated at 1000–5000 lb/in.[2] Retention times of known and unknown compounds were compared when they were assayed either separately or together. Peaks were recorded on a Hewlett-Packard

[1] The use of trade, firm, or corporation names in this publication is for the information and convenience of readers. Such use does not constitute endorsement or approval by the Forest Service or the Department of Agriculture of any product or service to the exclusion of others that may be suitable.

model 7127A recorder. Fractions were collected as the peaks occurred and were then scanned by UVS or hydrolyzed to determine their constituents.

Ultraviolet Spectroscopy (UVS)

UVS was conducted according to procedures described by Mabry et al. (1970). Eluates from HPLC or papers were scanned in a Hitachi spectrophotometer from 210 to 420 nm. Any spectral shifts occurring after the addition of solutions of sodium methoxide or $AlCl_3$ were noted (Mabry et al., 1970).

Eluates from HPLC or papers were hydrolyzed by boiling with an equal volume of 3*N* HCl for 1 min. The hydrolyzate was spotted on silica-gel thin-layer chromatograms (TLC) and sprayed after development for sugars, or spotted on papers, as described in a previous paper (Parker, 1974). This procedure served to identify both sugars and aglycones. Leucoanthocyanins were identified only as substances that turned red on hydrolysis.

RESULTS

Bark

At least 39 different compounds were detected in bark when 2-D papers were sprayed with vanillin, $FeCl_3$, or silver nitrate reagents or placed under UV illumination (Tables 1 and 2). These compounds included several sugars (Nos. 23–25) and probably a number of polyhydric alcohols (Nos. 26–29). The rest had the various characteristics of phenolics. The most prominent bark phenolics were identified by all three methods as quercitrin (quercetin 3-*O*-rhamnoside), robinin (kaempferol 3-*O*-robinoside 7-*O*-rhamnoside), quercetin 3-methyl ether, catechin, several leucoanthocyanins, and hydrolyzable and condensed tannins (Table 1). Condensed tannin, when hydrolyzed, yielded catechin. Hydrolyzable tannin yielded gallic acid, catechol, pyrogallol, and glucose when hydrolyzed. Gallocatechins (Table 1) were identified on the basis of PC only.

Under UV illumination, at least nine fluorescing compounds were found in bark (Table 2). Two of the most prominent blue-fluorescing compounds were identified by all three methods as scopoletin and chlorogenic acid. A third was identified on the basis of hydrolysis and PC as the glucoside skimmin.

Qualitative differences in bark phenolics among different trees were slight. There was as much variation between the branches of one tree as between branches from different trees. Quantitative differences in bark phenolics

TABLE 1. COLORS OF EXTRACTS OF BLACK OAK BARK OR LEAVES BROUGHT OUT ON 2-D PAPER CHROMATOGRAMS SPRAYED WITH THREE DIFFERENT REAGENTS[a]

Spot No.	Name	R_f values		Colors elicited by			Present in	
		BWA	HAc	Vanillin	$FeCl_3$	$AgNO_2$	Bark	Leaves
1.	—	0.86	0.36			Yellow	+	
2.	—	0·83	0.59			Grey	+	
3.	Robinin	0.82	0.38	Yellow	Olive	Yellow	+	
4.	Quercitrin	0.74	0.39	Yellow	Olive	Grey	+	+
5.	Catechin	0.68	0.39	Red	Olive	Brown	+	+
6.	Quercetin 3-methyl ether	0.74	0.25	Yellow			+	+
7.	—[b]	0.60	0.38	Yellow		Yellow		+
8.	—	0.32	0.28	Yellow				+
9.	Rutin (?)	0.52	0.41	Yellow			+	
10.	—	0.70	0.45	Yellow			+	
11.	Leucoanthocyanin	0.53	0.25	Red		Brown	+	
12.	*l*-Gallocatechin	0.52	0.28	Red		Brown	+	
13.	*d*-Gallocatechin	0.52	0.32	Red		Brown	+	
14.	Leucoanthocyanin	0.52	0.39	Red			+	
15.	—	0.51	0.43	Orange			+	
16.	Malvidin glucoside	0.50	0.75	Blue				+
17.	—	0.43	0.49	Yellow			+	
18.	—	0.47	0.63	Yellow			+	
19.	—	0.55	0.65	Blue			+	
20.	—	0.57	0.66	Blue			+	
21.	Leucoanthocyanidin glucoside	0.36	0.40	Red		Brown	+	+
22.	Leucoanthocyanin	0.30	0.52	Red		Brown	+	+
23.	Fructose	0.29	0.74	Bluish			+	+
24.	Glucose	0.25	0.73			Brown	+	
25.	Sucrose	0.15	0.72	Bluish			+	+
26.	—	0.12	0.42			Grey	+	
27.	—	0.07	0.43			Grey	+	
28.	—	0.10	0.35			Grey	+	
29.	—	0.10	0.27			Grey	+	
30.	Hyrolyzable tannin	0–0.70	0–0.40	Red		Brown	+	+
31.	Condensed tannin	0	0–0.50	Red	Grey	Brown	+	+
32.	Cyanidin diglucoside[c]	0.15	0.15	—[d]				+
33.	—	0	0.42		Blue	Brown		+

[a] Development solvents (first direction)—5% acetic acid (HAc), (second direction) *n*-butanol:water:acetic acid (BWA) (4:5:1). Presence of a compound is indicated by +.
[b] In leaves disappeared after May. Possibly dihydroxyflavone 7-*O*-rhamnoglucoside.
[c] Disappeared after May.
[d] Pink—no spray.

TABLE 2. COLOR OF EXTRACTS OF BLACK OAK BARK OR LEAVES ON 2-D PAPER CHROMATOGRAMS UNDER UV ILLUMINATION[a]

Spot No.	Name	R_f values BWA	R_f values HAc	Color under UV	Present in Bark	Present in Leaves
1.	—	0.85	0.50	Blue–green		+
2.	Scopoletin	0.75	0.45	Blue	+	+
3.	Quercitrin	0.74	0.39	Deep purple	+	+
4.	Quercetin 3-methyl ether	0.74	0.25	Deep purple		+
5.	Chlorogenic acid	0.55	0.52	Purple–blue	+	+
6.	—	0.52	0.55	Blue		+
7.	—	0.53	0.31	Blue	+	+
8.	—	0.51	0.42	Blue		+
9.	—	0.52	0.72	Blue		+
10.	—	0.50	0.73	Blue–green		+
11.	—	0.37	0.73	Light blue		+
12.	Skimmin (?)	0.23	0.79	Blue	+	+
13.	—	0.15	0.72	Blue	+	+
14.	—	0.09	0.75	Blue		+
15.	—	0.0	0.57	Blue		+
16.	—	0.0	0.03	Blue		+
17.	—	0.24	0.12	Blue		+
18.	—	0.54	0.12	Blue		+
19.	—	0.63	0.10	Blue		+
20.	—	0.22	0.04	Yellow	+	+
21.	—	0.12	0.03	Yellow	+	
22.	—	0.0	0.0	Yellow	+	

[a] Chromatograms developed as described in Table 1. Presence of compound indicated by +.

were judged only by visual estimation of spot density. Quantities varied as much among different branches of the same tree as among different trees.

Leaves

The number and quantities of phenolics in leaves changed appreciably with the season, whereas those of bark remained essentially unchanged. A strong anthocyanin spot occurred on papers in May (No. 32, Table 1), no doubt accounting for the pink color of young leaves. The parent compound had the correct R_f values on paper, and the products of its hydrolysis had the correct characteristics for cyanidin diglucoside. This spot and spot 7 (Table 1) represented the only substances that disappeared by midsummer. Catechin (No. 5, Table 1) was present in trace amounts in May, then increased throughout the summer and into autumn. Tannins (Nos. 30 and 31, Table 1) appeared

by June and continued to increase into autumn. Most prominent in leaves in late summer were catechin, epicatechin, quercitrin (although much less than in bark), and several compounds that turned yellow with vanillin, one of which (No. 6, Table 1; No. 4, Table 2) was identified as quercetin 3-methyl ether.

Under UV, fluorescent compounds in leaves were more numerous than in bark (Table 2). Chlorogenic acid and scopoletin were again identified. As the summer progressed, more fluorescent compounds were present than in May. There were at least 17 blue, green, or purple–blue-fluorescing compounds present by August (Table 2). Variation in leaf phenolics among trees followed the same qualitative and quantitative patterns as described earlier for bark.

DISCUSSION

The literature about phenolics of common North American oaks is very sparse, consisting principally of occasional reviews of specific compounds (Freudenberg and Weinges, 1962) and paper chromatographic surveys of a single species (Hathway, 1952; Feeny, 1970). Our work verified the findings of Hathway and Feeny for certain compounds, although the oak they studied was *Quercus robur* L. For example, Feeny had also found a marked increase in leaf tannins and catechin from spring to autumn. But there are certain inconsistencies between Hathway's results and those of Feeny. One of the commonly occurring phenolics found in *Q. robur* was called "gallocatechin" by Hathway; it corresponds to "catechin with gallic acid" on Feeny's 2-D chromatograms for the same species. Also, Feeny gave different coordinates for gallocatechin in 2-D chromatograms than did Hathway. We tentatively identified (+)- and (−)-gallocatechins from *Q. velutina* bark at about the same chromatogram coordinates as those given by Feeny.

We found no free gallic acid in black oak bark or leaves, as was the case for Seikel et al. (1971) in their analysis of *Q. rubra* L. bark, which we have found somewhat similar to that of *Q. velutina*. Yet gallic acid has been found in *Q. robur* leaves (Feeny and Bostock, 1968), *Rhus* leaves (El Sissi et al., 1972), and *Q. alba* L. heartwood (Chen, 1970). Esters of gallic acid are known to occur commonly in plants (Dewick and Haslam, 1969). The extraction methods used by some workers may cause a split in the ester bond of these compounds, resulting in the liberation of free gallic acid.

Whatever the source of these phenolics, it is probable that gallic acid, certain tannins, or both have some effect in preventing fungal invasions of plants. Many years ago, it was suggested that tannins act as inhibitors of fungi (Cook and Taubenhaus, 1911). Recently, a tannin, as well as chloro-

genic and caffeic acids, were found to inhibit cellulase enzymes extracted from a common parasitic fungus (Russel, 1974). Chlorogenic acid has been related to the fungal defense mechanism of potato tubers (Kuć et al., 1956) and of tobacco roots (Gayed and Rosa, 1975). The strong presence of chlorogenic acid in both bark and leaves of black oak is, then, pertinent to the subject of fungal inhibition.

Phenolics in plants may also affect the feeding behavior of insects. Gallic acid and D-catechin have been found to inhibit feeding by gypsy moth larvae, whereas quercetin, in certain concentrations, stimulated it (T. ODell, 1976, personal communication).

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EFFECT OF FORMULATIONS AND DISPENSERS ON ATTRACTIVENESS OF VIRELURE TO THE TOBACCO BUDWORM[1–3]

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Abstract—Formulations of virelure, the synthetic sex pheromone of the tobacco budworm, *Heliothis virescens* (F.), were developed, bioassayed in traps in field cages, and used in traps to survey populations in plant hosts. Virelure (10 mg) laminated between thin sheets of vinyl polymer plastic was attractive to male moths for 21 days, and attractiveness did not regress appreciably for the first 7 days. Another dispenser was an 8 × 30-mm cigarette filter encased in a glass shell vial. A mixture of 10 μl (Z)-11-hexadecenal and 0.5 μl (Z)-9-tetradecenal, the pheromone components, formulated with wheat germ oil and *d*-alpha tocopherol (9:1) in 0.5 ml CH_2Cl_2 (WGOE) was still attractive to males after 5 days. Cottonseed oil, polyethylene glycol 600 distearate (peg-600-d), and peg-6000-d also inhibited excessive vaporization and oxidation of virelure. Virelure (21.25 μl) formulated on filters with WGOE and used as bait (replaced weekly) in survey traps caught 27,263 males in 1975; analysis of catches each day for 7-day periods showed that most males were caught within 3 days after baiting, and attractiveness regressed considerably during the last 3–4 days of each week. Four ratios of virelure components were tested as bait (20 mg) in saucer-type pheromone traps, and the 16:1 ratio caught significantly more male moths than ratios of 24:1, 32:1, or 40:1.

[1] Lepidoptera : Noctuidae.
[2] In cooperation with the Texas Agricultural Experiment Station, Texas A & M University, College Station 77843.
[3] This paper reports the results of research only. Mention of a pesticide in this paper does not constitute a recommendation for use by the USDA nor does it imply registration under FIFRA as amended. Also, mention of a commercial or a proprietary product in this paper does not constitute an endorsement by the USDA.

Key Words—Tobacco budworm, virelure additives, *Heliothis virescens*, pheromone antioxidants, sex pheromone, pheromone dispensers.

INTRODUCTION

A sex pheromone of the female tobacco budworm, *Heliothis virescens* (F.), was isolated and identified as a combination of the two aldehydes, (Z)-11-hexadecenal (Z-11-HDAL) and (Z)-9-tetradecenal (Z-9-TDAL) (Roelofs et al., 1974; Tumlinson et al., 1975). The ratio of the two components in extracts of live females was 16 parts Z-11-HDAL to 1 part Z-9-TDAL. However, in the field-cage bioassays made during the isolation (Hendricks and Tumlinson 1974), 20 female equivalents (FE) of crude extract in ether or 500 ng of the 16:1 ratio of the synthetic components (virelure) in hexane were only attractive for 3–4 hr. Also, when higher doses (53 μg) of virelure (16:1) were tested that were competitive with live females in trapping native male moths, they too were attractive for only 4 hr at night (Tumlinson et al., 1975). In fact, as much as 50 mg of virelure or 500 FE dispensed from filter paper were not attractive more than 6 hr during a single night and were completely unattractive by the second night.

Aldehydes such as the components of virelure are readily oxidized (Fieser and Fieser 1958). Since these compounds rapidly become ineffective when dispensed in pure form, our objective was to develop formulations and dispensers to prolong the attraction of virelure.

METHODS AND MATERIALS

Four series of bioassays were made in 3 screen field cages ($12 \times 17 \times 3$ m) that were stocked daily with male tobacco budworm pupae. The cages were planted with pigeonpea, *Cajanus cajan* (L.) Millsp., a suitable cover crop and a flowering host on which the emerging male moths could feed and where they could shelter. Seven electric grid traps were installed at least 6 m apart in each of the screen cages, and pheromone dispensers were secured in the center of the trap grids. Trap grids were simultaneously energized (4500 V, 4 MA) by a time clock switch at 9 PM CDT and remained on until 4 AM the next morning. Numbers of males caught in each trap were counted.

The prospective formulations, baits containing virelure plus 5 or 10% oil additives in dichloromethane (CH_2Cl_2) or hexane, were injected into an 8×30-mm cigarette filter made of cellulose acetate fibers, as one type of dispenser. Also, dispensers were made by laminating virelure between 2 layers of vinyl polymer plastic sheets giving a total thickness of 50–55 mil

(Herculite Protective Fabrics Corp., New York, New York). The relative attractiveness of formulations or dispensers was determined after several replicated bioassays in the field cages. Adjustment was made for captures in unbaited check traps or in traps baited with filters treated with solvent. The ratios of Z-11-HDAL to Z-9-TDAL in both the filter and plastic dispensers were monitored periodically by extracting with CH_2Cl_2 and detecting relative residual quantities of each attractive compound by gas chromatographic techniques.

In the first bioassay series, virelure was formulated with 5% (v/v) cottonseed oil (CSO) or wheat germ oil mixed 9:1 with *d*-alpha tocopherol (WGOE), both possible inhibitors of vaporization, in 0.4 ml CH_2Cl_2. The total pheromone dose was 10.5 μl/trap (20:1, Z-11-HDAL:Z-9-TDAL). The baits were exposed for 14 hr in field conditions before bioassays at night. In some trials, the filters were not shielded in any way, or no oils were added. In others, filters were placed in glass shell vials (9 × 30 mm) that were open at one end. There were 8 replicates (4 nights of testing). In the second series, attractiveness of virelure (same total dose and ratio) alone or virelure formulated in CH_2Cl_2 with 10% (w/v) polyethylene glycol 600 distearate (peg-600-d), peg-6000-d, or CSO was compared. All formulations were prepared with 0.5 ml of solution. All baits were shielded in open-end vials and exposed for a total of 68 hr, four 17-hr nights. Between test periods in the daytime, baits were stored at $-5°$ to $-3°$C.

In the third series, virelure as before was formulated with 10% w/v peg-6000-d or WGOE in CH_2Cl_2; wheat germ oil was used to inhibit vaporization and *d*-alpha tocopherol as an antioxidant. All formulations were prepared in 0.5 ml of solution and dispensed from open-end glass vials, but exposed for 5 consecutive days and nights (112 hr). The test was replicated 10 times.

The fourth bioassay series was made to compare attractiveness of filter dispensers (treated with 10.6 μl virelure in WGOE with either CH_2Cl_2 or hexane) with attractiveness of plastic laminate dispensers containing 10.6 μl virelure. Dispensers were prepared and exposed to field conditions 14 hr before testing the first night, after which they were held to age outside in typical weather conditions. These dispensers were bioassayed again when they were 7 days old, and relative attractiveness of the filter vs. plastic dispensers was determined from field cage tests.

Laminated plastic dispensers containing 10 mg virelure were cut (1.3 × 1.3 cm) at 7-day intervals and sequentially aged for as long as 21 days in field conditions. Different ages of dispensers (1, 7, and 21 days old) were bioassayed simultaneously in the field cages. Each age of dispenser was used as bait in 2 grid traps per cage each test night, and numbers of male tobacco budworms caught during 20 replicates were compared.

In field tests, May–August 1976, four different ratios of the pheromone

components (Z-11-HDAL:Z-9-TDAL) were used as bait in saucer-type pheromone traps (Hendricks et al., 1973) to determine which ratio was most attractive. The ratios tested were 16:1, 24:1, 32:1, and 40:1. Even though the ratios between baits were different, the total pheromone dosage in the (1.3 × 2.5 cm) laminated plastic dispenser was 20 mg/trap. There were 25 traps baited with dispensers containing each ratio of pheromone components. Most traps were installed near cotton fields, but six of each bait ratio (24 traps) were set up near tomatoes. Traps were spaced at least 20 m apart and were adjusted to the height of the crop canopy, 0.5–1 m. Dispensers were replaced every 2 weeks and were rotated so that adjacent traps contained dissimilar bait ratios.

During the 1975 and 1976 cotton-growing seasons in Burleson County, Texas, dispensers treated with virelure were used in a tobacco budworm survey program. This survey involved the use of electric grid traps installed at sites near different cultivated and native host plants. In 1975, 11 traps were baited each week with shell vial-filter dispensers treated with 21 μl of virelure

TABLE 1. CATCH OF MALE TOBACCO BUDWORMS IN FIELD CAGES WITH ELECTRIC GRID TRAPS BAITED WITH VIRELURE[a] ALONE OR MIXED WITH 5% w/v COTTONSEED OIL (CSO) OR WGOE (*d*-ALPHA TOCOPHEROL MIXED 1:9 v/v WITH WHEAT GERM OIL) IN CH_2Cl_2 AND EXPOSED WITH OR WITHOUT VIALS (8 REPLICATES, EACH EXPOSED IN FIELD CONDITIONS 14 hr BEFORE BIOASSAY IN FIELD CAGES)

Formulations and dispenser[b]	Males caught	
	% of total	Average/night[c,d]
Virelure + WGOE in vial	23.26	29.1 o
Virelure + WGOE	21.45	26.6 op
Virelure + CSO in vial	16.20	19.4 p
Virelure + CSO	14.75	17.4 ps
Virelure in vial	13.21	15.3 s
Virelure	8.96	9.4 s
Empty trap (check)	2.17	-adj.-

[a] Virelure consisted of 10 μl Z-11-HDAL and 0.5 μl Z-9-TDAL/trap.

[b] All formulations were prepared in 0.4 ml solvent/trap, applied to 8 × 30-mm cigarette filters and (where indicated) placed in 9 × 30-mm glass shell vials.

[c] Numbers followed by same letters were not significantly different ($P = 0.05$, t test).

[d] Numbers of males were adjusted for random catch by subtracting numbers caught in unbaited (check) traps.

with 10% WGOE in 0.5 ml CH_2Cl_2. Numbers of male tobacco budworms attracted to and caught in each trap were tabulated daily from March 23 to October 27. In 1976, 7 traps were used in the survey and were baited with laminated plastic dispensers containing 20 mg of virelure; budworm males were counted from March 31 to September 28.

RESULTS AND DISCUSSION

Addition of either the CSO or WGOE to the virelure plus solvent increased the catch of males for 1 night (Table 1) compared with catches by virelure alone. Also, virelure alone on filters in vials caught 4.2% more males than virelure with no vial, but 3–10% less than virelure in vials with either WGOE or CSO. Thus addition of either CSO or WGOE to the virelure plus solvent either reduced the vaporization rate during the 14-hr pretest period or prevented rapid oxidation and breakdown of the two basic aldehydes and increased attractiveness.

Virelure dispensers with either peg-600-d or peg-6000-d (Table 2) attracted

TABLE 2. CATCH OF MALE TOBACCO BUDWORMS IN FIELD CAGES WITH ELECTRIC GRID TRAPS BAITED WITH VIRELURE[a] ALONE OR MIXED WITH 10% w/v COTTONSEED OIL, POLYETHYLENE GLYCOL 600 DISTEARATE (peg-600-d), OR peg-6000-d IN CH_2Cl_2 AND EXPOSED IN VIALS (8 REPLICATES IN 4 NIGHTS, 68 hr EACH)

Formulation[b]	Males caught	
	% of total	Average/night[c]
Virelure + peg-6000-d	36.9	83.0 t
Virelure + peg-600-d	36.3	81.8 t
Virelure + CSO	17.8	40.1 u
Virelure alone	4.4	10.0 v
CH_2Cl_2 + peg-6000-d (check)	1.9	4.2 v
CH_2Cl_2 + CSO (check)	1.8	4.1 v
CH_2Cl_2 + peg-600-d (check)	0.9	2.0 v

[a] Virelure consisted of 10 μl Z-11-HDAL and 0.5 μl Z-9-TDAL/trap.

[b] All formulations were prepared in 0.5 ml CH_2Cl_2/trap and were applied to 8 × 30-mm cigarette filters in 9 × 30-mm glass shell vials.

[c] Numbers followed by same letter were not significantly different ($P = 0.05$, t test).

TABLE 3. CATCH OF MALE TOBACCO BUDWORMS IN FIELD CAGES WITH ELECTRIC GRID TRAPS BAITED WITH VIRELURE[a] ALONE OR MIXED WITH 10% W/V POLYETHYLENE GLYCOL 6000 DISTEARATE (peg-6000-d) OR WGOE (*d*-ALPHA TOCOPHEROL MIXED 1:9 V/V WITH WHEAT GERM OIL) IN CH_2Cl_2 (10 REPLICATES OF 5 NIGHTS, 112 hr OF CONTINUOUS EXPOSURE)

Formulation[b]	Males caught	
	% of total	Average/night[c]
Virelure + WGOE	45.1	57.1 j
Virelure + peg-6000-d	32.2	36.9 k
Virelure alone	18.6	21.2 l
CH_2Cl_2 + WGOE (check)	1.9	2.2 m
CH_2Cl_2 + peg-6000-d (check)	1.0	1.2 m
CH_2Cl_2 only (check)	1.2	1.4 m

[a] Virelure was composed of 10 μl Z-11-HDAL and 0.5 μl Z-9-TDAL/trap.
[b] All formulations were prepared in 0.5 ml CH_2Cl_2/trap and applied to 8 × 30-mm cigarette filters placed in 9 × 30-mm glass shell vials.
[c] Numbers followed by the same letter were not significantly different ($P = 0.05$, t test).

and caught significantly more males than dispensers with virelure + CSO. Dispensers with virelure alone (no additive) caught significantly fewer males than formulations with any of the three additives tested. Nighttime temperatures during this test ranged from 30° to 32° C. Virelure mixed with WGOE attracted significantly more males than the formulation prepared with peg-6000-d (Table 3). Also, the WGOE formulation, being exposed 5 consecutive days when temperature ranged from 24° to 32°C, attracted substantial numbers of males during all 5 nights. Thus, addition of WGOE to the CH_2Cl_2–virelure system extended the attractive period and prevented rapid oxidation of the basic pheromone aldehydes.

During the first night of field-cage bioassays, cigarette filter–vial dispensers treated with 10.6 μl virelure in either CH_2Cl_2 or hexane WGOE attracted significantly more males than either the laminated plastic dispensers or filter dispensers without vials (Table 4). However, after the dispensers were held for 7 days in field conditions and again tested, the laminated plastic dispenser attracted significantly more males (68%) than all other filter dispensers combined. Hence, apparently the virelure dissipated from the laminated plastic dispenser at a relatively slow but constant rate and was

TABLE 4. CATCH OF MALE TOBACCO BUDWORMS IN FIELD CAGES WITH ELECTRIC GRID TRAPS BAITED WITH VIRELURE[a] IN LAMINATED PLASTIC DISPENSERS OR FORMULATED WITH WGOE IN HEXANE OR CH_2Cl_2 AND PLACED ON CIGARETTE FILTERS WITH OR WITHOUT SHELL VIALS (4 REPLICATES/BAIT/NIGHT). DISPENSERS WERE EXPOSED TO FIELD CONDITIONS BETWEEN NIGHTS 1 AND 7

Formulation and dispenser[b]	Males caught			
	1st night		7th night	
	% of total	Average/night[c,d]	% of total	Average/night[c,d]
Virelure + hexane WGOE in vial	28.4	39.62 e	6.3	6.75 g
Virelure + CH_2Cl_2 WGOE in vial	27.7	38.62 e	6.6	7.75 g
Virelure in laminated plastic	13.8	17.38 f	68.0	179.25 h
Virelure + hexane WGOE	12.8	15.87 f	8.4	12.75 i
Virelure + CH_2Cl_2 WGOE	12.6	15.62 f	3.0	−2.5 j
Hexane WGOE (check)	2.4	-adj.-	3.6	-adj.-
CH_2Cl_2 WGOE (check)	2.3	-adj.-	4.1	-adj.-

[a] Virelure consisted of 10 μl Z-11-HDAL and 0.625 μl Z-9-TDAL/trap.
[b] All formulations, except laminated plastic, were prepared in 0.4 ml of solvent with 10% WGOE (*d*-alpha tocopherol mixed 1:9 v/v with wheat germ oil), applied to 8 × 30-mm cigarette filters, and (where indicated) placed in 9 × 30-mm glass shell vials.
[c] Numbers followed by same letter were not significantly different ($P = 0.05$, modified *t* test).
[d] Numbers of males were adjusted for random catch by subtracting numbers caught in solvent (check) traps.

substantially attractive as a trap lure for budworms after 7 days of exposure. Also, this relationship was illustrated by catches of male tobacco budworms taken from the survey traps operated in 1975 and 1976 in Burleson County, Texas (Figure 1). In 1975, when the traps were baited weekly with filter dispensers in vials, 21 μl virelure in CH_2Cl_2 WGOE, 27,263 males were caught. Data analyzed on the basis of males caught during each night after baiting (days 1–7) showed that most males were caught during the first 3 nights. This effect was expected since the numbers of males caught are dependent upon the rate of dissipation of the pheromone and amounts available in the air during nighttime hours.

The slope of Figure 1 (1975 filter) indicates that the pheromone dissipated readily during the first and second nights; lower quantities dispersed during nights 3–7. However, in 1976 when the survey traps baited with 20-mg virelure laminated in plastic (Figure 1, 1976 laminated) caught 10,419 males, no regression slope was detected indicating steady dissipation of the phero-

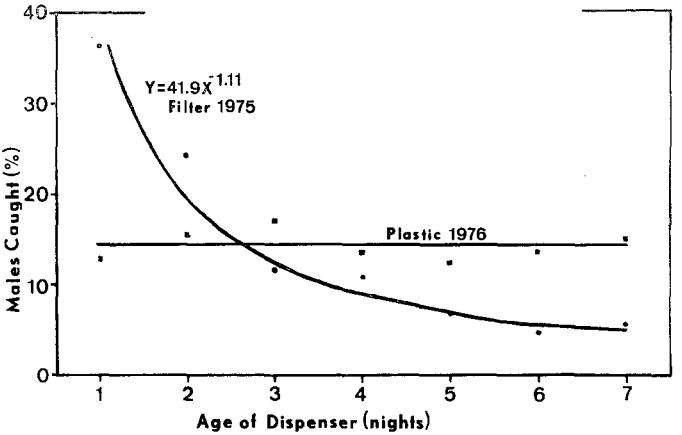

FIG. 1. Percentages of males caught in electric grid traps during each of 7 nights after baiting. In 1975, 11 traps were baited weekly with 21.25 μl virelure formulated on a filter dispenser (March 23–October 27); a total of 27,263 males were caught. In 1976, 7 traps were baited weekly with 20 mg virelure laminated in a plastic dispenser (March 31–September 28); 10,419 males were caught.

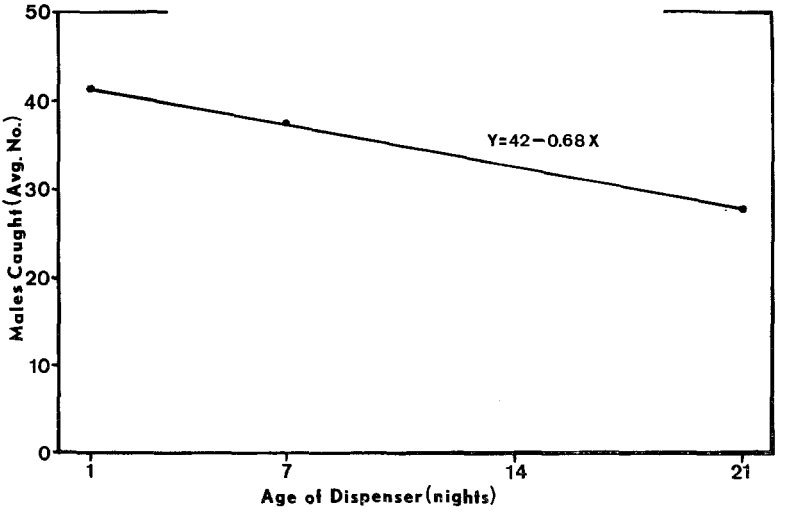

FIG. 2. Average numbers of males caught in field cages in electric grid traps baited with 10 mg virelure laminated in plastic dispensers of 3 ages (1, 7, and 21 days old) tested simultaneously, 20 replicates.

TABLE 5. CATCH OF MALE TOBACCO BUDWORMS IN SAUCER-TYPE TRAPS BAITED WITH SEX PHEROMONE MADE WITH 4 DIFFERENT RATIOS OF COMPONENTS[a] (TRAPS WERE SET IN 4 FIELD LOCATIONS NEAR COTTON AND TOMATOES)

Pheromone component ratio (Z-11-HDAL:Z-9-TDAL)	Males caught		
	% of total	Range/trap-night[b]	Average/night[c]
16:1	34.6	0–7	6.36 x
24:1	26.4	0–3	4.84 y
32:1	20.9	0–3	3.84 yz
40:1	18.1	0–5	3.32 z

[a] Virelure was dispensed from polyvinyl chloride laminated dispenser (1.3 × 2.5 cm) at a total dosage of 20 mg/trap.
[b] There were 25 traps baited with each ratio; data was used from 1675 trap-nights, May–August 1976.
[c] Numbers followed by same letter were not significantly different ($P = 0.05$, modified t test).

mone components between weekly baiting periods from March 31 to September 28. Additional cage bioassays using laminated plastic dispensers, aged from 1 to 21 days, did reveal a relative decrease in catch by the older dispensers (Figure 2). The regression, however, was linear and dispensers remained attractive and caught significant numbers of males even after 21 days of exposure to typical weather conditions.

Gas chromatographic analysis of extracts from both filter and plastic laminated dispensers after 3 and 5 days of exposure showed that the ratios of the components remained within 7% of the original. However, only a slight variation in the dissipation rate of Z-9-TDAL could reduce the attractiveness of the component mixture to males. In the test of component ratio (Table 5), a 16:1 ratio attracted significantly more males than all other ratios. Catch was significantly reduced by the 40:1 ratio, indicating either an excessive amount of Z-11-HDAL or too little Z-9-TDAL (0.49 mg/dispenser). Thus when virelure was formulated in laminated plastic, 50–55 mil thick, the most effective ratio was the same as found in the original female extracts (Tumlinson et al., 1975).

Many possible additives could be tested and infinite numbers of ratios bioassayed. Nevertheless, our tests showed that virelure (20 mg) laminated in vinyl polymer dispensers had a prolonged and consistently attractive span over a 7-day period that otherwise would have been significantly shorter and more regressive if virelure were dispensed from filters. However, the filter–vial dispenser treated with virelure in WGOE did prolong the attractive life of the

pure virelure aldehydes which would have been inactive after 5 hr (<1 night) of exposure in the field. The filter dispenser might be useful for research purposes or in survey or control projects where the use of short-interval baiting periods or high-dosage formulations is scheduled.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS VI. An Examination of the Kairomones for the Predator *Chrysopa carnea* Stephens[1] at the Oviposition Sites of *Heliothis zea* (Boddie)[2–4]

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Abstract—Evidence is presented that demonstrates the presence of kairomones for larvae of *Chrysopa carnea* Stephens in materials associated with egg deposition by female *Heliothis zea* (Boddie). These kairomones are involved in prey finding and/or acceptance.

Key Words—*Chrysopa carnea*, *Heliothis zea*, kairomones, predators, biological control, entomophagy.

INTRODUCTION

Lewis et al. (1977) demonstrated that the scales of adult *Heliothis zea* (Boddie) contain kairomone(s) that stimulate(s) the feeding of larval *Chrysopa carnea* Stephens on eggs of *H. zea*. The studies reported here were designed to

[1] Neuroptera : Chrysopidae.
[2] Lepidoptera : Noctuidae.
[3] In cooperation with the University of Georgia College of Agriculture Experiment Stations, Coastal Plain Station, Tifton, Georgia. Accepted for publication January 17, 1977.
[4] Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the USDA.

determine if other materials at the oviposition site of *H. zea* females are involved in prey finding and acceptance by *C. carnea*.

MATERIALS

The *C. carnea* used in these studies were purchased from Rincon Insectary, Inc., Oak View, California. Newly emerged larvae were fed *H. zea* eggs that had been processed with a sodium hypochlorite wash, as described by Burton (1969), and irradiated with 25 krad (^{60}Co source). The larvae were held with food for at least 2 days at approx. 26°C and 70% relative humidity (RH) prior to their use in bioassays.

Pairs of *H. zea* moths were placed in 3.8-liter cardboard cartons (3 pairs/carton), lined with Whatman no. 1 filter paper for oviposition. After approx. 24 hr, the filter paper was removed, and pieces approx. 6.5 cm^2 were cut that contained either one naturally deposited egg or no eggs (but which were assumed to contain most of the materials associated with the adult moth). Loose naturally deposited eggs were obtained by placing moths in similar cartons that had been lined with wax-coated paper[7] so that the eggs could easily be brushed off and collected. Control eggs were obtained from laboratory cultures and had been processed with a sodium hypochlorite wash (Burton, 1969), which was assumed to have removed any material associated with oviposition. The control eggs were irradiated with 25 krad (^{60}Co source) when 8–36 hr old to prevent eclosion and were stored at approx. 10°C.

The tests were conducted in 150 × 15-mm glass petri dishes, each containing 5 pieces of filter paper (approx. 6.5 cm^2) and 2 *C. carnea* larvae. Exposure time was 2 hr in all tests. Eggs were applied using a Plantgard®[8] solution (Nordlund et al., 1974). After exposure to the *C. carnea*, the number of eggs that had been fed on was determined with a dissecting microscope. Unless otherwise stated, statistical analysis was done by analysis of variance.

METHODS AND RESULTS

Test 1

To determine if stimuli other than those found in the moth scales of *H. zea* are important in the prey finding and/or acceptance behavior of *C. carnea*, dishes were set up using filter paper which had received one of the following treatments: control filter paper with a control egg applied (CCA), exposed filter paper with a control egg applied (ECA), or exposed filter paper

[7] 40/48 #D.W. Bleached, manufactured by Midwest Waxed Paper Co., Box 216, Fort Madison, Iowa 52627.

[8] Manufactured by Polymetrics Intl., New York, New York.

with a naturally deposited egg (EN). It was assumed that EN would be the optimum condition; that is, it would contain all of the materials associated with a naturally deposited egg. This test was replicated 9 times with 20 dishes/replication.

The mean percentage of fed eggs in EN (27.3%) was significantly higher ($P < 0.05$) than that in either ECA (20.1%) or CCA (17.0%). The mean for ECA was not significantly different from that of CCA. These results demonstrate that factors associated with natural oviposition are involved in the prey finding and/or acceptance behavior of *C. carnea* and indicate that factors, in addition to those found in the moth scales (Lewis et al., 1977), are involved.

Test 2

This test was designed to demonstrate that the response shown in test 1 was due, at least in part, to factors associated with oviposition as opposed to differences in the quality of the control and natural eggs. Dishes were set up with either control filter paper with a control egg applied (CCA), exposed filter paper with a control egg applied (ECA), or exposed filter paper with a control egg applied, as a replacement for a natural egg, on a naturally deposited egg spot (ECAN). The test was replicated 28 times with 20 dishes/replication.

The mean percentage of fed eggs for ECAN was 16.9% which was significantly higher ($P < 0.05$) than the mean of 13.6% for CCA. The mean of 15.3% for the ECA treatment was not significantly different from that of either ECAN or CCA. These results suggest that there is a kairomone(s), or other stimulus, for *C. carnea* associated with the oviposition site of *H. zea*.

Test 3

This test was conducted to determine specifically if a factor important to the prey finding and/or acceptance by *C. carnea* is associated with the natural egg itself but is removed by the sodium hypochlorite wash. Dishes were set up with either control filter paper with a control egg applied (CCA), exposed filter paper with a control egg applied (ECA), or control filter paper with a natural egg applied (CNA). This test was replicated 10 times with 20 dishes/replication.

The mean percentage of fed eggs for CNA was 22.5%, which was significantly higher ($P < 0.05$) than the mean for ECA of 14.9% or for CCA of 14.2%. The mean for ECA was not significantly different from that for CCA. These data indicate that the natural egg itself has a kairomone(s), or other stimulus, associated with it that is involved in prey finding and/or acceptance by *C. carnea* larvae.

Test 4

In each of the preceding tests, predation of control eggs on exposed filter paper (ECA) was greater than the predation of control eggs on control filter paper (CCA), but the differences were not significant. This test was conducted to confirm that materials left on the substrate surrounding the egg are important in the prey finding and/or acceptance behavior of *C. carnea.* Natural eggs were used to ensure that all conditions were as natural as possible. Dishes were set up with exposed filter paper with a natural egg applied (ENA) or control paper with a natural egg applied (CNA). This test was replicated 10 times with 20 dishes/replication. Statistical analysis was done by the paired t test using arcsin transformations.

The mean percentage of fed eggs for ENA (18.5%) was significantly higher ($P < 0.05$) than the mean for CNA (15.2%). These data demonstrate that stimuli left by the adult moth on the surrounding substrate are important in prey finding and/or acceptance by *C. carnea* larvae. The kairomone found in the moth scales (Lewis et al., 1977) is at least one of these stimuli, if, in fact, there is more than one.

DISCUSSION

The data presented demonstrate that there is a factor, probably a kairomone, in addition to the kairomone(s) found in the moth scales (Lewis et al., 1977), which is important to the feeding of *C. carnea* larvae on *H. zea* eggs. These data also demonstrate that the stimulus (or stimuli) is closely associated with the naturally deposited *H. zea* egg.

We suggest that it is found in the accessory gland secretion used to adhere the eggs to the substrate (Chapman, 1971). Vinson (1975) reported that *Chelonus texanus* Cresson females failed to recognize, as hosts, the eggs of *Heliothis virescens* (F.), *Spodoptera exigua* (Hübner), and *Spodoptera frugiperda* (J.E. Smith) from which the accessory gland cement had been removed with a 10% Chlorox® solution. However, the stimulus involved was not removed with various other solvents (which did not remove the cement) nor were the accessory glands themselves active.

It is reasonable to assume that the kairomone found in the moth scales is a search stimulant and that the stimulus (or stimuli) associated with the egg mediates prey acceptance. The data also suggest that the search stimulant has a primer effect that is necessary before the predator becomes responsive to the acceptance component since neither the search stimulant nor the prey acceptance stimulus (or stimuli) works as well alone as when both are present.

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KAIROMONES AND THEIR USE FOR MANAGEMENT OF ENTOMOPHAGOUS INSECTS VII. The Involvement of Various Stimuli in the Differential Response of *Trichogramma pretiosum* Riley[1] to Two Suitable Hosts[2,3]

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Abstract—The response of *Trichogramma pretiosum* Riley to stimuli from *Heliothis zea* (Boddie) and from *Trichoplusia ni* (Hübner) was examined in the laboratory. Kairomones were responsible, at least in part, for the greater response to naturally deposited *H. zea* eggs.

Key Words—kairomone, parasitoid, *Trichogramma pretiosum*, biological control, host finding, host acceptance, *Heliothis zea*, *Trichoplusia ni*.

INTRODUCTION

In previous papers (Lewis et al., 1972, 1975a, b, 1976), the importance of kairomones in host finding by female *Trichogramma* spp. has been discussed. Ashley et al. (1974) found that female *Trichogramma pretiosum* Riley produced more progeny, parasitized more host eggs, and lived longer when they were provided naturally deposited eggs of *Heliothis zea* (Boddie) than when

[1] Hymenoptera : Trichogrammatidae.
[2] In cooperation with the University of Georgia College of Agriculture Experiment Stations, Coastal Plain Station, Tifton, GA 31794. Accepted for publication January 19, 1977.
[3] Mention of a commercial or proprietary product in this paper does not constitute endorsement by the USDA.

they were provided naturally deposited eggs of *Trichoplusia ni* (Hübner). They suggested that these differences reflected differences in stimuli received by the parasite. Nordlund et al. (1976) demonstrated that the presence of kairomones found in the moth scales of *H. zea* did increase the percentage of parasitization, the number of progeny produced, and the longevity of *T. pretiosum* females in the laboratory. A study was therefore undertaken to examine the reason for the differential response of *T. pretiosum* females to the two hosts.

METHODS AND MATERIALS

The *Trichogramma* stock used in the study originated from Hermosillo, Mexico. It was found to cross successfully with a stock from Los Mochis, Mexico (Gonzales and Allen, 1975; Division of Biological Control, University of California, Riverside, California 92501, unpublished results) that was identified as *T. pretiosum* (Oatman et al., 1970). These parasites were reared in our laboratory in treated *H. zea* eggs at approx. 26°C and 70% relative humidity (RH).

The treated *H. zea* eggs used in the studies (and also for rearing the *T. pretiosum*) were obtained from laboratory cultures, 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 approx. 10°C.

H. zea and *T. ni* pupae of comparable age were obtained from laboratory cultures. The pupae were sexed, and 5 pairs were placed in each of a number of 3.8-liter cardboard containers lined with wax-coated paper.[6] Sugar water was provided. After eclosion and after the females began ovipositing, the paper liners were changed daily. Disks of the carton liners were cut with a 1/2-in.-diameter cork borer. These disks contained 1 naturally deposited egg or no egg, as desired.

The tests were conducted in 150 × 15-mm glass petri dishes with 8 eggs (1 egg per disk of carton liner) and 2 (1-day-old) female *T. pretiosum* per dish. Different dishes were used for each host species. Exposure time was 1 hr. Percentage of parasitization was determined by dissecting the host eggs according to the procedure of Lewis and Redlinger (1969).

Statistical analysis was done by the paired *t* test by using arcsin transformations.

RESULTS

Test I

This test was designed to demonstrate the difference in the response of

[6] 40/48 #D.W. Bleached, manufactured by Midwest Waxed Paper Co., Box 216, Fort Madison, Iowa 52627.

T. pretiosum females to naturally deposited *H. zea* and *T. ni* eggs as reported by Ashley et al. (1974). Disks which contained 1 naturally deposited egg were cut as previously described. Eight disks were placed in each dish with the egg on top. Seven replications of this test, with 10–15 dishes per replication, were conducted.

The mean percentage of parasitization of the *H. zea* eggs was 45% vs. 19% of the *T. ni* eggs (means were significantly different at the 5% level). These data conclusively demonstrate that *T. pretiosum* females are more able to find or willing to accept naturally deposited *H. zea* eggs than they are *T. ni* eggs.

The following 3 tests were designed to examine the eggs and associated materials for sources of the stimuli which produce this differential response.

Test II

Dishes were set up with disks from which a naturally deposited egg had been removed and a treated *H. zea* egg substituted (Nordlund et al., 1974). Disks on which an egg had ruptured were discarded. Five replications of this test, with 10–15 dishes each, were conducted.

The mean percentage of parasitization of the eggs on *H. zea* disks was 47% vs. 37% of the eggs on *T. ni* disks (means were significantly different at the 5% level).

Test III

In this test, disks were cut from portions of cage liner which contained no naturally deposited eggs. One treated *H. zea* egg was applied (Nordlund et al., 1974) to each piece. These pieces were assumed to possess the chemicals associated with the adult moths but not with oviposition. Dishes were set up with 8 disks and 2 female *T. pretiosum* for 1 hr. Seven replications of 10–15 dishes each were conducted.

The mean percentage of parasitization for the eggs on *H. zea* liners was 42% vs. 28% for those on *T. ni* liners (means significantly different at the 5% level).

Test IV

Naturally deposited eggs were removed from cage liners, with a small camel's hair brush (moistened slightly with water), and placed directly on the petri dishes. Two female *T. pretiosum* were placed in each dish and allowed to search for 1 hr. Seven replications of this test, with 10–15 dishes per replication, were conducted.

The mean percentage of parasitization of the *H. zea* eggs was 30% vs. 19% for the *T. ni* eggs (means were significantly different at the 5% level).

DISCUSSION

The differential response of *T. pretiosum* females, as previously reported by Ashley et al. (1974), is demonstrated by the results of test I. The results of tests II and III indicate that trails of the adult moth on the surrounding substrate and possibly materials left at the egg site during oviposition provide important stimuli for the stronger response of the female parasitoid to *H. zea* eggs. Previously, Nordlund et al. (1976) demonstrated that *T. pretiosum* females, as with other *Trichogramma* species (Lewis et al., 1972), respond to kairomones found in the scales of adult *H. zea*. Subsequently, Nordlund et al. (1977) demonstrated that stimuli important to the prey finding and/or acceptance behavior of *Chrysopa carnea* Stephens are associated with the oviposition materials of *H. zea*. Vinson (1975) reported that *Chelonus texanus* Cresson females failed to recognize as hosts the eggs of *Heliothis virescens* (F.), *Spodoptera exigua* (Hübner), or *Spodoptera frugiperda* (J.E. Smith) after their being washed with Chlorox®, a process that removes the accessory gland secretion which binds the eggs to the substrate. Vinson postulated that the stimulus involved was the rough surface provided by the cement because extractions with various solvents that did not remove the cement did not reduce the attractiveness of the eggs and the accessory glands themselves were not attractive. It is possible that the surface texture provided by the accessory gland secretion is important in the differential response by *T. pretiosum* females to these two species. But, because the mean percentage of parasitization of the treated *H. zea* eggs in test II was as high or higher than that of the naturally deposited eggs in test I, this does not seem to be probable. A kairomone from the accessory gland secretion is a possible factor.

The results of test IV demonstrate a significant difference in the acceptability of the eggs of *H. zea* and *T. ni* (without a sodium hypochlorite wash). A number of stimuli sources, such as kairomones from the oviposition material, the size of the egg, or the physical or chemical composition of the egg itself, could be responsible for this difference.

In other comparative calculations, we find that the mean percentage of parasitization for naturally deposited *H. zea* eggs (45%) in test I is higher ($P < 0.1$, as determined by the unpaired t test using arcsin transformations) than that for natural *H. zea* eggs placed directly on the petri dishes (30%) in test IV. This is an additional demonstration of the importance of the materials left by the adult moth on the surrounding substrate. In addition, the mean percentage of parasitization of the naturally deposited *T. ni* eggs (19%) in

test I is significantly lower ($P < 0.05$, as determined by the unpaired t test using arcsin transformations) than the mean for the treated *H. zea* eggs on *T. ni* disks (37%) in test II. These data indicate that the *H. zea* egg itself is more acceptable to *T. pretiosum* females.

In summary, then, stimuli from various sources are involved in the differential response of *T. pretiosum* females to these two suitable host species. Because of the previously demonstrated importance of chemical stimuli in the host-selection process of this and other parasitoids, one can reasonably assume that at least some of the stimuli involved are kairomones. Undoubtedly, these stimuli are important, at various points of the host selection sequence described by Lewis et al. (1976), in governing the extent and intensity at which various host species are parasitized. It will be necessary to consider several stimuli sources when attempting to use kairomones to manage parasitization of less preferred host species by parasitoids.

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A PRECISION LOW-FLOW-RATE AIR DELIVERY SYSTEM FOR USE WITH OLFACTOMETERS

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Abstract—A precision air delivery system was adapted for use with insect olfactometers where a regulated low flow rate is desirable. The system employs hypobaric flow regulated by a barostat tower and a series of capillary flowmeters. Air flow is regulated by a balance of pressures on either side of the capillary. The regulated air flow is fed through Teflon lines to the olfactometer chambers. Flow-rate adjustments ranging from 5 ml to 5 liter/min can be made by changing the pressure on the system through the barostat tower or by changing the size of the capillary.

Key Words—air-flow control, olfactometers, insect attractants, insect repellents, flow rates, bioassay apparatus.

INTRODUCTION

Insect olfactometers have been widely used in entomological research since the early work of Barrows (1907) utilizing the Y-tube principle. Many types of olfactometers have since been developed and described, each having advantages for specific uses. Change was brought about partially through technical advancement and development of new materials and partially through necessity. As each new species was tested, it became apparent that bioassays must be built around the natural habits and idiosyncrasies of each species before meaningful results could be obtained (Beroza and Jacobson, 1963).

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McIndoo (1926) constructed an apparatus similar to that of Barrows (1907), and with modifications this device is still used today. Hoskins and Craig (1934) designed a more versatile model, incorporating more regulatory equipment and modifying the device to accommodate flying insects. Other improvements over the years included improved air-flow, temperature, and light control refinements and elimination of contaminated air from the system (Wilson and Bean, 1959; Kinzer et al., 1970; Schuster and Starks, 1974).

The insect pheromone work by Shorey and Gaston (1964), Toba et al. (1968), Sower et al. (1971), and Mayer (1973) show that detection and response to pheromones is largely concentration dependent. Shorey et al. (1967) estimated the lower threshold for behavioral response by males of *Trichoplusia ni* Hübner to be about 60 molecules/mm^3 of air, based on bioassay responses to a pheromone placed on filter paper in a closed air system.

The rate of gas distribution in a moving air mass varies greatly with wind speed and turbulence. At higher wind speeds, the initial concentration of a volatile near the emission source will be reduced by more rapid gas dispersion due to greater turbulence (Sutton 1953). Shorey (1970) gave a complete review and discussion of pheromone research and made the point that air movement is of prime importance in studies of this type. In general, the air delivery method employed on these devices, whether hyper- or hypobaric flow, has been treated as a secondary consideration. These systems were designed either with little control over the flow rate or with reasonable control but at considerable expense.

The following system was adapted from an air-flow delivery system used to measure very low levels of endogenously evolved volatiles from plants. Its simplicity, accuracy, flexibility, and low cost (under $100) makes it highly desirable for use in entomological experimentation. It is portable for field use and has the potential for mixing gases for a gradient in concentration prior to entering the test chambers.

METHODS AND MATERIALS

The system for supplying and administering the regulated air flow to a series of olfactometers was modified from the apparatus described by Pratt et al. (1960) and Claypool and Keefer (1942). Figure 1 illustrates the complete air-flow system with air source, flow control board, and connected olfactometer series. Figure 2 shows the schema for the gas-flow regulating mechanism. Air is supplied to the system by a large commercially available compressed gas cylinder containing breathable air. The tank was equipped with a single-stage pressure regulator and in-line flowmeter for gross air adjustment which causes a static pressure to develop on the system. Stable

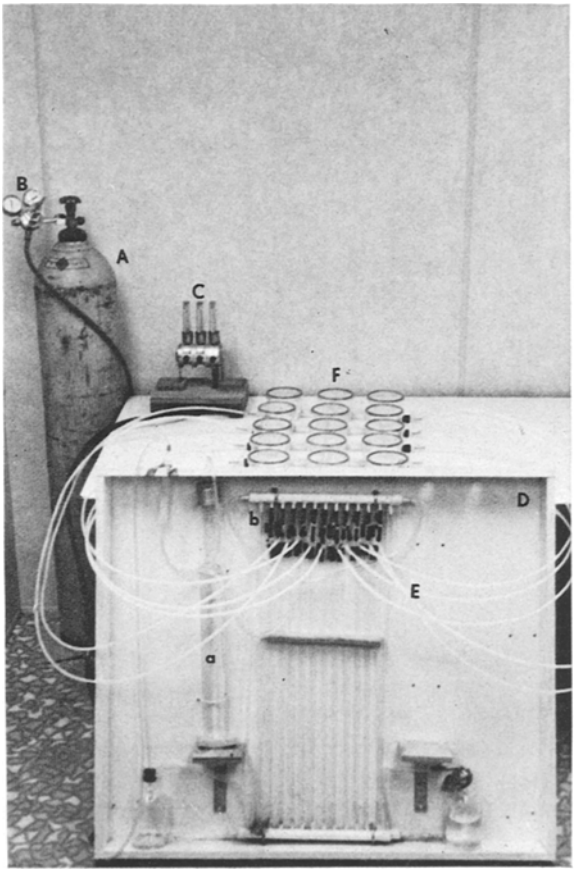

FIG. 1. The complete air flow control and olfactometer apparatus: (A) compressed air cylinder; (B) pressure regulator; (C) constant rate flow controller; (D) mounting board with (a) barostat tower and (b) capillary flowmeters; (E) air lines from individual outlet manometers to olfactometer outer chambers; (F) two-choice olfactometers.

and accurate regulation of this main air stream, over a wide range of flow rates, is accomplished by use of a barostat tower (B, Figure 2). The internal effective pressure (h) is independent of the volume of liquid in the barostat tower and is adjusted by raising or lowering the air inlet tube (C). Air flow should be adjusted to generate a steady release of air bubbles in the liquid of the tower (B) and excess air is released through outlet (D). The water reser-

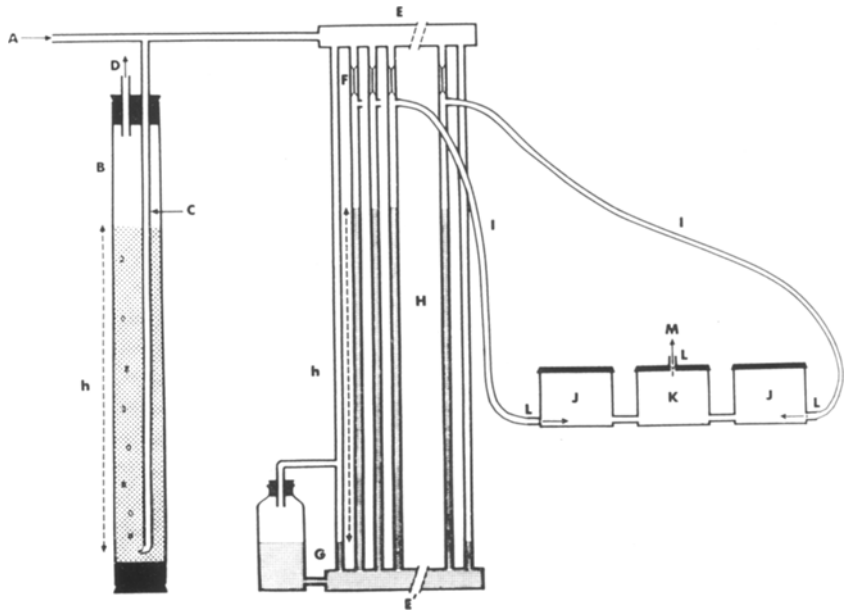

FIG. 2. Schema for controlled air flow system: (A) air inlet from a pressure regulator; (B) barostat tower; (C) tube C is adjusted upward or downward to alter the internal pressure (h) (cm of H_2O); (D) excess air outlet; (E) upper manifold; (E′) lower manifold; (F) individually calibrated precision-bore capillary tubes; (G) water reservoir and high-pressure arm of the manometer manifold; (h) actual internal pressure (cm of H_2O) at the capillaries; (H) individual outlet manometers (low-pressure arm); (I) Teflon lines from capillary outlets to olfactometer; (J) outer olfactometer chambers; (K) inner olfactometer chamber; (L) screen grids to restrict insect movement from olfactometer; (M) exhaust air from olfactometer.

voir and outside tube (G) attached to the manometer manifolds (E and E′) acts as the high-pressure arm to all of the flowmeters and causes water to rise in the individual manometers (H, low-pressure arm). The height of the rise (h) is dependent upon the difference in pressure on either side of the capillary portion (F) of the manometers (H). Once adjusted, the pressure on the manometers will remain constant even with a considerable variation in amount of excess air released through the barostat. The regulated air flow passing through each capillary flowmeter is led through a Teflon line (I) to the outer olfactometer chambers (J). The outer chambers of each olfactometer were connected to the inner chamber (K) by short pieces of Teflon tubing. Chamber (K) was fitted with a Plexiglass partition which extended to within 25 mm of the bottom of the chamber. This minimized mixing of gases

in the chamber without restricting insect movement. Screen grids (L) were placed at the olfactometer air intake and at the exhaust port (M) to restrict insect movement from the olfactometer.

The air-flow control system including all parts shown in Figure 2 was mounted on a plywood board. A series of 10 flowmeters (manometers + capillaries) was used which permitted operation of 5 olfactometers at a time. Manifolds were constructed from 19-mm ID copper tubing with approx. 25-mm inserts soldered in place. Manometer arms were attached to the manifolds with black rubber tubing. The barostat tower should be approx. 50 mm in diameter. The capillaries used were 0.008-in. ID Trubore tubing, 25 mm long, obtained from Ace Glass Co., Inc., Vineland, New Jersey. These gave a flow rate of 20 ml/min at 30 cm water pressure.

Calibration and adjustment of air-flow systems of this type have been described by Claypool and Keefer (1942). To test the accuracy and precision of the system, an experiment was conducted using a bubble meter which measured the time required for a bubble of air to rise a given height. Each of the 10 capillary lines (I) was sampled 5 times at 26, 30, and 34 cm of water height in the barostat tower. The results are shown in Table 1. The data were subjected to an analysis of variance with height of water column serving as blocks and sample lines serving as treatments. If the treatments were judged not significantly different, then it was evident that the lines were performing in a comparable manner. The F test showed that the ratio, 2.056 with 9 and 18 *df*, was not significant at the 5% level. The precision of the individual measurements was 0.1 sec at the 95% confidence level.

DISCUSSION

Bioassays using olfactometers for detection of pheromones, kairomones,

TABLE 1. FLOW RATES FOR EACH OF 10 AIR-OUTLET LINES FROM A FLOW-CONTROL APPARATUS AS SHOWN IN FIGURES 1 AND 2

Height (cm) of water column in barostat tower	$\bar{X}$ time (sec.) required for a CO_2 bubble to rise 25 ml in a pinch-cock buret attached to each of the following lines[a]									
	1	2	3	4	5	6	7	8	9	10
26	87.2	88.4	87.8	89.0	88.2	88.0	89.6	88.2	86.2	86.2
30	78.4	79.2	80.0	79.0	79.2	78.8	79.8	78.8	78.6	78.4
34	72.0	71.6	72.0	71.0	71.0	70.2	72.0	71.0	71.8	71.0

[a] SEM = 0.82 sec. Means of 5 runs on each line.

other attractants, and repellents by insects are largely concentration dependent. Under experimental conditions, there are two simple ways to alter the concentration of a particular molecule (whether of known or unknown identity). One technique is to alter the concentration at the source (e.g., greater quantity of source material or greater output from the source). The other is to alter the flow rate of air moving through an apparatus (i.e., olfactometer). For example, if source A has a fixed production rate of molecules of a particular pheromone, kairomone, etc., then altering the flow rate of air through the system from 10 ml/min to 20 ml/min would decrease the concentration of the volatile at the point of detection by 50%. It can readily be seen that small changes in flow rate can result in major changes in the potential for detection by an organism. Thus erroneous results can easily be obtained if the flow rate is not completely uniform through all arms of the olfactometer.

With a vacuum system, air movement is generally not calibrated and moves via the path of least resistance. As a consequence, small changes in shape or size of outer chamber openings can make substantial differences in the total volume of air that moves through one chamber as opposed to another. The use of a low-positive-pressure system, such as the one we describe here, gives a constant, known flow rate of air at each entry point. Interchamber mixing due to excess turbulence is minimized. With this system, the flow rate may be altered within a given range by making either of two different adjustments: changing the height of the air-intake tube in the barostat tower thus altering the pressure on the system (h) or changing the size of the capillaries in the manometer arms (F). These adjustments will allow for a total range in flow rates from approx. 5 ml/min to 5 liters/min.

Care should be taken to ensure that accepted techniques of olfactometer study such as minimization of outside effect have been followed.

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AN ESTIMATE OF THE EFFECTIVENESS OF PHEROMONE-BAITED TRAPS FOR THE SUPPRESSION OF *Scolytus multistriatus* (COLEOPTERA : SCOLYTIDAE)

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Abstract—We attempted to suppress a population of *Scolytus multistriatus* (Marsham), the principal vector of Dutch elm disease, by trapping flying beetles on sticky traps baited with synthetic pheromone. The estimated catch on 421 traps distributed throughout a 1-km^2 plot in Detroit, Michigan, was nearly 1 million beetles. Because an estimated 5 million beetles emerged in the plot during the study period, we conclude that this preliminary trapping study had no appreciable effect on suppressing the population. Studies that employ improved materials and techniques are continuing.

Key Words—*Scolytus multistriatus*, Dutch elm disease, pheromone, Multilure, mass-trapping, *Ulmus*.

INTRODUCTION

The smaller European elm bark beetle, *Scolytus multistriatus* (Marsham), is the principal vector of Dutch elm disease (DED) and aggregates in large numbers when it colonizes the phloem tissue of decaying elms (*Ulmus* spp.). Beetles are initially attracted to odors produced by host elms (Meyer and Norris, 1967), but the mass attack on the decaying wood is triggered by pheromones released by virgin female beetles that tunnel in phloem tissue (Peacock et al., 1971). The components of the attractant released from the host and the virgin female beetles have been isolated, identified, and syn-

thesized, and the effectiveness of the synthetic beetle attractant has been confirmed in field tests (Pearce et al., 1975). We made a small-scale trapping study in Detroit, Michigan, in 1974 to estimate the number of beetles that were caught on pheromone-baited traps and the total population that emerged in the plot.

METHODS AND MATERIALS

Plot Description

A square plot about 1 km^2 was established in a residential district of Detroit, Michigan. It contained 1360 elms (*Ulmus americana*) during 1974, most of which were 50–65 cm in diameter at breast height (dbh), 11–18 m tall, and spaced 12 m apart along streets (except for gaps where trees had been removed). The elms in the plot were close to other elms to the south and east, but there was an elm-free strip 300–500 m wide to the north and west. The annual DED rate in the plot increased gradually from about 5% in 1969 to more than 10% in 1974.

DED control techniques applied from 1969 to 1974 were: (1) dormant mist-blower spraying of 1/4 kg technical methoxychlor per tree to prevent beetles from feeding on healthy elms, and (2) sanitation cutting of diseased or decaying wood to remove beetle breeding sites. City forestry crews removed most of the diseased elms during the winter, but little, if any, diseased wood was removed from April 1 to November 1.

Trap Description and Placement

The suppression traps were 30×30 cm squares of 0.62-cm-mesh hardware cloth coated with Stikem Special® (Bedard et al., 1969).[1] Each trap was baited with 10^6 beetle-hour equivalents (Pearce et al., 1975) of a synthetic pheromone, Multilure, in a closed 2.5-ml polyethylene vial. For 2–3 weeks, the pheromone was released at a rate of about 2000 beetle-hour equivalents per hour, but the rate diminished greatly thereafter. The vials were replaced on June 7, July 11, August 15, and September 12 to coincide with the beginnings of peak emergence periods.

We placed 421 baited traps about 50 m apart in a grid pattern throughout the plot. We also deployed 10 unbaited check traps. The traps were attached at a height of about 3 m on the boles of trees along streets from May 18 to May 23, 1974. After each generation emerged (July 11, September 12, Octo-

[1] The use of trade, firm, or corporation names in this paper is for the information and convenience of the reader. It does not constitute an official endorsement or approval by the Forest Service or the USDA.

ber 31), the sticky screens from all the traps were removed to determine the number of trapped beetles.

To obtain estimates of the number of trapped beetles, we soaked groups of about 80 traps in a kerosene bath for 1 week to loosen beetles and extraneous material from the Stikem. The beetles and extraneous materials were washed off the traps with additional kerosene and were collected in a 0.08-cm-mesh screen net. These collected materials were then rinsed with acetone to remove the kerosene and any remaining Stikem and to dry the material.

Soil sieves were used to separate the beetles from most of the extraneous material; a 0.13-cm-mesh sieve retained both beetles and beetle-sized particles. The total volume of this sample was measured, and the beetles in three separate 25-ml subsamples were counted for each 1000 ml of sample collected. We estimated the total catch by multiplying the total volume by the mean number of beetles per unit volume. Because some beetles were broken during the cleaning process and passed through the sieve, we calculated the bias of this estimate. The bias was the difference between the actual counts of beetles and the estimated number of beetles for a sample of 80 traps. Actual counts exceeded estimates by about 20%.

We also deployed 15 traps baited with virgin female beetles. Ten traps were placed on diseased elms and 5 were placed on healthy elms. The traps on healthy elms provided a comparison between the attractiveness of the synthetic and natural pheromone and, because the traps released a relatively constant amount of pheromone, we were able to monitor fluctuations in beetle population through the summer. Traps on diseased elms allowed us to determine if the additional pheromone produced by a natural infestation would affect the trap catches.

These 15 traps were similar to those described by Peacock et al. (1971). The source of pheromone for each trap was 160 virgin female beetles that were confined on 4 elm bolts (40 per bolt). Each week we replaced the oldest elm bolt with another prepared bolt to maintain a relatively constant level of pheromone production throughout the trapping season. Traps were placed on elms from May 18 to May 20. Stikem-coated screens on these traps were replaced weekly.

Sources of Beetles

Elm bark beetles can colonize any decaying phloem/outer xylem tissue of any species of *Ulmus* larger than 5 cm in diameter (diseased trees, wind-damaged branches, wood piles, etc.) (Wallace, 1940). However, in the Detroit study plot, the amount of brood wood that resulted from sources other than diseased trees was insignificant for the following reasons: (1) forestry crews generally removed wind-damaged trees and broken branches

promptly and the use of elm as firewood was prohibited; (2) decaying limbs on healthy elms not removed by city crews did not seem to be an important source of beetles. Only 11 of 68 healthy elms (5% of the population, randomly selected) had decaying limbs that beetles might have colonized (total area was 15 m^2), and none of these limbs contained beetle brood.

However, a single diseased elm that is 60 cm dbh has 50–100 m^2 of bark surface area, although usually only a fraction of that area is colonized in a single year. Since 100–200 elms in the plot became diseased each year, and 20–40 (through oversight) remained in the plot for two summers, it seemed that diseased trees were the most important source of beetles because they contained so much more brood wood than any other source. Therefore, our study aimed at determining the number of beetles in diseased elms.

We used a two-stage sampling system to estimate the beetle population. In the first stage, we constructed a stratified random sample of diseased elms based on two DED surveys in 1973 and two in 1974. Preliminary studies indicated that beetle populations in trees varied widely and depended on the date of initial infection and the severity of symptoms. In an attempt to reduce variance between trees, we grouped the trees according to these variables. The symptoms were classified as light when less than 3 m of only 1 limb was affected; medium when more than 3 m of only 1 major limb (30 cm basal diameter) was affected; and severe when more than 1 major limb was affected.

Later examinations indicated that trees with symptoms classed as light were not colonized, and there was no apparent difference in the extent of beetle emergence between medium or severely diseased trees. Therefore we grouped medium or severely diseased elms according to the time of initial infection. In the second stage, we took a systematic sample of the beetle population that emerged from elms within each tree group.

Dutch Elm Disease Surveys

Two DED surveys were made in 1973. The first survey was in mid-June, and the second was in mid-August; these are the periods when symptoms are most easily detected (Neely, 1970). From these surveys, and city records of tree removals during the winter of 1973–1974, we were able to identify elms that were diseased in 1973 but not removed during the winter. These trees were potential sites for overwintering beetles (group I).

The first survey of 1974 was also conducted in mid-June, just after full leaf expansion. The survey corresponded with the peak emergence of the overwintering generation of beetles. Elms that showed initial symptoms at this time were likely to be colonized by the overwintering beetles and thus were potential sites for emerging beetles in mid- and late summer (group II).

The second survey of 1974 was made in mid-August to locate elms that first showed disease symptoms after the June survey. Although some of these trees (for example, those that developed symptoms shortly after the June survey) could have been colonized by overwintering beetles, they were more likely to be attacked by the second-generation beetles that emerged during midsummer; thus they would be potential emergence sites for a partial third generation (group III).

Within-Tree Sampling

Twenty-five elms with medium or severe symptoms (17% of the total) were selected for sampling. Elms with light symptoms were not selected because they did not have limbs that were suitable for beetle colonization. Based on our expectations of variability within a group, 9 elms were selected at random from group I, 11 from group II, and 5 from group III. These diseased elms were felled and systematically sampled to assess: (1) the number of beetles that emerged, (2) the surface area of bark where there was emergence, and (3) the number of maternal galleries in areas of emergence.

The sampling technique that we used was similar to the technique described by Truchan.[2] The trees were felled after emergence was complete (December, 1974), and each limb was examined for emergence holes. The emergence holes were distinguished from attack holes or parasite holes by size and angle of entry. If beetles emerged, the limb was cut into sections at intervals where it was forked, or where the diameter was 5 cm smaller than it was at the previous cut. Only the areas of limbs with emergence holes were sampled; the rest of the tree, which usually had numerous attack holes, was discarded. The mean diameter and length of all sections with emergence holes were recorded to calculate the total emergence area within the tree.

We cut samples from each of the limb sections, counted the emergence holes in each sample, measured the area, and calculated the emergence density. We cut at least 5% of the section but up to 100% if the section was small; 28% of the total wood with emergence was sampled. The emergence density in each sample multiplied by the area of the appropriate section gave us the estimate of emergence from that section. Total emergence from a tree was obtained by adding these estimates for tree sections. Multiple emergence is not considered important for this insect, so each emergence hole was counted as only one beetle. The bark was then stripped from the samples, and the maternal galleries were counted. Estimates of the number of maternal galleries per tree were calculated the same way. We used the mean and vari-

[2] Truchan, J.G. 1970. Field evaluation of *Dendrosoter protuberans* as a biological control agent for *Scolytus multistriatus*, the primary vector of Dutch elm disease. Ph.D. thesis. Mich. State Univ., East Lansing, 97 pp.

ance of these total estimates for individual trees within each tree group to calculate population estimates.

RESULTS AND DISCUSSION

Beetle Response to Traps

The estimated catch on the 421 suppression traps was 991,000[3] beetles. Based on the catches at virgin-female-baited traps, we estimated that 240,000 were caught during the first emergence period (June 1 to July 11), 506,000 during the second period (July 12 to September 12), and 245,000 during the third emergence period (September 13 to October 31). The beetles began flying to the traps during the first week in June and continued through October. Weekly fluctuations in population levels are shown in Figure 1 by mean catches on the 15 traps baited with virgin female beetles. The peak catch periods coincided with peak emergence periods for this insect (Wallace, 1940). However, weather conditions apparently caused some fluctuations;

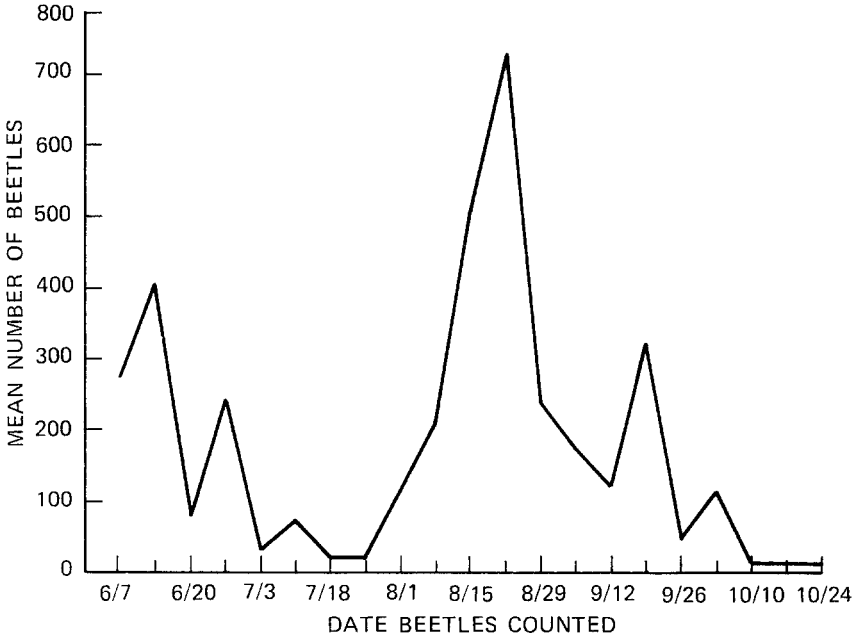

FIG. 1. Weekly mean catch on 15 traps baited with virgin female beetles boring in elm bolts.

[3] The 20% bias is included in this estimate.

TABLE 1. MEAN AND TOTAL ESTIMATES OF EMERGENCE OF ELM BARK BEETLES FROM 142 DISEASED ELMS AND THEIR STANDARD ERRORS (SE) BY TREE GROUP

Tree group	Date of initial symptoms	Per elm		Number of elms[a]	Per group	
		Mean	SE		Total	SE
I	August 1973	18,370	4,119.1	38	698,060	156,526
II	June 1974	65,318	8,229.3	63	4,115,034	518,446
III	August 1974	3,047	475.9	41	124,927	19,512
Total		34,774.8[b]	3,816.3	142	4,938,021	541,911

[a] Does not include 49 elms that developed only light disease symptoms by August 1974.
[b] Weighted average.

fewer beetles were caught during a cool and windy period from June 13 to June 20.

The mean catch on female-baited traps that were placed on healthy elms was 1520 beetles; on diseased elms the mean catch was 4942 beetles. The relationship was the same for mean catches on unbaited check traps. Two hundred thirty beetles were caught on one check trap that was on a diseased elm that became symptomatic in August 1974. The mean catch for check traps on healthy elms was 34 beetles. Pheromone produced by the natural beetle infestations in the diseased elms very likely accounts for the higher beetle catches on these elms.

Beetle Population Estimates

We estimated that nearly 5 million beetles emerged from 142 diseased elms (with medium or severe symptoms) during 1974 (Table 1). However, less than 15% (698,060) were overwintering beetles (group I)—considered the most important generation for the transmission of DED (Parker et al., 1941). More than 80% of the beetles (4,115,034) emerged from elms that first developed symptoms during June 1974 (group II). The estimated emergence from elms that became symptomatic during July and August 1974 (group III) was practically negligible ($<5\%$ of total) compared to the emergence from elms in other groups.

Beetles emerged only from limited areas of less than half the diseased elms in the plot during 1974. None emerged from 11 of the 25 trees in the subsample (Table 2). We were particularly surprised that there was no emergence from 3 of the 9 trees sampled in group I because all of these trees had been symptomatic for more than a year. As expected, the percentage of

TABLE 2. ESTIMATES OF BEETLE ATTACK AND EMERGENCE IN SUBSAMPLE OF 25 DISEASED ELMS

Tree group and number	Area with emergence holes (dm^2)	Maternal galleries per dm^2[a]	Emergence holes per dm^2	Replacement rate for female beetles
I 3	0	—	—	—
14	0	—	—	—
17	0	—	—	—
7	165	0.59	8.09	6.86
18	875	2.12	1.77	0.28
12	1,293	1.50	4.94	1.65
19	2,385	1.51	18.95	6.25
6	3,032	5.78	17.87	1.55
9	2,907	3.18	19.58	3.08
Per group	10,657	3.22	15.51	2.41
II 10	0	—	—	—
5	0	—	—	—
4	0	—	—	—
20	0	—	—	—
1	287	4.61	0.82	0.09
16	61	1.64	21.48	6.55
21	1,332	1.97	9.70	2.46
15	1,592	3.61	9.39	1.30
22	5,928	4.07	10.86	1.33
8	6,940	3.85	42.61	5.53
11	6,722	2.68	48.93	9.13
Per group	22,862	3.44	31.43	4.57
III 13	0	—	—	—
23	0	—	—	—
24	0	—	—	—
2	0	—	—	—
25	2,174	6.18	7.01	0.57
Per group	2,174	6.18	7.01	0.57
All groups	35,693	3.54	25.19	3.56

[a] Includes only galleries in areas where beetles emerged.

trees with no emergence was even higher among those trees that became diseased later (groups II and III).

In the elms from which beetles had emerged, usually only 1 or 2 major limbs were affected. Only 3 elms (all in group II) had emergence holes over their entire surface area. The total area with emergence holes in the 25 sampled trees was about 20% of their total bark surface area.

The emergence per unit of area in a tree appeared to be directly proportional to area with emergence in that tree (Table 2). The few trees with the most emergence area in each group accounted for nearly all the estimated emergence. In group I, 95% of the beetles emerged from 3 trees; 87% emerged from 2 trees in group II; and 100% emerged from 1 tree in group III.

Although beetles emerged from only about 20% of the bark surface of diseased elms during 1974, they had successfully colonized a much greater proportion by the end of the summer. An examination of the 25 sampled trees showed that beetles had attacked nearly all the bark surface of limbs with disease symptoms and that these limbs contained live larvae. Also, most limbs from which beetles had emerged during the summer still contained many live larvae when the trees were felled. We are not sure whether these larvae represent a diapausing population or the progeny of a succeeding generation. From the counts of beetles that emerged from sample log sections in the laboratory, we estimated that tree 11 and tree 22 (Table 2) still contained about 52,000 and 372,000 larvae, respectively, in the limb sections where beetles had emerged during the summer. Despite incomplete emergence at the time of sampling, the average replacement rate for female beetles (1/2 the emergence divided by the maternal galleries) in areas with emergence holes was 3.56 (Table 2). But the actual increase in the total population over the summer, including larvae in areas of diseased elms that were not sampled, was far greater than was indicated by either trap catches or estimates of adult emergence. However, where sanitation cutting is practiced, most of these larvae are destroyed during the winter.

Effect of Trapping on the Beetle Population

We could not make a conclusive evaluation of the effect of trapping on population suppression because of the large standard errors of the population estimates. But on the basis of the population trend and the proportion of beetles trapped, we concluded that trapping did not significantly affect the population. Although the number of beetles trapped during the first emergence period was nearly 35% of the total estimated emergence, we estimated that the succeeding generation of beetles was 5 times greater than the first generation. Also, the percentage of the emerging population that was trapped during

the second generation was even lower than that of the first generation. The total number of beetles trapped (991,000) during the summer was only 20% of the estimated number that emerged (4,938,021)—clearly not enough to suppress the population.

Several factors contributed to the failure of trapping to suppress the population during 1974. First, beetles probably migrated into the plot from nearby areas. When suitable feeding and breeding sites are available, the flight range of beetles is generally less than 600 m (Collins, 1938; Wolfenbarger and Jones, 1943; Wallace, 1940) but studies have shown that they can fly at least 3 km. Since the plot was small compared to the flight range of beetles and the elms in the plot were close to other elms on two sides, we concluded that beetle immigration lessened the impact of trapping.

Second, the incidence of DED in Detroit was very high during 1974 (14% of the elm population in the study plot), so the potential brood sites and beetles also were abundant. Thus the effect of trapping was reduced because of the large number of naturally attractive sites that competed with our traps.

Third, the release rate of the three pheromone components from the plastic vials may have been too low initially, and it diminished below minimum attractive levels after exposure for 2–3 weeks. Because the vials were changed only every 5 or 6 weeks, the traps were ineffective about 50% of the time. The prevalence of DED in Detroit during 1974 presented a severe test for the use of pheromone-baited traps to suppress the beetle population. In view of these problems, future tests will be conducted with improved pheromone dispensers in areas where the DED rate is considerably lower and beetle migration is less likely.

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ISOLATION AND IDENTIFICATION OF A MAJOR SEX-ATTRACTING COMPONENT OF *Attagenus elongatulus* (CASEY) (COLEOPTERA : DERMESTIDAE)

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Abstract—A major sex-attracting component in extracts of virgin females of *Attagenus elongatulus* Casey was isolated by various chromatographic techniques. The chemical was identified as (Z,Z)-3,5-tetradecadienoic acid by spectroscopic analyses.

Key Words—Coleoptera, Dermestidae, *Attagenus elongatulus*, sex-attracting pheromone, identification, black carpet beetle, (Z,Z)-3,5-tetradecadienoic acid, (E,Z)-3,5-tetradecadienoic acid, megatomoic acid.

INTRODUCTION

A pheromone of the black carpet beetle, *Attagenus megatoma* (F.), identified as (E,Z)-3,5-tetradecadienoic acid by Silverstein et al. (1967), was given the trivial name megatomoic acid. Several other species of *Attagenus* have been reported from Wisconsin (Bayer et al., 1972) and the United States (Beal, 1970). One of the most important of these species appears to be *Attagenus elongatulus* Casey (Barak and Burkholder, 1977a), widespread in houses in the northern United States and occurring also in peanut warehouses in Georgia (Beal, 1970).

Pheromone studies with *A. elongatulus* by Barak and Burkholder (1977b) have shown that females produce a pheromone which excites and attracts

males. The response by *A. elongatulus* males is greater to extracts of their own females than to those of *A. megatoma* females; this indicates a possible difference in the pheromones. Recently we have also identified the major pheromone of *Anthrenus flavipes* (Fukui et al., 1974) to be (Z)-3-decenoic acid.

To identify the sex pheromone of *A. elongatulus*, we isolated and tested the activity of various components of female beetle extracts, and identified one major component of the *A. elongatulus* female sex pheromone. We now report this work.

METHODS AND MATERIALS

A. elongatulus adults used in this study were from a stock culture originally collected from homes in Madison, Wisconsin, in 1959. They were reared on the same diet and under the same conditions used for *Trogoderma* species reared in the Stored Product Insects Laboratory (Hammack et al., 1973).

The crude megatomoic acid used in this study was synthesized at the Stanford Research Institute. The material consisted of 76% megatomoic acid or its isomers in the following amounts: (E,Z) 53%; (Z,E) 20%, (Z,Z) 18%; (E,E) 9%. These isomers were isolated by repeated preparative gas–liquid chromatographic separation (GLC) on an SE52 column. Throughout the experiment, the biological activity of the acids or their corresponding methyl esters was monitored by a small-vial test described by Vick et al. (1970).

The purification method for the extracts was essentially identical to the one employed for the sex pheromone of *Anthrenus flavipes* Le Conte (Fukui, et al., 1974). Virgin females (1500 individuals) of *A. elongatulus*, along with the filter papers from the holding jars, were used as the source of pheromone. The insects and filter papers were extracted with three portions of 30-ml aliquots of *n*-hexane. These extracts were combined, condensed to 5 ml through evaporation by using a Snyder column, then quantitatively transferred to a chromatographic column with silicic acid powder (Mallinckrodt) 100–200 mesh.[3] The column was successively eluted with (1) 200 ml of *n*-hexane, (2) 100 ml of benzene, (3) 200 ml of chloroform, and (4) 100 ml of ethanol. The biologically active chloroform fraction was saved and concentrated to approximately 1 ml. This residue was spotted on HF254 silica gel thin-layer chromatographic plates (activated at 120°C for 2 hr, thickness, 0.25 mm; plate dimension, 20×20 cm). The plates were developed with a mixture of benzene and acetone (6:1) till 15 cm from the origin. The biologically active zone ($R_f = 0$–0.3) was collected and eluted with acetone. After distillation of the acetone, the residue was redissolved in 10 ml of anhydrous

[3] Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the USDA.

ether containing excess diazomethane and allowed to stand overnight. The solvent was evaporated, and the residue was spotted on another silica gel plate, which was developed with benzene. The active zone (R_f = 0.3–0.6 as assayed directly in the form of methyl esters) was again collected and eluted with acetone. This fraction was used for the final gas chromatographic purification, which is described in the following section.

The primary gas–liquid chromatographic system used was a Beckman GC-4 with dual flame ionization detectors. For purification attempts, the effluent gas was split 9 to 1; the larger portion was collected in ether traps at 0°C, while the latter was sent to the detector (Matsumura et al., 1969). In all cases, glass columns (180 cm, ID 3 mm) packed with SE52, NGA (neopentyl glycol adipate), and Carbowax 4000 (monostearate, all on chromosorb P) were used. For capillary GLC analysis, a Varian aerograph system (model 1824) with a 50-ft SE30 capillary column (Perkin-Elmer Co.) was used with a flame ionization detector. An essentially identical capillary–GLC system on another Varian aerograph system (a modified model 1700) was connected to the Finnigan (model 1015) mass-spectrometer system for GLC-MS analysis of the purified sample.

Ozonolysis of the pheromone in its methyl ester form was carried out in 0.5 ml of *n*-pentane at −18°C for 6 min.

All proton magnetic resonance (PMR) spectra were recorded on a Bruker Spectrospin 90-Hz model using 5-mm tubes with deuterochloroform as the carrier solvent. The infrared spectra were recorded on a Beckman IR-33 in micro-KBr pellets. The UV spectra in anhydrous ether were taken manually on a Beckman DU or by scanning on a Varian-Techtron 650 D spectrometer.

RESULTS

As a result of the silicic acid column and the first thin-layer chromatographic (TLC) separation, it was noted that there are at least two biologically active components in the original extract: a highly polar material (R_f = 0.3 on TLC) and a less polar material (R_f = 0.6–0.8). Since the latter component was present only in a small quantity, it was decided that we first examine the former component.

After the first TLC purification, the polar pheromone fraction was treated with diazomethane, and the product was further purified on the second TLC process. The R_f value of the active fraction was found to be higher than the original polar pheromone as expected by the methylation of an acid. This fraction gave a relatively simple peak pattern on SE52–GLC analysis (Figure 1). As a result of repeated preparative SE52 purification, the biologically active zone was purified to yield a single peak on SE52, NGA,

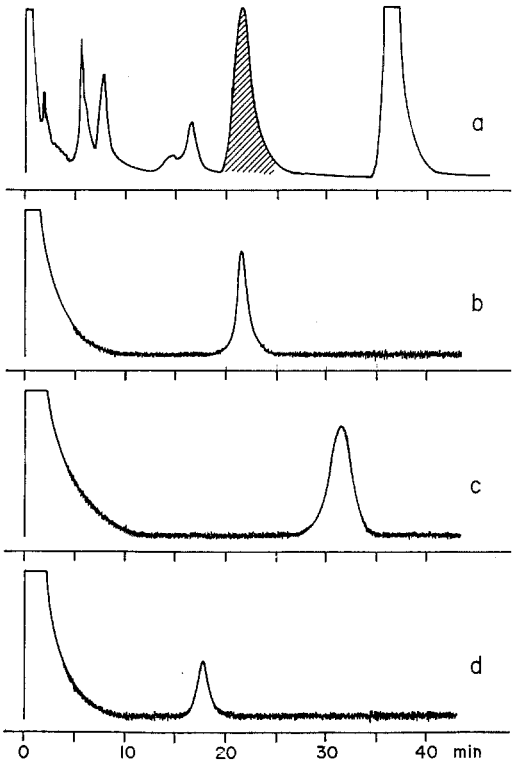

FIG. 1. GLC patterns of the methyl ester of *Attagenus elongatulus* pheromone. (a) Partly purified active fraction injected on GLC (SE-52). (b) Active component collected on (a) injected on SE-52. (c) Natural compound on NGA. (d) Natural compound on Carbowax 4000.

or Carbowax 4000. By these methods, 150 μg of the pure pheromone were obtained.

GLC-MS analyses (Figure 2a) established that the molecular weight of the pheromone's methyl ester was 238, the identical mass number as the methyl ester of megatomoic acid. To ascertain the positions of the double bonds, a small fraction (5 μg) of the *A. elongatulus* pheromone was subjected to ozonolysis. The resulting major fragment was identified by GLC on SE52 (Figure 3) as nonanal; therefore, one of the double bonds is at the carbon-5 position. There are two pieces of evidence to show that the other double bond is located at the carbon-3 position. The results of UV spectroscopic analyses show that the pheromone has an absorption maximum at 234 nm

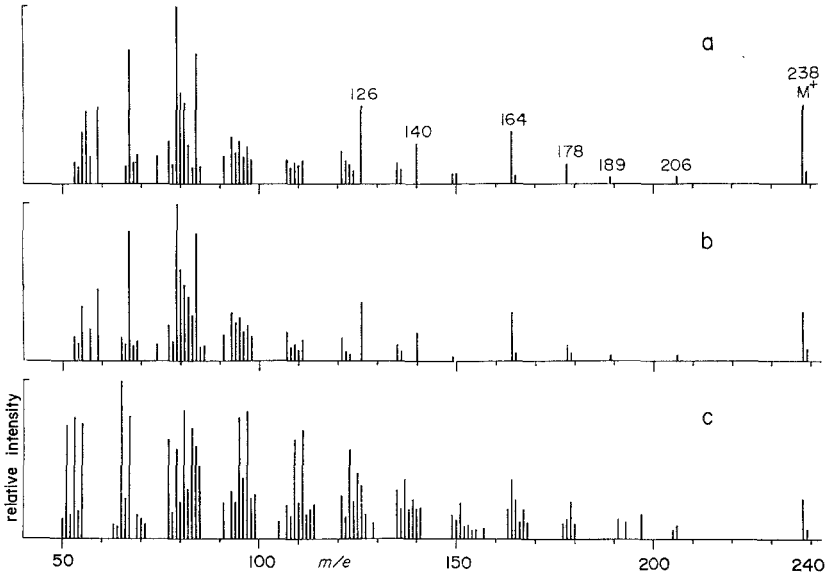

FIG. 2. Mass spectra of methyl esters of *Attagenus elongatulus* pheromone. (a) *Attagenus elongatulus* methyl ester (GC-MS). (b) Synthetic Z-3, E-5 methyl ester (GC-MS). (c) *Attagenus elongatulus* methyl ester (via direct inlet method).

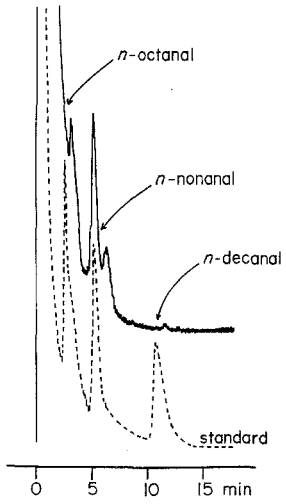

FIG. 3. GLC pattern of ozonolysis product of methyl ester of *Attagenus elongatulus* pheromone. Ozonolysis products were injected on SE-52 and compared with standards. Arrows refer to the standard aldehydes.

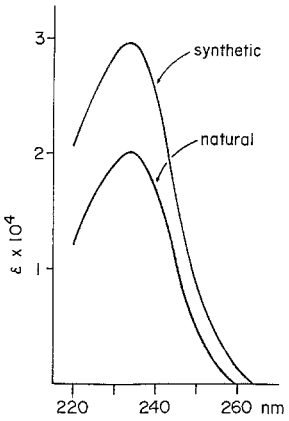

FIG. 4. Ultraviolet absorption spectra of the purified pheromone and the synthetic (Z,Z)-3,5-tetradecadienoic acid (solvent: absolute ether).

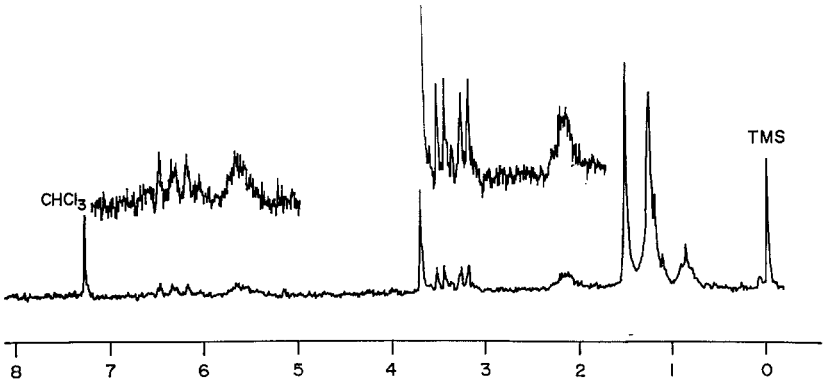

FIG. 5. PMR spectrum of the methyl ester of the major sex attracting component of *A. elongatulus*. The presence of a perturbed triplet (3H) at 0.87δ showed that this compound has a long carbon chain, whose signal is exhibited at 1.26δ. Furthermore, four conjugated olefinic protons (5.22–5.75δ and 5.85–6.60δ, multiplet), carbomethyoxyl protons (3.69 singlet), two allylic protons (2.13δ, multiplet) and the other two allylic protons (3.21δ doublet), which are deshielded by a carboxyl group, are recognized in the proton magnetic resonance spectrum (90 MHz, $CDCl_3$). The signals at 3.47δ (quartet) and at 1.20δ (triplet) are derived from ether. The signal at 1.52δ (singlet) is assigned to water. See also Silverstein (1968) for reference.

(Figure 4) with an extinction coefficient of approximately 20,000, indicating the presence of a conjugated diene, and not an α,β-unsaturated ester. The PMR spectrum (Figure 5) for the methyl ester of the pheromone clearly shows the conjugated double-bond arrangement (i.e., 2 protons each at 5.3–5.8 and 6.0–6.6) and 2 adjacent protons for $-CH{=}CH-CH_2C(O)OCH_3$ at $\delta = 3.22$. Taking the mass spectroscopic and ozonolysis data into consideration, we can conclude the basic skeleton of the *A. elongatulus* pheromone structure is $CH_3(CH_2)_7CH{=}CH-CH{=}CHCH_2COOH$, a formula identical to megatomoic acid.

The geometrical configurations of the double bonds of the pheromone are apparent when the infrared spectrum of its methyl ester is examined in comparison to that of megatomoic acid. The characteristic peaks due to the conjugated double bonds are the 1015, 985, 955 cm^{-1} peaks (Figure 6c) for methyl megatomic while only the 1015 cm^{-1} peak (Figure 6a) is predominant for the methyl ester of the pheromone. This 1015 cm^{-1} peak must represent a cis–cis conjugated double bond. Indeed the IR spectrum of the authentic methyl ester of (Z)-3,(Z)-5-tetradecadienoic acid (Figure 6b) is identical to that of the methyl ester of the *A. elongatulus* pheromone. All GLC (Table 1) and spectroscopic comparisons of the natural and the synthetic pheromones (Figures 2, 4–6) unequivocally confirm this diagnosis.

In addition, the methyl ester of the pheromone was critically compared

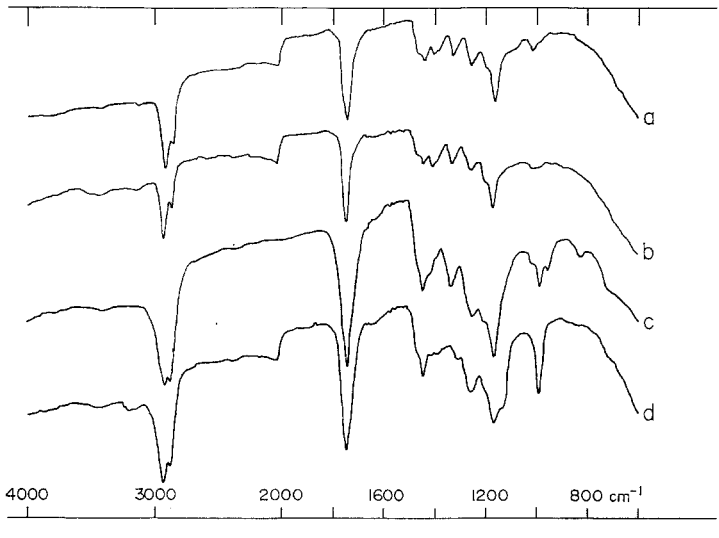

FIG. 6. IR (KBr) spectrum of the methyl ester of the pheromone and its isomers. (a) *Attagenus elongatulus* methyl ester. (b) Synthetic Z-3, Z-5 methyl ester. (c) Synthetic E-3, Z-5 methyl ester. (d) Synthetic E-3, E-5 methyl ester.

TABLE 1

	SE-52 (130° C, 1.8-m glass column, N_2)	NGA (130° C, 1.8-m glass column, N_2)
Z-3, Z-5	13.4[a]	14.5[a]
Z-3, E-5	12.7	13.7
E-3, Z-5	12.2	13.4
E-3, E-5	14.4	16.8

[a] Identical retention time as the methyl ester of the *A. elongatulus* pheromone.

TABLE 2. BIOLOGICAL ACTIVITIES OF FOUR GEOMETRIC ISOMERS OF MEGATOMOIC ACID IN *A. elongatulus* MALES[a]

Isomers	concentration (μg/assay)					
	1×10^{-1}	3×10^{-2}	1×10^{-2}	3×10^{-3}	1×10^{-3}	3×10^{-4}
Methyl esters						
Z-3, Z-5[b]	60%	50%	48%	24%	14%	8%
E-3, Z-5[b]	4%	6%	8%	10%	4%	0
Z-3, E-5[b]	0	8%	14%	10%	0	0
E-3, E-5[b]	10%	28%	8%	6%	6%	6%
Natural pheromone[c] methyl ester	57%	37%	7%	20%	13%	7%
Free acids						
Z-3, Z-5[c]	53%	20%	37%	3%	7%	0
natural pheromone[c]	23%	13%	17%	0	10%	3%

[a] Data are expressed in % of males responding to the chemical at the given concentration.
[b] 50 males per assay.
[c] 30 males per assay.

to the methyl ester of the synthetic (Z)-3,(Z)-5 isomer on the carbowax 4000 (90°C, 1.8 m, R_t = 11.1; methyl pentadecanotate, 11.7 min) and the capillary SE30 column (190°C, N_2 2 ml/min, R_t = 9.8 min).

Male responsiveness to these isomers was compared to the natural pheromone. In the experiment summarized in Table 2, we found that the cis–cis isomer elicits the same level of reaction in male *A. elongatulus* as the natural pheromone. We also ascertained that the male behavioral response elicited by the synthetic pheromone was qualitatively identical to that produced by the natural pheromone. Biological activities of the free acids were compared by the same methods (Table 2).

DISCUSSION

There are several examples of species differentiation by the use of different geometric isomers of pheromones, particularly in lepidopterous insects (Klun et al., 1973). In coleopterous insects we know of only one other example; the major sex-attractant of *Trogoderma inclusum* Le Conte has shown to be (Z)-14-methyl-8-hexadecenal, while in another related species, *T. glabrum* (Herbst) it is (E)-14-methyl-8-hexadecenal (Cross et al., 1976). The geographical distributions of *A. elongatulus* and *A. megatoma* considerably overlap each other (Beal, 1970), and it is therefore likely that the geometrical difference in the configuration of their respective pheromone molecules may serve as a means of reproductive isolation.

An interesting finding is that males of *Attagenus* species respond favorably to the methyl esters of their respective natural pheromone(s). Similar observations were made by Fukui et al. (1974) on *Anthrenus flavipes*. In our study we could see similarities in the activities of the acid and the methyl ester. In the case of *A. elongatulus* we have noted the presence of at least another active fraction with medium polarity (i.e., less polar than acid) in the untreated original extract of the females, although the quantity was small. Because of this quantity difference and the precedent of olefinic acid acting as pheromone in coleopterous insects (Silverstein et al., 1967, Fukui et al., 1974), the balance of evidence suggests the acid to be the major pheromone. Why the males show equal sensitivity towards the acid and the methyl ester forms remains to be answered, for the special requirements of chemoreceptors for insect pheromones are known to be very strict and it is highly unusual to find such impartiality.

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ISOLATION AND TENTATIVE IDENTIFICATION OF LINEATIN, A PHEROMONE FROM THE FRASS OF *Trypodendron lineatum* (COLEOPTERA : SCOLYTIDAE)[1]

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Abstract—An attractant compound was isolated from frass produced by *Trypodendron lineatum* female beetles boring in Douglas fir. The proposed structure is one of two isomeric tricyclic acetals, to which the trivial name lineatin is assigned.

Key Words—*Trypodendron lineatum*, lineatin, pheromone, attractant, tricyclic acetal, Scolytidae, aggregation pheromone, ambrosia beetle.

INTRODUCTION

As a major ambrosia beetle pest of timber throughout the northern hemisphere, *Trypodendron lineatum* (Olivier) has been the subject of considerable research (Nijholt, 1975). Much of this research has been concerned with "secondary attraction," resulting from compounds emanating from the beetles, usually in conjunction with compounds from the host (Borden et al., 1975), with the objective of eventually developing a capacity to manipulate

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pest populations with behavior-modifying chemicals. Secondary attraction involving pheromones produced by virgin or mated female *T. lineatum* was originally demonstrated in the field (Rudinsky and Daterman, 1964a,b; Chapman, 1966) and verified in the laboratory (Borden et al., 1968; Borden and Slater, 1969).

This paper reports the isolation and tentative identification from frass of the principal attractant component of secondary attraction in *T. lineatum.*

METHODS AND MATERIALS

Biology

Litter and duff containing overwintering beetles (Dyer and Kinghorn, 1961) were collected in March–May, 1968–1970 from forest margins adjacent to heavily infested slash fields on southern Vancouver Island. At Simon Fraser University, the beetles were allowed to emerge from the litter and duff in heated rooms and were collected from screened windows to which they had flown.

Approximately 25,000 unmated females were allowed to attack 1-m-long logs from Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] trees, felled in the previous fall or winter. Since Graham (1968) reported that anaerobic metabolism rendered host material more attractive to *T. lineatum,* all logs were immersed in water for 24 hr before being exposed to beetles. From mid-June through August 1970, when the majority of frass[6] was produced, all logs were soaked for 24 hr in 10% ethanol, a primary attractant for *T. lineatum* (Moeck, 1970). An unreplicated experiment with 3 logs from the same tree demonstrated increased frass productivity from females in ethanol-soaked logs (Table 1). The frass was caught in containers cooled with dry ice and held at −40°C until shipped in dry ice to the chemical laboratory in Syracuse, New York. Approximately 450 g of attractive frass was produced and extracted.

All chemical isolation procedures and tests of isolated or synthetic pure compounds were monitored with a runway olfactometer precisely as reported by Borden et al. (1968). In this olfactometer, walking beetles characteristically respond to pheromone odor by rapid turning movements with increased speed of walking when they pass over a perforated area in the runway, beneath which the test stimulus is placed on a cotton wick. The assumption was made that positive responses by walking beetles in the laboratory were equivalent to flight attraction responses of beetles in the field. This assump-

[6] Frass in the Scolytidae usually means a mixture of boring fragments and fecal pellets. We have used the term here even though solid feces do not appear until after about a week in the new host, when *T. lineatum* begins to feed on the symbiotic fungus.

TABLE 1. COMPARATIVE PRODUCTION OF FRASS BY FEMALE *Trypodendron lineatum* IN LOGS PREVIOUSLY IMMERSED FOR 24 HR IN WATER OR 10% ETHANOL IN WATER

Treatment	No. females	Percent producing frass	Total frass production for 7 days (g)	Frass production/♀ for 7 days (g)
Water-soaked log, beetles held near preformed entrance holes by gelatin capsules	544	59.6	3.5	0.011
Ethanol-soaked log, unconfined beetles allowed to attack log with preformed entrance holes	553	42.5	11.6	0.049
Ethanol-soaked log, beetles held near preformed entrance holes by gelatin capsules	508	82.1	15.1	0.036

tion proved valid for *Scolytus multistriatus* (Marsham) for which a similar laboratory bioassay was used (Peacock et al., 1973; Pearce et al., 1975). To avoid observer error, the identity of test stimuli was made known to the experimenter only after all bioassays in a test series were completed. Only males were used, as females were less responsive in the olfactometer (Borden et al., 1968) and were in critical demand for the production of frass.

Each fraction within a given series was bioassayed at approximately 0.01 g equiv[7] with at least 16 male beetles (2 groups of 8 beetles each). If any attraction was apparent, the number of test beetles used was increased to at least 24, and usually 32. The recombined fractions and benzene solvent controls were tested in addition to each individual fraction. The most attractive fraction for each of 7 series identified the starting material for the next chemical isolation procedure. A total of 71 extracts and/or fractions was tested with 440 groups of 8 beetles each during the isolation procedure.

Chemistry

Frass produced by female beetles was extracted with benzene in a water-cooled Waring blender, and the extract, after concentration to about 5% of the volume with a water aspirator, was transferred to a short-path distillation apparatus; the remaining benzene was collected on a condenser (cold finger) cooled with dry ice. The residue was then slowly distilled (with magnetic stirring) at 0.1 Torr up to a bath temperature of 110°C, and the distillate was collected on the cold finger.

[7] One gram equivalent is the amount present in 1g of frass.

This biologically active distillate was chromatographed on a silica gel column (Gallard-Schlesinger, 90–200 mesh), and the active fraction, which was eluted with 20% ether in benzene, was subjected to gas chromatography on a Varian 1740 instrument equipped with dual flame ionization detectors. Initial fractionation was achieved on a 4.7-mm ID × 1.5-m aluminum column of 4% SE-30 on 45/60 mesh Chromosorb G. Batches of 400 μl were injected at an oven temperature of 75°C; after 5 min, the oven temperature was increased at a rate of 6°/min to 150°C. Collection of the chromatographic effluent was carried out with a variable ratio effluent splitter (split ratio approx. 75:1) and a thermal gradient collector (Brownlee and Silverstein, 1968). All material eluting between 5 and 30 min (the active fraction) was rechromatographed in 80-μl batches on a 4.7-mm ID × 1.5-m aluminum column of 4% Carbowax 20M on 60/80 mesh Chromosorb G. The oven temperature of 110°C was held for 3 min after injection, then increased 2°/min to 150°C. This final temperature was held until 60 min had elapsed since injection; then the column was cooled for the next cycle. Under these conditions, the active fraction eluted between about 12 and 19 min postinjection. This fraction was rechromatographed on a 2.4-mm OD × 6.7-m stainless-steel column of 4% FFAP on 60/80 mesh Chromosorb G. At 135°C, the active component eluted between 24 and 28 min. Final purification was achieved on a 2.4-mm OD × 3-m stainless-steel column of 5% DEGS on 60/80 mesh Chromosorb G. The pure, active material was collected between 25 and 28 min. About 200 μg of pure attractant was obtained from 200 g of frass. On both Carbowax 20M and DEGS support-coated open tubular columns (approximately 15,000 theoretical plates), the active compound gave a single symmetrical peak.

The IR spectrum of the active compound was recorded on a Perkin-Elmer Model 621 instrument equipped with a beam condenser and an ultramicro NaCl cell. The solvent absorption (CCl_4) was balanced out with a variable-path-length cell in the reference beam. The NMR spectrum in CCl_4 was recorded on a Varian HA-100 instrument; experiments using the paramagnetic shift reagent Eu(fod)$_3$ (Rondeau and Sievers, 1971) in CCl_4 were also performed with this instrument. The reagent–substrate molar ratio was 1.3. The unit-resolution mass spectrum was recorded on a Hitachi-Perkin-Elmer RMU-6 instrument; the high-resolution mass spectrum was obtained on a CEC 21-110B instrument.

Hydrogenolysis (Beroza, 1970) of the isolated compound was carried out by injecting a pentane solution of the unknown onto a 3-cm column of 1% Pd on Gas Chrom P (washed with 5% Na_2CO_3 solution) in a 2.4-mm OD stainless-steel tube connected to the injection port of the gas chromatograph and maintained at 250–280°C. Hydrogen was used as the carrier gas at a flow rate of 20 cm^3/min. Hydrogenolysis products were identified by co-

injection with reference compounds on two columns: 2.4-mm OD × 7-m stainless-steel packed with 100/120 mesh Porasil C at 120°C and a column of the same dimensions packed with 5% SE-30 on 60/80 mesh Chromosorb W at 80°C.

RESULTS AND DISCUSSION

Biology

Laboratory bioassays used to monitor the sequential isolation procedure disclosed increasing attraction as the purity of the attractant component increased. This suggests that there was a slight masking effect of the principal component by other constituents of the extract. There was evidence of trace attraction to some additional components, but no evidence of synergism between components. The recombined fractions were never more attractive than the single most attractive fraction. Thus, we appear to be dealing with a single-component attractant for all practical purposes.

The isolated and synthetic (see below) pure compounds were highly attractive to male beetles in the laboratory bioassay (Table 2). A solution containing 4.5 ng of the synthetic compound deposited on the cotton wick was sufficient to induce a positive response in the laboratory bioassay. This amount is similar to that required to elicit a threshold response for *Gnathotrichus sulcatus* Le Conte, another ambrosia beetle that apparently employs a single pheromone component, sulcatol, in its secondary attraction (Byrne et al., 1974). However, a definitive statement that the compound produced by *T. lineatum* by itself will elicit aggregation of both sexes in the field cannot be made until the synthetic compound is available in quantity for extensive field tests.

TABLE 2. RESPONSE OF MALE *Trypodendron lineatum* TO ISOLATED AND SYNTHETIC LINEATIN IN A LABORATORY OLFACTOMETER (ALL STIMULI IN 0.05 ML BENZENE)

Stimulus description	Stimulus concentration	No. Beetles tested	No. responders	Percent response[a]
Lineatin from ♀ frass	0.038 g equiv.	35	14	40.0 a
	0.0038 g equiv.	49	10	20.4 ab
	0.00038 g equiv.	35	1	2.9 c
Lineatin, synthetic	0.045 μg	50	20	40.0 a
	0.0045 μg	63	11	17.5 b
Benzene control	0.05 ml	18	0	0.0 c

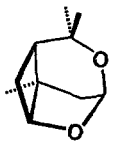
[a] Percents followed by the same letter not significantly different, χ^2 test, $P < .05$. Fisher's (1954) exact test for 2 × 2 contingency tables used when expected values fell below 5.

TABLE 3. SIGNIFICANT PEAKS IN THE HIGH-RESOLUTION MASS SPECTRUM OF LINEATIN

Nominal *m/e*	Measured mass	Calculated mass	Composition
43	43.0196	43.0184	$C_2H_3O^+$
43	43.0559	43.0548	$C_3H_7^+$
55	55.0195	55.0184	$C_3H_3O^+$
55	55.0557	55.0548	$C_4H_7^+$
83	83.0491	83.0497	$C_5H_7O^+$
83	83.0852	83.0861	$C_6H_{11}^+$
85	85.0639	85.0653	$C_5H_9O^+$
96	96.0582	96.0575	$C_6H_8O^+$
96	96.0946	96.0939	$C_7H_{12}^+$
107	107.0861	107.0861	$C_8H_{11}^+$
109	109.0655	109.0653	$C_7H_9O^+$
111	111.0440	111.0446	$C_6H_7O_2^+$
125	125.0967	125.0966	$C_8H_{13}O^+$
135	135.0795	135.0810	$C_9H_{11}O^+$
150	150.1051	150.1045	$C_{10}H_{14}O^+$
153	153.0920	153.0915	$C_9H_{13}O_2^+$
168	168.1163	168.1150	$C_{10}H_{16}O_2^+$

Chemistry

We propose two possible structures (**1** and **2**) from the results of chemical tests and consideration of the spectral information [unit-resolution mass spectrum (Figure 1), high-resolution mass spectrum (Table 3), infrared spectrum (Figure 2), NMR spectrum (Figure 3) and NMR spectrum with $Eu(fod)_3$ shift reagent (Figure 4)]; we further propose the trivial name lineatin for the active compound.



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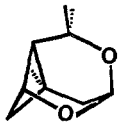

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The mass spectra (Figure 1 and Table 3) suggest $C_{10}H_{16}O_2$ as the molecular ion; the facile loss of 15 mass units (to *m/e* 153) confirms that *m/e* 168 is indeed the molecular ion peak and suggests methyl branching. The IR spectrum (Figure 2) is devoid of hydroxyl or carbonyl absorption; in addition, the molecule was not affected by treatment with a silylating agent (a mixture of trimethylchlorosilane, hexamethyldisilazane, and pyridine) or lithium aluminum hydride. These data exclude hydroxyl, carbonyl, epoxy, and

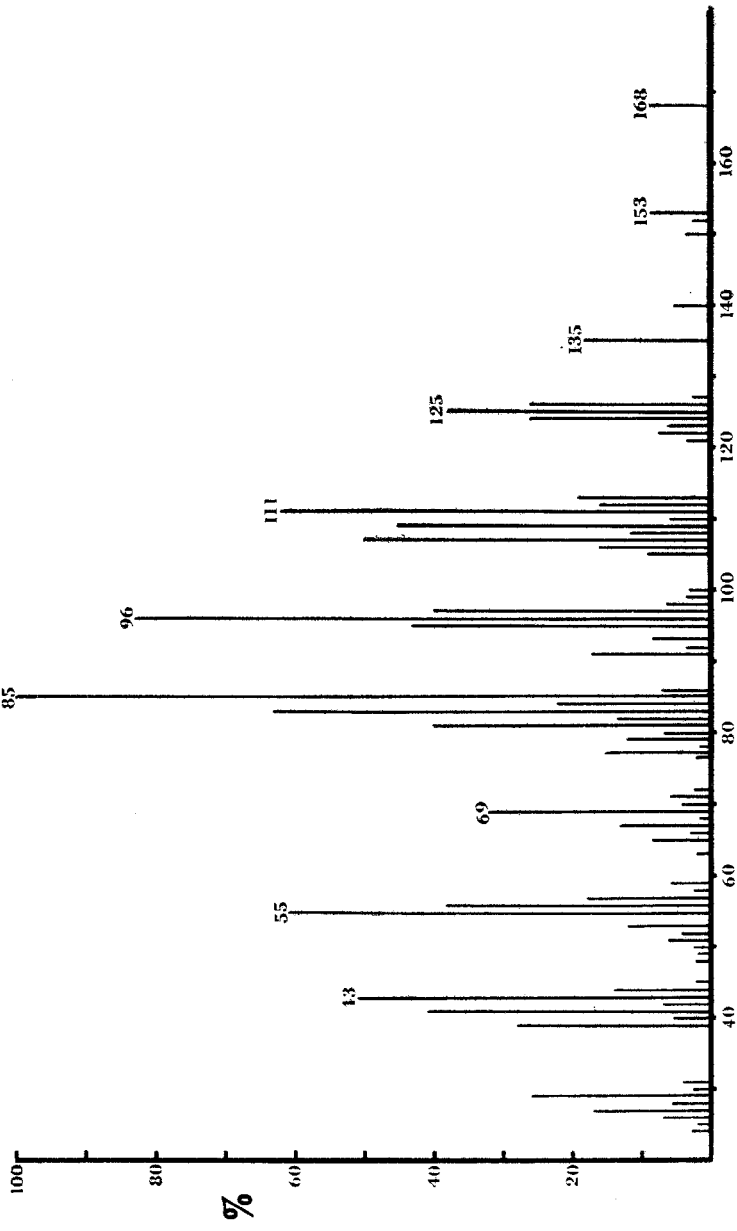


FIG. 1. Unit resolution mass spectrum of lineatin.

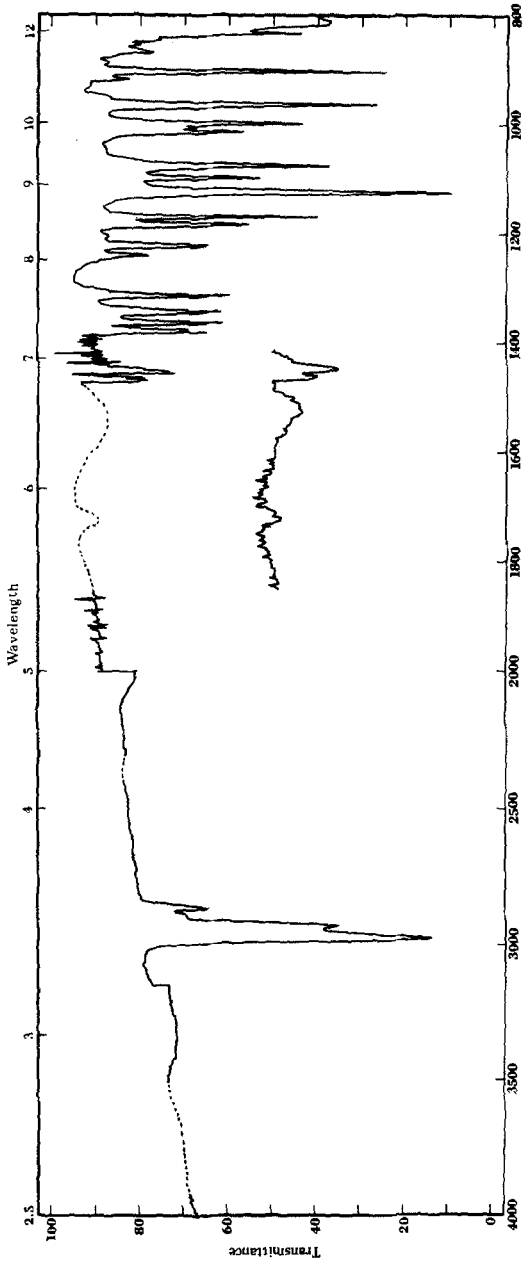


FIG. 2. Infrared spectrum, recorded on about 70 μg of pure lineatin in CCl_4.

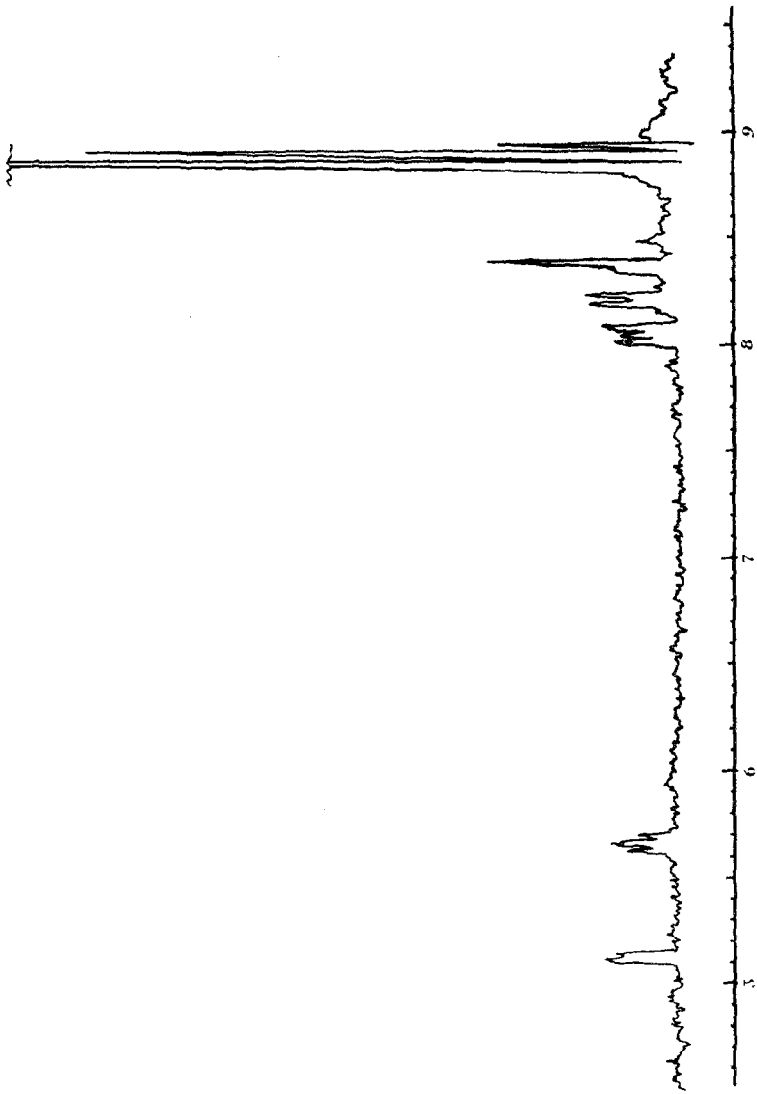


FIG. 3. 100-MHz NMR spectrum, recorded on about 200 μg of lineatin in CCl_4.

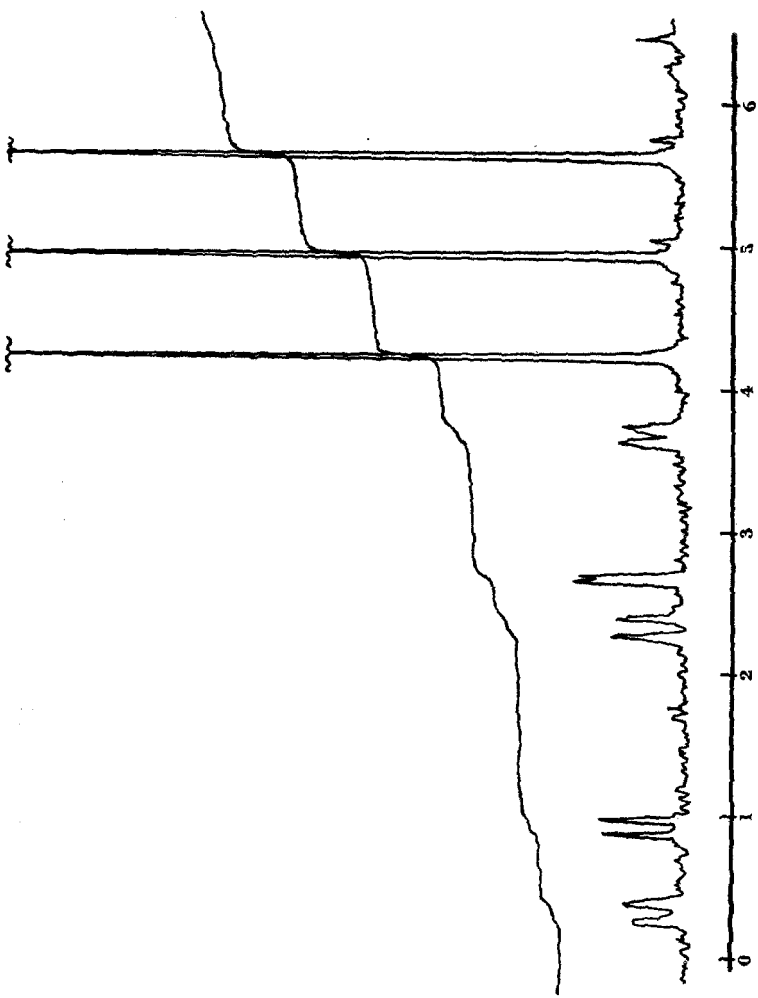


FIG. 4. 100-MHz Eu(fod)$_3$-shifted NMR spectrum of lineatin. Eu(fod)$_3$/substrate molar ratio = 1.3, in CCl_4.

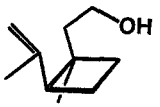
peroxy groups. We consider two C—O—C linkages. Since the index of hydrogen deficiency is 3, we consider appropriate combinations of double and triple bonds and rings. The absorption in the NMR spectrum (Figure 3) at τ 5.15 might represent one proton of a $=CH_2$ group, but confirmation is lacking in the IR spectrum. On the other hand, the IR spectrum bears some resemblance to that of the bicyclic ketal *exo*-brevicomin, isolated from *Dendroctonus brevicomis* Le Conte, and the absorption at τ 5.65 is reminiscent of the *H*C—O protons of *exo*-brevicomin (Silverstein, 1970). The absorption at τ 5.15 might then represent a proton deshielded by two oxygen atoms (O—C*H*—O), and we could be dealing with a tricyclic acetal structure. We suspected that two methyl groups are under the absorption at τ 8.85, and one under that at τ 8.91. The signal at τ 8.95 is an artifact.

The $Eu(fod)_3$ spectrum (Figure 4) shows three distinct methyl singlets. The absorptions (1 proton each) at τ 5.15 and τ 5.65 in the normal spectrum are now shifted far downfield to $\tau - 11.77$ and $\tau - 5.37$, respectively (off-scale to the left in Figure 4). These extreme shifts argue for proximity to oxygen atoms rather than for olefinic character. Double-irradiation experiments on the shifted spectrum showed that the signals at τ 3.65 and τ 0.91 are coupled (J = 10.5 Hz) as are those at τ 2.32 and τ 0.30 (J = 13 Hz). These two sets must represent the protons of two nonadjacent methylene groups. The single proton at τ 2.64 was shown to be coupled (J = 3.5 Hz) to the single proton at τ −5.37. Irradiation of the most highly shifted proton at τ −11.77 gave inconclusive results.

Hydrogenolysis produced a number of hydrocarbon products, one of which was identified as 2,6-dimethyloctane.

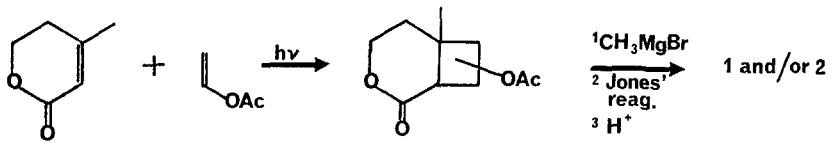
To accommodate this information, we had to propose a structure within these constraints: (1) the general structure is a tricyclic acetal; (2) there are three methyl groups on quaternary carbons, probably in an isoprenoid arrangement; (3) two nonadjacent methylene groups must be included; (4) only two hydrogen atoms are permitted on carbons bearing oxygen atoms; (5) because the acetal moiety can account for no more than two rings, a small carbocyclic ring must be present that can be hydrogenolytically opened to yield 2,6-dimethyloctane. Structures **1** and **2** satisfy these requirements. We cannot unequivocally assign structure **2** because of the coupling observed between the single protons at $\tau - 5.37$ and τ 2.64 in the shifted spectrum; long-range coupling of this magnitude (3.5 Hz) could occur across the four-membered ring in structure **1**.

These proposed structures and that of grandisol (structure **3**), one of the pheromone components of the cotton boll-weevil (Tumlinson et al., 1971), have the same carbon skeleton. Indeed, both compounds gave very similar hydrogenolysis product patterns on GLC, including in both cases the production of 2,6-dimethyloctane.



3

The following synthetic scheme was attempted to confirm one or the other of the postulated structures.



Since separation of the individual components of the mixture of isomeric intermediate compounds was not successful, the sequence was carried through on the crude mixtures, and a small amount of a product was isolated that was indistinguishable in chromatographic, spectral, and biological properties from the natural product. Other syntheses that will constitute a rigorous proof of structure of the attractant molecule are under study.

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NERAL AND GERANIAL: COMPONENTS OF THE SEX PHEROMONE OF THE PARASITIC WASP, *Itoplectis conquisitor*

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Abstract—A laboratory investigation of the *Itoplectis conquisitor* sex pheromone was initiated to elucidate the structure(s) of the active component(s) of the system. Functional group tests performed on crude virgin female extract classified the pheromone as unsaturated aldehyde(s) and/or ketone(s). Isolation of active fractions by gas–liquid chromatography (GLC) suggested the existence of a multicomponent pheromone system. Analysis by gas chromatography–mass spectrometry indicated the presence of saturated and unsaturated aldehydes including neral and geranial. When mixtures of neral and geranial were bioassayed independently and in combination with other active GLC fractions, they elicited male sexual activity of intensity comparable to that released by the GLC fraction (DEGS E) from which they were isolated. The authors conclude that neral and/or geranial are components of the sex pheromone system of this species.

Key Words—*Itoplectis conquisitor* (Say), sex pheromone, citral, neral, geranial.

INTRODUCTION

Although nearly 200 insect sex pheromones and other synthetic attractants have been identified since the first pheromone structure was elucidated 17 years ago (Butenandt et al., 1959), most of this work has been concentrated on the orders Lepidoptera and Coleoptera. Other insect orders, including the Hymenoptera, have remained relatively untouched by the recent progress in sex pheromone identification. Reasons for the apparent lag in progress in the

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latter orders almost certainly include their economic status with regard to man's endeavors to control his environment.

Where Hymenoptera have occupied economically important positions, significant advances have been made in determining the structures and mechanisms of operation of their pheromones. Sex pheromones have been identified for the honeybee (Butler and Fairey, 1964), the Oriental hornet (Ikan et al., 1969), and the pine sawfly (Jewett et al., 1976).

In a previous publication, Robacker et al. (1976) reported that the hymenopteran *Itoplectis conquisitor* (Say) (Hymenoptera: Ichneumonidae) possesses an intriguing sex pheromone system in which chemicals issuing from both newly emerged males and females elicit sexual behavior from older males. The purpose of the present work is to study the chemical nature of this pheromone system.

METHODS AND MATERIALS

Biological

Rearing and Pheromone Extraction. Laboratory rearing and extraction of pheromone from virgin females were conducted in the same manner as described previously (Robacker et al., 1976), except that methylene chloride (redistilled pesticide grade) was substituted for methanol as the extraction solvent.

Bioassays. They were also handled in a fashion similar to that described before (Robacker et al., 1976). In general, males used in bioassays were 2–7 days old and a particular chamber of males was used once (or less frequently) each day for biological testing. Two changes were made in the bioassay procedure, however, to help eliminate the occurrence of accidental negative responses. These changes were: use of 10 male insects per chamber per replicate and extension of the time allowed for response from 3 min to 5 min. To assist in interpretation of male responses, blank sample cups were tested just prior to each authentic sample. In the event that two or more insects were attracted to the blank cup or showed any other form of sexual behavior during these blank test periods, the chamber was excluded from further bioassays.

Activity Coefficient. Because of the tedium involved in statistically analyzing the number of attractions, abdomens up, and copulations independently, a measurement called an activity coefficient (Ac Co) was devised. An activity coefficient is defined as follows:

$$\text{Ac Co} = \frac{(1 \times \text{no. of attractions}) + (2 \times \text{no. of abdomens up}) + (3 \times \text{no. of copulations})}{\text{no. of replicates}}$$

In this system, each type of response is arbitrarily weighted according to the authors' judgment of the importance of that response as an indication of sexual arousal.

Statistical Analyses. All statistical analyses were performed using a standard two-sample *t* test assuming independent means (Games and Klare, 1967).

Chemical

Extraction with Sodium Bicarbonate. Three female equivalents (FE) of crude virgin female extract in 10 ml of ether were extracted with 10 ml of 5% sodium bicarbonate solution. The aqueous layer was separated from the neutral ether layer and acidified with 1 M hydrochloric acid to pH 2. The acidified aqueous layer was then extracted with a second 10-ml portion of ether. Each ether solution was concentrated to 0.05 ml and bioassayed. The procedure was repeated four times.

Extraction with Hydrochloric Acid. Three FE of crude virgin female extract in 10 ml of ether were extracted with 10 ml of 1 M hydrochloric acid. The aqueous layer was separated from the neutral ether layer and 10% sodium hydroxide solution was added to pH 10. The basic aqueous layer was then extracted with a second 10 ml of ether. Each ether solution was concentrated to 0.05 ml and bioassayed. The procedure was repeated four times.

Ozonolysis. A solution of 2 FE of crude virgin female extract in 0.1 ml of methanol was ozonized for 10 sec using an apparatus modified from that described by Beroza and Bierl (1969). The resulting ozonide was decomposed with triphenylphosphine and the solution filtered and bioassayed. A control test was run using a solution of 2 FE and 0.1 g of triphenylphosphine as the bioassay sample. Three replicates were performed.

Hydrogenation. One FE of crude virgin female extract in 3 ml of methanol and 0.5 g of 5% palladium catalyst on barium sulfate were placed into a small flask fitted with a reflux condenser. Hydrogen gas was bubbled through the stirring mixture for 2 hr at room temperature. The mixture was filtered, concentrated to 0.05 ml, and bioassayed. A control test was conducted using a mixture of 1 FE and 0.1 g of palladium on barium sulfate as a bioassay sample. The experiment was replicated three times.

Reaction with Acetyl Chloride. Two FE of crude virgin female extract in 2 ml of anhydrous ether were placed into a small flask fitted with a reflux condenser. Fifteen drops of acetyl chloride were added, and the solution was refluxed for 10 min. The solution was then extracted with 2 ml of 1 M sodium hydroxide, concentrated to 0.1 ml, and bioassayed. Three replicates were conducted.

Saponification with Potassium Hydroxide. To 2 ml of a 25% solution of potassium hydroxide in methanol was added 4 FE of crude virgin female

extract. This solution was refluxed for 2 hr, then neutralized to pH 7 with 1 M hydrochloric acid. The neutralized solution was next extracted with 5 ml of ether and the ether layer separated from the aqueous layer. The ether layer was subsequently concentrated to 0.1 ml and bioassayed. The procedure was performed in duplicate.

Reduction with Lithium Aluminum Hydride. Five FE of crude virgin female extract were added to a slurry of lithium aluminum hydride in 2 ml of anhydrous ether. The resulting suspension was refluxed for 10 min followed by decomposition of excess lithium aluminum hydride with sodium sulfate decahydrate. The reaction mixture was filtered, concentrated to 0.05 ml, and bioassayed. Five FE of crude extract were tested as a control. Three replicates of this experiment were carried out.

Reduction with Sodium Borohydride. Five FE of crude virgin female extract and a suspension of sodium borohydride in 2 ml of ether were placed into a small flask fitted with a reflux condenser. This mixture was stirred for 1 hr at room temperature, filtered, concentrated to 0.1 ml, and tested in the usual manner. A control test was conducted using 5 FE as the bioassay sample. The preceding experiment was performed in triplicate.

Stability to Molecular Oxygen. To a small flask fitted with a reflux condenser was added 5 FE of crude female extract in 2 ml of methanol. Oxygen was bubbled through the extract for 6 hr before the solution was concentrated to 0.1 ml and bioassayed. Two replicates were conducted.

Isolation of Active Fractions by Gas–Liquid Chromatography. Crude virgin female extract was fractionated using a Finnigan 9500 gas chromatograph (GC) equipped with an effluent splitter for collection of approximately 60% of the injected sample. Collection of effluent was accomplished by inserting cooled capillary glass tubing into the splitter. The collected material was then either bioassayed, saved for further fractionation, or used for analysis by mass spectrometry.

Extract was sequentially fractionated on two different gas–liquid chromatography (GLC) substrates. The first fractionation was carried out on a nonpolar substrate: 3% OV-1 on 100–120 mesh gas chrom Q. This substrate was packed into a 1.5-m glass U-tube column with a 2-mm inside diameter. The column temperature was initially 70°C but was programmed at 4°/min immediately following sample injection. Helium carrier gas flow rate was 40 ml/min.

Various 3% OV-1 fractions which elicited sexual activity were fractionated on a polar GLC substrate: 10% DEGS (diethylene glycol succinate) on 100–120 mesh chromosorb WAW. The substrate was packed into a 1.7-m glass U tube column with a 2-mm inside diameter. Column temperature was initially 80° C and was programmed at 4°/min as before. Helium carrier gas flow rate was 40 ml/min.

Besides effluent collection for biological testing, various compounds were analysed on each type of column under standard conditions for the purpose of demarcating the retention times of biologically active fractions. Additionally, retention times of standards provided much essential information concerning the identity of the pheromone.

Standardization of GLC Bioassay Data. Because of the variations in insect responsiveness on successive days of testing (due to changing temperature, insect aging, quantity of pheromone, etc.), it was necessary to standardize all GLC bioassay data. Only data which were recorded on the same day from a single GLC analysis could be compared directly with reasonable accuracy. Therefore, each fractionation process had to be mediated by continually comparing the biological activities of the individual fractions either to the activity of their combination or to that of another fraction capable of serving as a common denominator. Standardization of various fractions collected at different times could then be accomplished simply by multiplying their mean activities by ratios which appropriately described their relationships to such a common denominator. Furthermore, since 3% OV-1 and 10% DEGS fractions obviously could not be compared with each other by this method, it was assumed that the activity elicited by the 3% OV-1 fraction injected onto 10% DEGS must in fact be equal to the summation of the activity of the combined 10% DEGS fractions.

Spectral Analyses. Biologically active 3% OV-1 fractions were collected and injected on a gas chromatograph–mass spectrometer (GC–MS) with an interactive model 6000 computer data system. The complex consisted of two Finnigan 3200 mass spectrometric units: a chemical ionization (CI) system and an electron impact (EI) system. Chemical ionization spectra were recorded at approximately 100 electron volts (eV) over a mass range of 50–300 atomic mass units (amu). Methane served the dual function of carrier and reagent gas. Electron impact spectra were recorded at 67 eV over a mass range of 35–350 amu with helium as carrier gas.

Both CI and EI samples were injected on a 10% DEGS column as described in the previous section. Carrier gas flow rates varied but were generally between 20 and 30 ml/min as measured at the effluent end of the column.

Quantitation of Citral (Neral and Geranial). Because neral and geranial were identified from extracts of virgin females (see Results and Discussion), the amount of each present in the extracts was estimated. This quantitation was conducted by comparing GC traces of known quantities of standard neral and geranial to traces obtained from injection of 10 FE of extract.

Bioassay of Citral. Testing of citral for biological activity was carried out by directly comparing male behavior elicited by three different bioassay samples (each with a fresh cage of insects). These samples were: (1) 30 ng of

citral; (2) DEGS ABCD; (3) 30 ng of citral plus an aliquot of DEGS ABCD [equal to and taken from the same GLC collection as in (2)]. In addition, seven replicates were performed in which citral alone was compared to blanks. All samples of citral contained 10 ng of neral and 20 ng of geranial (prepared from 97% citral; Supelco Inc., Bellefonte, Pennsylvania).

RESULTS AND DISCUSSION

Chemical Characterization of Functional Groups

The results of the functional group tests conducted on crude virgin female extract appear to present a relatively clear picture of the type of molecule(s) which comprise the *I. conquisitor* sex pheromone system (Table 1). Extraction with ionizing reagents such as sodium bicarbonate and hydrochloric acid apparently failed to convert the pheromone into water-soluble

TABLE 1. RESULTS OF CHEMICAL CHARACTERIZATION TESTS ON SEX PHEROMONE OF *I. conquisitor*

Test	Sample	Attractions[a]	Abdomens up[a]	Copulations[a]
Extraction with sodium bicarbonate[b]	Neutral layer	7.2	5.0	4.0
	Basic layer	1.0	0.0	0.0
Extraction with hydrochloric acid[b]	Neutral layer	8.5	6.5	5.0
	Acidic layer	1.0	0.0	0.0
Ozonolysis[c]	Product	3.0	0.0	0.0
	Control	7.3	6.0	5.3
Hydrogenation[c]	Product	2.7	0.0	0.0
	Control	7.0	5.0	4.8
Reaction with acetyl chloride[c]	Product	9.0	3.7	5.0
	Blank	1.3	0.0	0.0
Saponification with potassium hydroxide[d]	Product	6.5	5.5	2.5
	Blank	1.0	0.0	0.0
Reduction with lithium aluminum hydride[c]	Product	1.0	0.3	0.3
	Control	7.3	6.3	4.3
Reduction with sodium borohydride[c]	Product	1.3	0.0	0.0
	Control	4.3	2.7	1.3
Stability to molecular oxygen[d]	Product	8.0	7.0	8.0
	Blank	1.3	0.0	0.0

[a] Reported results are means of the number of replicates indicated.
[b] Based on four replicates.
[c] Based on three replicates.
[d] Based on two replicates.

ions. Thus it appears that neither carboxylic acids nor amines are significant components of the system.

The results of the ozonolysis and catalytic hydrogenation reactions, however, strongly suggest the presence of carbon–carbon unsaturation(s). On the basis of the low incidence of carbon–carbon triple bonds and aromatic systems previously identified as insect sex pheromones, the most probable type of unsaturation is the olefinic linkage (Mayer and McLaughlin, 1975). In addition, the chemical evidence favors double bonds over aromatic systems since catalytic hydrogenation of the latter generally requires more rigorous conditions than those employed in this experiment (March, 1968).

At the reflux temperatures of ether and methanol, no observable reaction takes place with either acetyl chloride or potassium hydroxide. This is effectively demonstrated by the high biological activity evoked by the reaction products obtained in these experiments. Therefore, it seems likely that alcohol and ester functionalities are not present or at least not requisite for pheromone detection by the parasite.

Reaction products from treatment with metal hydrides elicited significantly less sexual activity than the immediately following control tests performed on the same insects. Since ester and acid functional groups were eliminated from contention by experiments described formerly, reduction by lithium aluminium hydride implies the presence of keto or aldehydic groups. Inasmuch as sodium borohydride does not react with ester or carboxylic

TABLE 2. RESPONSES OF *I. conquisitor* MALES TO VARIOUS FRACTIONS COLLECTED FROM A 3% OV-1 GLC COLUMN

Retention time (min)[a]	Symbol	Replicates	Attractions[b]	Abdomens up[b]	Copulations[b]	Ac Co[c]
0.0–2.2	OV-1 A	6	3.8	1.7	0.0	7.2[1]
2.2–3.5	OV-1 B	8	0.8	0.0	0.0	0.8[2]
3.5–4.5	OV-1 C	11	3.5	2.5	0.3	9.4[1]
4.5–5.5	OV-1 D	11	8.5	7.5	2.2	30.0[3]
5.5–6.5	OV-1 E	10	2.8	1.6	0.0	6.0[1]
6.5–7.5	OV-1 F	9	1.2	1.8	0.6	6.6[1]
0.0–5.5	OV-1 ABCD	5	9.6	9.4	5.4	45.0[3]
7.5–12.5		2	1.0	0.5	0.0	2.0[2]
12.5–20.0		2	0.0	0.0	0.0	0.0[2]
20.0–49.0		2	0.5	0.0	0.0	0.5[2]

[a] Crude Virgin female extract was the injected sample for all 3% OV-1 fractions listed. Results are means of the number of replicates indicated.
Entries followed by the same number are not significantly different from each other at the 5% level.

acid groups, the absence of biological activity from this product further indicates a ketone or aldehyde. Lithium aluminum hydride and sodium borohydride do not ordinarily reduce carbon–carbon double bonds except in special cases of conjugation with carbonyl groups (Nystrom and Brown, 1947; Kadin, 1966).

The final chemical test, stability in the presence of molecular oxygen was performed to clarify the observation that pheromone extract rapidly loses its potency (5–10 min) during laboratory bioassays. From the results of the experiment, it appears that this loss of potency is more likely due to pheromone volatility rather than lability.

In summary, the active component(s) of the *I. conquisitor* sex pheromone appear to be oxygen-stable aldehyde(s) and/or ketone(s) containing one or more carbon–carbon double bonds.

Active Fractions from 3% OV-1 GLC

Table 2 summarizes the biologically active fractions isolated from a 3% OV-1 GLC column. By themselves, five low-retention-time and probably low-molecular-weight fractions elicited significantly more sexual activity than blank sample cups. The retention times of these fractions, with designations in parentheses, are: 0.0–2.2 min (OV-1 A), 3.5–4.5 min (OV-1 C), 4.5–5.5 min (OV-1 D), 5.5–6.5 min (OV-1 E), and 6.5–7.5 min (OV-1 F). Of these, fraction OV-1 D was significantly more active than the others. Indication that more than one compound is involved follows from the increase in biological activity which resulted when fractions OV-1 A, OV-1 C, and OV-1 D were added to another low retention fraction, 2.2–3.5 min (OV-1 B). The Ac Co obtained from this combination fraction (OV-1 ABCD) is approximately equal to the sum of the Ac Co's of its constituent fractions.

Fractions of retention time greater than 7.5 min elicited no significant activity and were not tested further. Fractions OV-1 E and OV-1 F, although highly active, were also not subjected to additional fractionation. Fraction OV-1 ABCD was collected and further fractionated on a 10% DEGS column as previously described.

Active Fractions from 10% DEGS GLC

The highly active fraction OV-1 ABCD was utilized as the injection sample for all 10% DEGS fractionation. The results of these experiments are recorded in Table 3. As with the 3% OV-1 results, it appears that the highest biological activity is associated with fractions of relatively low retention time and presumably low polarity. In particular, the fraction with retention time 1.2–2.2 min (DEGS 1) was responsible for 30% of the total

TABLE 3. RESPONSES OF *I. conquisitor* MALES TO VARIOUS FRACTIONS COLLECTED FROM A 10% DEGS GLC COLUMN

Retention time (min)[a]	Symbol	Replicates	Attractions[b]	Abdomens up[b]	Copulations[b]	Ac Co[c]
0.0–1.2		Untested	—	—	—	—
1.2–2.2	DEGS A	10	3.3	3.5	1.1	13.6[1]
2.2–3.5	DEGS B	10	0.5	0.7	0.0	1.9[2]
3.5–4.5	DEGS C	10	2.0	1.2	0.0	4.4[3]
1.2–4.5	DEGS ABC	20	5.9	4.9	4.1	28.0[4]
4.5–7.5	DEGS D	18	2.6	1.3	0.2	5.8[3]
7.5–10.0	DEGS E	18	2.1	1.8	0.2	6.3[3]
1.2–7.5	DEGS ABCD	18	6.1	4.0	2.8	22.5[1,4]
1.2–4.5 + 7.5–10.0	DEGS ABCE	18	8.3	5.8	4.5	33.4[4,5]
4.5–10.0	DEGS DE	17	5.1	3.2	2.0	17.5[1]
1.2–10.0	DEGS ABCDE	19	8.9	7.0	7.4	45.0[5]
10.0–22.5	DEGS F	4	1.8	1.8	0.0	5.4[3]
22.5–32.5[d]		2	1.0	0.0	0.0	1.0[2]

[a] Fraction 0.0–5.5 min from 3% OV-1 collection was the injection sample for all 10% DEGS fractions listed.
[b] Results are means of the number of replicates indicated.
[c] Entries followed by the same letter are not significantly different from each other at the 5% level.
[d] Column temperature was isothermal 170°C.

sexual response observed. Other fractions which elicited significant activity were 3.5–4.5 min (DEGS C), 4.5–7.5 min (DEGS D), 7.5–10.0 min (DEGS E), and 10.0–22.5 min (DEGS F). Once again, combinations of the various fractions were consistently more active than any of the constituent fractions in a nearly additive manner. One exception to this rule is fraction 1.2–7.5 min (DEGS ABCD) which has a lower Ac Co than the sum of the Ac Co of its constituent fractions.

With these and the 3% OV-1 results as foundation, the hypothesis that more than one compound may serve as sex pheromone components seems quite plausible.

Relative Retention Times of Standards and Active GLC Fractions

Gas chromatographic analysis of various aldehyde and ketone standards and subsequent comparisons of their retention times to active fractions afforded useful information concerning the probable molecular weight span of the pheromone compounds. The active range on 3% OV-1 extends from

0.0 to 7.5 min and on 10% DEGS from 0.0 to 10.0 min. Compounds which lie within these boundaries include aldehydes and ketones up to 12 carbons in chain length.

Computerized Chemical Ionization and Electron Impact Mass Spectra

Various biologically active 3% OV-1 fractions including OV-1 A, OV-1 C, OV-1 D, and OV-1 ABCD were analyzed by CI and EI as described previously. Reinjection of fraction OV-1 D onto GC-MS gave rise to two large peaks within the limits of fraction DEGS E. Because of identical retention times of these peaks to neral and geranial (MacLeod et al., 1966) on both 3% OV-1 and 10% DEGS columns, background-subtracted CI mass spectra of each were recorded and compared to the spectra of authentic neral (Figure 1) and geranial (Figure 2). Examination of these data reveals the near

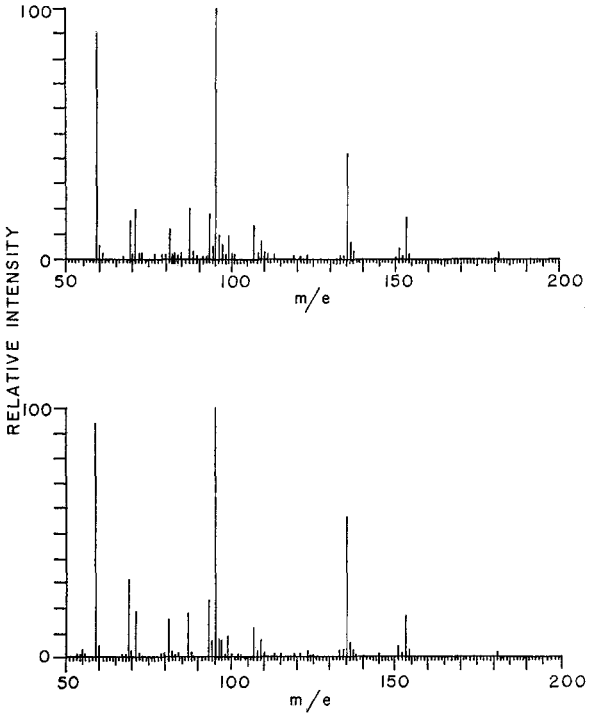


FIG. 1. Computerized background-subtracted CI mass spectra of standard neral (top) and a compound from fraction DEGS E (bottom).

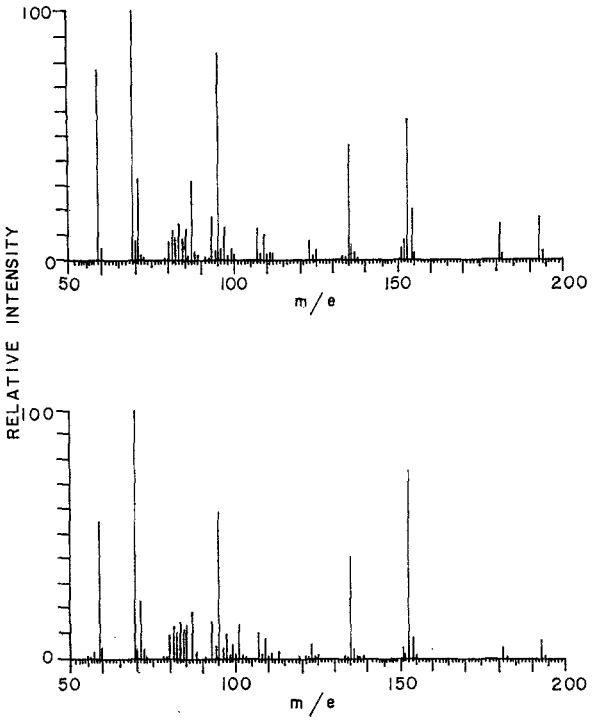
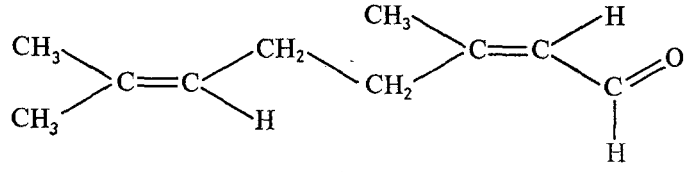


FIG. 2. Computerized background-subtracted CI mass spectra of standard geranial (top) and a compound from fraction DEGS E (bottom).



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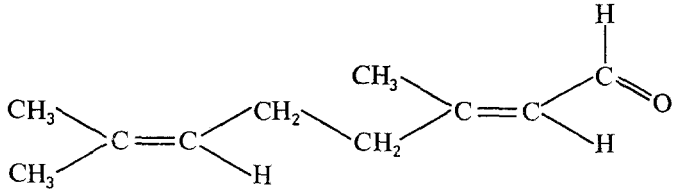

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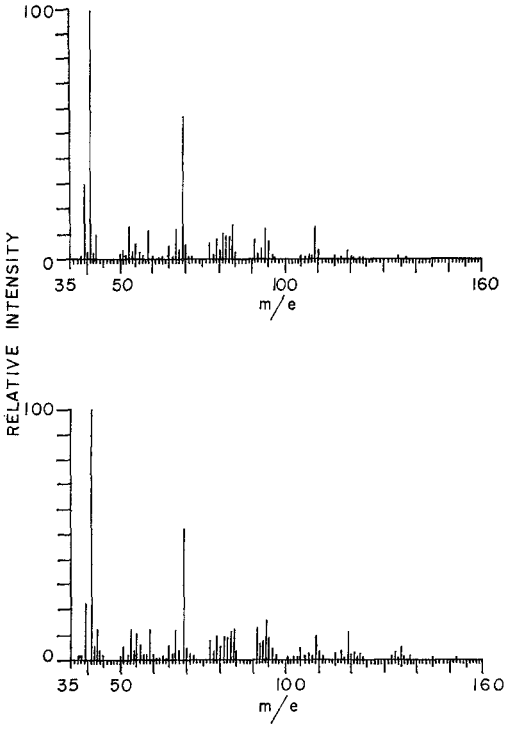

FIG. 3. Computerized background-subtracted EI mass spectra of standard neral (top) and a compound from fraction DEGS E (bottom).

equivalency of the mass spectra of neral and geranial to those of the lower and higher retention compounds, respectively.

Active fraction OV-1 D was analysed by EI in the same manner as described for CI. The background-subtracted EI spectra of these peaks were recorded and compared to spectra of the two standard compounds. As before, the spectrum of the lower retention peak closely matches that of neral (Figure 3), and the spectrum of the peak of higher retention matches that of geranial (Figure 4). Based on these findings, the CI spectra and identical GC retention times using both 3% OV-1 and 10% DEGS, the authors conclude that these two compounds are indeed neral (structure I) and geranial (structure II).

Quantitation of Neral and Geranial

By comparisons of GC peak areas, it was determined that between 5 and 50 ng of both neral and geranial were present per female extracted. Further

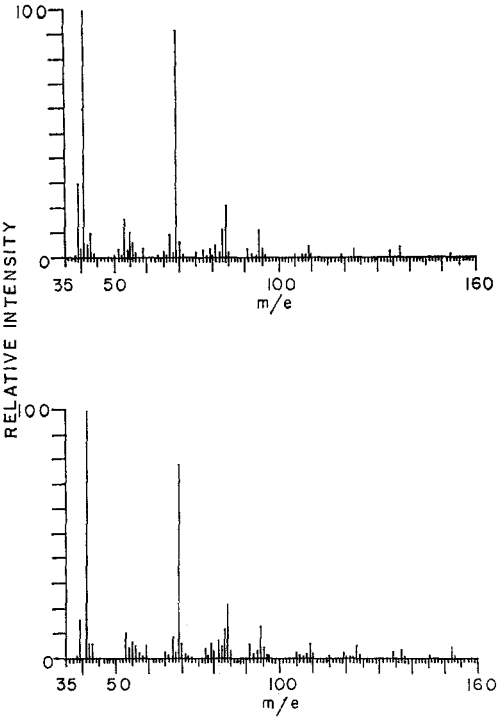

FIG. 4. Computerized background-subtracted EI mass spectra of standard geranial (top) and a compound from fraction DEGS E (bottom).

TABLE 4. RESPONSES OF *I. conquisitor* MALES TO CITRAL

Bioassay sample	Replicates	Attractions[a]	Abdomens up[a]	Copulations[a]	Ac Co[b]
Blank	7	1.2	0.0	0.0	1.2[1]
30 ng citral	13	1.9	1.9	0.0	5.7[2]
DEGS E[c]	18	2.1	1.8	0.2	6.3[2]
DEGS ABCD[d]	6	5.9	4.7	2.4	22.5[3]
DEGS ABCD[d]+ 30 ng citral	6	9.0	9.0	6.7	47.1[4]
DEGS ABCDE[c]	19	8.9	7.0	7.4	45.0[4]

[a] Results are means of the number of replicates indicated.
[b] Entries followed by the same letter are not significantly different from each other at the 2% level.
[c] This data was taken from Table 3.
[d] Fraction OV-1 ABCD (0.0–5.5 min) from 3% OV-1 collection was the injected sample for these 10% DEGS fractions.

calculation showed that the ratio of geranial to neral ranged from 1.3 to 1.8 in all extracts tested.

Bioassay of Neral and Geranial

The above findings taken into consideration, citral was tested using *I. conquisitor* males. The results of this experiment are listed in Table 4; all data were standardized so that the Ac Co of fraction DEGS ABCD equalled 22.5, the value given in Table 3. Comparison of the responses released by authentic citral to those elicited by fraction DEGS E (from Table 3) provides evidence that citral may indeed be responsible for the biological activity residing in this fraction. Furthermore, the combination of citral and DEGS ABCD evoked approximately the same sexual response as fraction DEGS ABCDE (from Table 3).

It should be noted, however, that citral does not elicit the same sexual response as fraction OV-1 D. This finding is probably due to the presence of other active compounds in OV-1 D which have retention times (on 10% DEGS) which do not coincide with fraction DEGS E.

While this work is not the first report of citral acting as a mediator of insect behavior (Beroza, 1970), it has seldom been implicated in the role of a sex pheromone. Whether or not citral functions in the sexual behavior in other parasitic Hymenoptera and whether these terpenoid components originate with the parasite, the host, or some other source remains to be established.

Acknowledgments—The authors wish to express appreciation to Karen Robacker and Joseph Wichmann for technical assistance. This research was supported through grants from the USDA CRA (4012), the Rockefeller Foundation (3726), and the Environmental Protection Agency (7450).

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SOLDIER FRONTAL GLANDS OF THE TERMITE *Macrotermes subhyalinus:* MORPHOLOGY, CHEMICAL COMPOSITION, AND USE IN DEFENSE

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Abstract—Soldiers of the East African fungus-growing termite *Macrotermes subhyalinus* (Rambur) (Termitidae : Macrotermitinae) employ both mechanical and chemical defenses. Soldiers release a chemical secretion composed of long-chain saturated and monounsaturated hydrocarbons into wounds inflicted by their powerful mandibles. Chemical analysis of the secretion shows the paraffin fraction to consist primarily of *n*-tricosane, *n*-pentacosane, 3- and 5-methylpentacosane, and 5-methylheptacosane. The major olefins were identified as (Z)-9-heptacosene and (Z)-9-nonacosene. The secretion originates from the frontal glands of both major and minor soldiers; however, the hypertrophied gland of the major soldiers contains 500-fold more secretion than that of the minor soldiers. This secretion appears to impair the healing of wounds in test ants, and thus could represent a valuable supplement to the mechanical defense mechanism.

Key Words—*Macrotermes subhyalinus*, chemical defense, termite soldier, frontal gland, fungus-growing termites, long-chain hydrocarbons.

INTRODUCTION

Defensive techniques vary widely among termites. Indeed, the morphological variety of the soldiers' armaments and the chemical variety of the

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so-called "defensive secretions" reflect this range of techniques (Quennedey, 1973, 1975a; Prestwich, 1975). Defensive substances are generally associated with the frontal glands of the termite soldiers; however, this is not always true. In the fungus-growing termites of the subfamily Macrotermitinae (Isoptera : Termitidae), secretions from the soldier salivary glands are most frequently used in defense (Noirot, 1969; Quennedey, 1975b). Soldiers of the Oriental termite *Macrotermes carbonarius* discharge an aqueous solution of benzoquinone and toluquinone from massive labial glands (Maschwitz et al., 1972). Five other species of the Oriental macrotermitine genera *Microtermes*, *Macrotermes*, *Odontotermes*, and *Hypotermes* were shown to release defensive secretions, most containing quinones, from labial glands (Maschwitz and Tho, 1974). An aqueous mixture of benzoquinone and protein has been reported by Wood et al. (1975) as the defensive secretion of the East African termite *Odontotermes badius*. Macrotermitine soldiers of the genera *Protermes*, *Pseudocanthotermes*, *Macrotermes*, *Odontotermes*, and *Ancistrotermes* are also known to produce defensive secretions from extended salivary reservoirs (cf. Noirot, 1969). Partial chemical analysis of two of these species has been reported by Howse (1975). *Macrotermes bellicosus* was reported to produce a mixture of toluquinone and an unidentified volatile solvent, and *Ancistrotermes cavithorax* may produce four oxygenated sesquiterpenes. Although the glandular source of these chemicals was not specified, it was implied that they originated from labial glands.

Frontal-gland development in macrotermitine soldiers is the exception rather than the rule. Quennedey (1975b) reported that only *Ancistrotermes*, *Sphaerotermes*, *Acanthotermes*, *Syncanthotermes*, and *Macrotermes* have frontal glands and that these are generally small (with the exception of *Acanthotermes*). We now report a hypertrophy of the frontal-gland reservoir of major soldiers of the East African termite *Macrotermes subhyalinus* (Rambur), and we describe herein the morphology of the major and minor soldier frontal gland reservoirs, the chemical analysis of the secretions produced, and the utilization of the secretion in defense.

METHODS AND MATERIALS

Collection Procedures

Major and minor soldiers of *Macrotermes subhyalinus* (Rambur) were collected from excavated dome-shaped mounds in Kajiado District, Kenya, East Africa. Frozen and live specimens were dissected under water. Use of alcohol was contraindicated in the case of this termite, since immersion in alcohol resulted in the release of the soldier's frontal-gland secretion as an insoluble white latex.

Secretions were obtained in three ways: (1) extraction of the extirpated major soldier frontal gland with distilled hexane, (2) microcapillary collection of the secretion discharged from the major soldier gland in response to severe irritation, and (3) hexane extraction of crushed heads of major or minor soldiers, followed by filtration of the crude extract through a short Florisil column using 5% ether–hexane. These three techniques gave secretions which were indistinguishable by TLC and GLC; consequently, the third method was employed for large-scale collections.

Ants were also collected in Kajiado District, Kenya. The small ants (*Crematogaster* sp.) were obtained from the base of a dead acacia bush. The large stridulating, termitophagic ponerine ant *Megaponera foetens* was collected from a subterranean colony and had been observed to conduct nocturnal raids on *M. subhyalinus* foraging parties (M. LePage, personal communication). Ants were used within two days of collection.

Instrumental Methods

Infrared (IR) spectra were determined as neat films using a Perkin-Elmer model 720 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were determined in deuteriochloroform solution containing 1% TMS using a Hitachi Perkin-Elmer model R-24A spectrometer.

Gas–liquid chromatography (GLC) was performed using a Hewlett Packard model 7620 gas chromatograph equipped with a flame-ionization detector (250°) and 91×0.6 cm OD copper columns packed with (1) 3% OV-1 on 100/200 mesh Gas Chrom Q and (2) 10% Silar 10C on 100/200 mesh Gas Chrom Q. Additional GLC was performed in Nairobi for screening purposes using a Hewlett Packard model 402 gas chromatograph equipped with a 90×0.5-cm OD glass column packed with 5% OV-1 on 80/100 Gas Chrom Q.

Mass spectra were obtained using a Finnigan 1015 quadrupole mass spectrometer (250 μA, 70 eV) interfaced with a Varian model 1200 gas chromatograph equipped with a 152×0.2-cm ID column packed with 3% OV-1 on 100/200 mesh Gas Chrom Q. A Systems Industries model 150 data system was employed to process the mass spectral data.

RESULTS

Morphology of the Glands

Dissection of the head capsules of major and minor soldiers revealed a vast difference in the sizes of the frontal-gland reservoirs (Figure 1). Located just beneath the fontanelle, the small pore through which the frontal gland

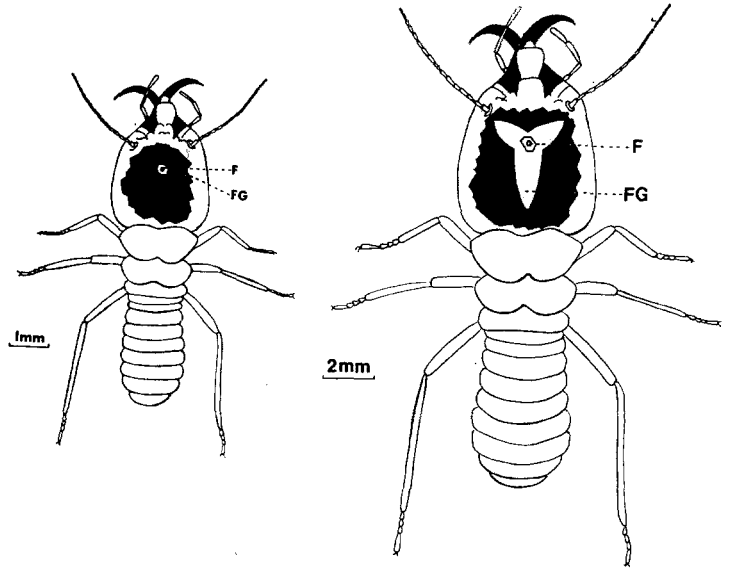

FIG. 1. Minor (left) and major soldiers of the termite *Macrotermes subhyalinus*. Frontal-gland reservoirs (FG) are shown as clear sacks within the cut-away cuticle and upper musculature of the head tapsules. The glands open to the outside through the fontanelle (F).

opens to the cuticle surface, is the frontal-gland reservoir. In minor soldiers, the reservoir is a roughly spherical sack with a volume of 0.03 mm^3. In major soldiers, the reservoir consists of a voluminous three-lobed sack with a capacity of 14 mm^3. The largest lobe of the reservoir runs posterior to the fontanelle along the median line of the head capsule; the two smaller lobes run anteriolateral to the fontanelle, reaching almost to the bases of the antennae. All three lobes are laterally compressed to some extent and are interspersed between various groups of musculatures within the head capsule.

Chemical Analysis of the Secretions

The GLC trace of the hexane extracts of crushed soldier heads is shown in Figure 2a. There were no differences observed between this trace and similar traces of secretions obtained by either crushing an extirpated frontal gland in hexane or by "milking" the secretion directly from the fontanelle. Furthermore, a GLC survey of individual soldier secretions from soldiers from ten closed-dome mounds selected at random within a 20-km radius of

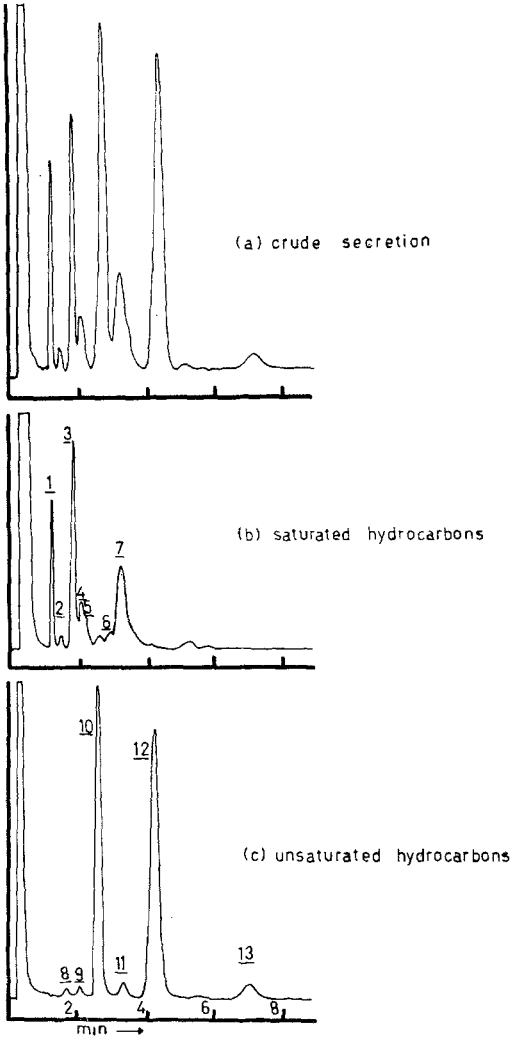

FIG. 2. GLC traces (HP 7620, 3% OV-1, 250°) of the major soldier frontal-gland secretion of *Macrotermes subhyalinus*: (a) before and (b, c) after separation by argentation chromatography. The identities of the peaks are indicated numerically as follows: 1, *n*-tricosane; 2, *n*-tetracosane; 3, *n*-pentacosane; 4, 5-methylpentacosane; 5, 3-methylpentacosane; 6, *n*-heptacosane; 7, 5-methylheptacosane with traces of 7- and 3- methylheptacosanes; 8, pentacosene; 9, hexa-cosene; 10, (Z)-9-heptacosene; 11, (Z)-9-octa-cosene; 12, (Z)-9-nonacosene; 13, (Z)-9-hentriacontene. Minor amounts of the corresponding 7-, 8-, and 10-alkenes were also detected in peaks 10 through 13.

TABLE 1. AVERAGE WEIGHTS OF *Macrotermes subhyalinus* SOLDIERS AND OF THEIR FRONTAL-GLAND SECRETIONS (VALUES DETERMINED FROM A SINGLE WEIGHING OF THE DESIGNATED NUMBER OF INDIVIDUALS OR POOLED SECRETIONS THEREFROM)

	Live wt. (n = 10) (mg)	Dry wt. (n = 10) (mg)	Secretion wt. (n = 100) (mg)	Secretion as % dry wt.
Major	129	31	2.6	8.4
Minor	26	3.6	0.005	0.14

Kajiado town showed that there was virtually no variation among soldiers of the same or of different colonies.

Minor soldiers possessed approximately 500-fold less secretion than major soldiers, but the GLC trace showed this secretion to be identical to that of the major soldiers. The relative weights of the soldiers and their respective secretions are summarized in Table 1.

Preliminary chemical and spectroscopic data indicated that the secretion was a mixture of long-chain hydrocarbons. Temperature-programmed GLC (HP 402, 5% OV-1, 60–250° at 5° min^{-1}) indicated the absence of more volatile components in the secretion. A sample of 50 mg of the crude secretion was passed through a column of 20 g of Hi-Flosil-Ag (Applied Science Laboratories) activated at 110° for 1 hr. Successive 10-ml fractions were collected using a total of 90 ml of 100% hexane and 50 ml each of 1, 5, 15, and 50% ether–hexane solutions. The saturated hydrocarbons eluted in fractions 4, 5, and 6; the monounsaturated hydrocarbons eluted in fractions 20 and 21. The GLC traces of the paraffin and olefin fractions are shown in Figures 2b and 2c. Components of these fractions were identified as described below.

The saturated hydrocarbons (Figure 2b) were identified on the basis of their GLC retention times (HP 7620, 3% OV-1, 230°) and their mass spectra. The normal hydrocarbons were easily identified on the basis of reference spectra and retention indices. Branched-chain hydrocarbons fragment adjacent to centers of branching. Thus, methyl-branched hydrocarbons exhibit a characteristic $M^+ - 15$ peak and fragment ions derived from the cleavage of the chain on either side of the methyl branch (cf. Lockey, 1976; Uebel et al., 1975b). The *Macrotermes* branched-chain hydrocarbons were therefore identified on the basis of their retention indices and their characteristic mass spectral fragments: 5-methylpentacosane (*m/e* 309, $M^+ - C_4H_9$ and 85, $M^+ - C_{20}H_{41}$), 3-methylpentacosane (*m/e* 337, $M^+ - C_2H_5$ and 57, $M^+ - C_{22}H_{45}$), and 5-methylheptacosane (*m/e* 337, $M^+ - C_4H_9$ and 57, $M^+ - C_{22}H_{45}$). Smaller quantities of 7- and 3-methylheptacosanes were also detected.

The components of the olefin fraction were identified by GLC retention times (HP 7620, 3% OV-1, 250°); mass, IR, and NMR spectra; TLC; and finally mass spectra of their epoxide derivatives. GLC retention data indicated that all of the components were straight-chain olefins with no indication of methyl branching. Mass spectra of the olefins provided molecular weights but no information concerning the position of the unsaturation. The IR spectrum of the alkene mixture showed no absorption at 10.4 μm, thus indicating the lack of a *trans*-disubstituted olefinic bond. NMR of the olefin mixture showed a triplet at δ 5.47 (J = 5 Hz), also supportive of a *cis*-disubstituted olefin. Finally, TLC of the olefin mixture on silver nitrate-impregnated silica gel, developed twice with 100% hexane, also showed the olefins to possess the cis configuration (R_f 0.11 v. R_f 0.24 for trans straight-chain alkenes).

The locations of the double bonds were determined by examination of the electron-impact mass spectra of their corresponding epoxides (Bierl et al. 1971; Bierl and Beroza, 1974). A portion of the olefin fraction was treated with *m*-chloroperbenzoic acid for 12 hr at 25°. The reaction mixture was washed with aqueous $NaHCO_3$ and saturated NaCl solutions and then dried over anhydrous Na_2SO_4. The GLC (HP 7620, Silar 10C, 200°) retention indices confirmed the cis configuration and straight-chain nature of the original olefins. Mass spectra of the epoxide mixture showed the preferred alpha cleavage fragments for: 9, 10-epoxyheptacosane (*m/e* 394, M^+, $C_{27}H_{54}O$; 376, $M^+ - H_2O$; 281, $M^+ - C_8H_{17}$; 155, $M^+ - C_{17}H_{35}$), 9,10-epoxyoctacosane (*m/e* 408, M^+, $C_{28}H_{56}O$; 390, $M^+ - H_2O$; 295, $M^+ - C_8H_{17}$; 155, $M^+ - C_{18}H_{37}$), 9,10-epoxynonacosane (*m/e* 422, M^+, $C_{29}H_{58}O$; 404, $M^+ - H_2O$; 309, $M^+ - C_8H_{17}$; 155, $M^+ - C_{19}H_{39}$), and 9, 10-epoxyhentriacontene (*m/e* 450, M^+, $C_{31}H_{62}O$; 432, $M^+ - H_2O$; 337, $M^+ - C_8H_{17}$; 155, $M^+ - C_{21}H_{43}$). Minor amounts of other positional isomers, including the 7,8-, 8,9-, and 10,11-epoxy compounds were also indicated in each epoxide peak based on the mass fragments. On the basis of the combined data, therefore, the two major olefins in the secretion are (Z)-9-heptacosene and (Z)-9-nonacosene, with lesser amounts of (Z)-9-octacosene and (Z)-9-hentriacontene also present. Trace amounts of the corresponding 7-, 8-, and 10-alkenes are also indicated.

The relative percentages of the paraffin and olefin components in the original secretion are presented in Table 2.

Use of Secretion in Defense

Preliminary observations were made in the laboratory with two species of ants in order to demonstrate the relative efficacy of the defensive capabilities of the termite soldiers. Against the smaller *Crematogaster* ants (length 3–4 mm), the *M. subhyalinus* major soldier (length 10–13 mm) may be an

TABLE 2. RELATIVE ABUNDANCES OF PARAFFIN AND OLEFIN COMPONENTS OF THE *Macrotermes subhyalinus* MAJOR SOLDIER FRONTAL-GLAND SECRETION (VALUES DETERMINED FROM THE INTEGRATED GLC TRACES OF SATURATED AND UNSATURATED FRACTIONS, ASSUMING EQUIMOLAR DETECTOR RESPONSE FACTORS; TRACES OF THE 7-, 8-, AND 10-POSITIONAL ISOMERS WERE DETECTED IN THE (*Z*)-9-ALKENES)

Component	Relative abundance (%)
n-Tricosane	4.5
n-Tetracosane	0.6
n-Pentacosane	9.2
Pentacosene	0.5
5- and 3-Methylpentacosanes	3.8
Hexacosene	0.6
n-Heptacosane	1.0
(Z)-9-Heptacosene	22.0
5-, 7-, and 3-Methylheptacosanes	11.0
(Z)-9-Octacosene	1.8
(Z)-9-Nonacosene	40.0
(Z)-9-Hentriacontene	5.0
	100.0

effective combatant. A major soldier placed in a 7-cm petri dish with six ants could kill the majority within 30 min. Attacks by soldier termites were only provoked by direct physical encounters with ants. Once cut by the termite mandibles, ants then adhered to the oily secretion of the frontal gland coating the termite soldier's head, and were thus easily cut into yet smaller pieces by subsequent mandibular movements.

The larger ponerine ants (length 12–14 mm) possess a poison sting, and two or more ants could immobilize a major soldier in less than 30 sec. In a similar setup, it required three or more major soldiers to disable one ant, often ending in the death of at least one soldier. In these experiments, major soldiers were able to bite through the ants' legs and abdomens, but no obvious damages to the head or thorax could be observed. Minor soldiers were unsuccessful in withstanding the ant attacks, and were invariably killed, even in a ratio of ten soldiers to one ant. Moreover, minor soldiers attacking ants immobilized with glue were unable to cut through the legs or abdomens of their noncombatant enemies.

The combined efficacy of the chemical and mandibular defenses was demonstrated using three test groups and one control group of eight or ten

ponerine ants each. In the first group ("cut only"), one front leg and one hind leg were amputated by cutting midway along the femur. In the second group ("secretion only"), approx. 2 mg of the crude major soldier secretion was applied ventrally to the thorax of the ant using a 3-μl Drummond microcapillary. In the third group ("cut and secretion"), two legs were amputated *and* 2 mg of the soldier secretion was applied as described above. In the preliminary tests, it was found that application of the secretion to the mouthparts or to the abdomen induced extensive cleaning behavior, but that the net effect on the viability of the treated ant was the same as for thoracic application.

After treatment, ants were placed in plastic jars containing moistened soil, and the status of the ants was assessed at intervals of 2, 6, 12, 22, and 32 hr. Ants which were alive, fast-moving, and which responded to irritation by stridulating were scored as "A". Ants which appeared slower in movement or did not stridulate were classed as "W." Ants which still responded to probing but which were incapable of locomotion were classed as "I." Finally, ants which did not respond to probing were classed as "D." The results of a representative test are presented in Table 3. Similar results were obtained in three replicate experiments.

Whereas mortality in the control, cut only, and secretion only groups was essentially nil after 32 hr, mortality and morbidity claimed 70% of the ants in the cut-and-secretion test group in the same time period. In separate tests, ants were placed in empty glass petri dishes. Mortality and morbidity was increased in all groups under these unnatural conditions, with 100% mortality in the cut-and-secretion group in 30 hr. Moreover, the dishes containing ants of this latter group showed excessive moisture indicative of an uncontrolled loss of hemolymph from the secretion-treated wounds.

TABLE 3. EFFICACY OF FRONTAL-GLAND SECRETION AGAINST PONERINE ANTS[a]

	2 hr				6 hr				12 hr				24 hr				32 hr			
Group	A	W	I	D	A	W	I	D	A	W	I	D	A	W	I	D	A	W	I	D
Control	10				10				10				10				10			
Cut only	10				10				10				10				10			
Secretion only	10				10				10				10				10			
Cut and secretion	6	1	2	1	4	2	2	2	4	2		4	3	1	1	5	3		1	6

[a] Symbols are interpreted as follows: A, active; W, weak; I, immobile; D, dead (see text for details).

DISCUSSION

Long-chain hydrocarbons are well-known constituents of the cuticular lipids of virtually all arthropods and may be functional in the waterproofing of the cuticle (Lockey, 1976). The occurrence of such massive quantities of hydrocarbons in a defensive gland is, however, unprecedented.

It is curious to note that the two major olefins, (Z)-9-heptacosene and (Z)-9-nonacosene, are closely related chemically (no biological connection is implied) to the sexually attractive cuticular hydrocarbons found in Diptera. (Z)-9-Tricosene was identified as the sex attractant pheromone of the common house fly, *Musca domestica* (Carlson et al., 1971). (Z)-9-Hentriacontene and (Z)-9-tritriacontene were among the cuticular hydrocarbons of the female stable fly *Stomoxys calcitrans* which elicited copulatory attempts by male flies (Uebel et al., 1975b). (Z)-13- and (Z)-14-Nonacosene and (Z)-13 heptacosene were identified as the sex pheromone components from the female face fly *Musca autumnalis* which stimulated mating strikes by male flies (Uebel et al., 1975a). Finally, the 3-methylalkanes are well-known components of the cuticle of cockroaches (Tartivita and Jackson, 1970; Jackson, 1970).

The soldier secretion is not ejected in a stream, but rather oozes from the fontanelle until the entire dorsal surface of the major soldier's head glistens with the oily secretion. The paraffin–olefin mixture is just at its melting point in the termite soldier and under normal hive temperatures (27–29°). Below 25°, the oily secretion begins to solidify into a wax. Its seems probable that since the frontal-gland reservoir is situated amidst the heavy musculature required for the operation of the mandibles, that the contractions in the surrounding tissues play some role in the exudation of the secretion from its reservoir.

The chemical composition of the secretion is specific for the subpopulation of *Macrotermes subhyalinus* found in the Kajiado District. All soldiers used in this study were obtained from closed-chimney mounds in reddish-brown soil. In surrounding areas where diatomite-type soil is more prevalent, mounds are built much differently (multiple open chimneys). The soldier secretions appear to be significantly different as well, with branched saturated and unbranched unsaturated hydrocarbons of chain lengths C_{27} to C_{35}. Further investigations into the correlation between mound type and soldier secretion are in progress and may prove valuable in the ecological and taxonomic studies of *Macrotermes* in this locale.

Filter paper disks (1 cm^2) impregnated with fresh major soldier secretion appeared to have little or no effect as a volatile or contact alarm signal for termite soldiers. No recruitment towards such a disk nor snapping movements of the mandibulae of soldiers walking over the disk could be observed.

Similarly, worker termites and ponerine ants exhibited no reaction to the secretion. Communication of alarm in this termite appears to be associated with the head-thumping by the soldiers and the lateral vibrations of both soldiers and workers.

Our results with live ants indicate that the chemical secretion of the major soldiers is an effective supplement to the traditional mandibular defenses. It causes small ants to adhere to the soldier head, allowing repeated attacks with the mandibles. With larger ants, the secretion increases mortality and morbidity in injured individuals, presumably by impairing the healing process.

The efficacy of *M. subhyalinus* soldier defenses in the field is further enhanced by the observation that during the nocturnal foraging activities of the major workers (accompanied by minor soldiers), the major soldiers rarely leave the foraging hole entrance or the foraging gallery entrance. In this way, the soldiers act as "dangerous doors," blocking the interfaces of the termite society with the outside world with a hard head, snapping mandibles, and a chemical weapon.

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THE CAUDAL GLAND IN REINDEER (*Rangifer tarandus* L.): ITS BEHAVIORAL ROLE, HISTOLOGY, AND CHEMISTRY

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Abstract—Behavior observations in European reindeer (*Rangifer tarandus*) showed a high frequency of sniffing the tails of conspecifics. A caudal gland was found, and it is the largest skin gland in *Rangifer*. Behavioral contexts of tail sniffing, the histology of the gland, and some aspects of the composition of the volatiles in the gland's secretion are described.

Key Words—caudal gland, cervids, deer, mammals, pheromones, *Rangifer tarandus*, reindeer, scent communication, scent gland, skin gland, tail gland.

INTRODUCTION

During observations on chemical communication in captive "forest" reindeer (*Rangifer t. tarandus* L.) in May and June 1975, one of the authors (D.M.-S.) noted that social interactions are often accompanied by sniffing another individual's tail.

Close examination of live reindeer showed a large amount of secretion on the basal third of the long white hair on the sides of the tail. This secretion was particularly obvious when the animals were shedding their tail hair in June. During that period, large bunches of old tail hair could easily be pulled out and used for extraction and chemical analysis of the deposited secretion. The secretion was present in both sexes, but more evident in females, and

particularly in a lactating cow. A large gland was found that had not been described for European reindeer.

After these observations we found that caudal glands had been discovered in study skins of the American caribou (*Rangifer tarandus arcticus*) by Lewin and Stelfox (1967). For the European reindeer (*Rangifer t. tarandus*) no caudal gland had been reported. The other, well-known skin glands in this species are the antorbital, tarsal, and interdigital glands (Quay, 1955).

METHODS AND MATERIALS

Behavior

Nine captive reindeer (4 males, 4 females, ranging in age from one to four years, and one calf born in June 1975) of the "forest" variety were observed at the field station of Umeå University in northern Sweden. The observations took place in two pens, each 100 × 100 m large, from May to September 1975. In addition, 90 free-ranging reindeer were observed for two days in Norra Storfjället, Swedish Lappland.

Histology

Tails from both sexes were collected in September 1975 at the annual reindeer slaughter at Klimpfjäll, Lappland, and preserved in Bouin's solution. The gland was studied histologically at 8 sites of caudal skin: middorsal and midventral at basal, distal (tail–tip), and intermediate points, and lateral at basal and intermediate points. Serial sections 6 to 8 μm thick were stained with Ehrlich's acid alum hematoxylin and eosin Y.

Chemistry

Tail hair from both sexes was collected at the field station of Umeå University in July 1975 and at Klimpfjäll (Lappland) in September 1975, immediately frozen in liquid nitrogen, and stored at -20°C in the dark until analyzed by methods described by Andersson et al. (1975).

We used GLC with 2.0 and 2.7-m glass columns, coated with 10% Reoplex 400 (isothermal at 58°C) and 3% OV-17 (isothermal at 100°C), respectively, on 100/120 mesh Chromosorb W-AW-DMCS, and nitrogen (40 ml/min) as carrier. Mass spectra were run on a LKB 9000 mass spectrometer with helium as carrier.

Acids were analyzed both as free acids and as methyl esters formed by methylation with diazomethane in ether.

RESULTS

Behavior

During social encounters, the tail and the areas immediately around it, were sniffed most often of all body parts. During the summer, 25% of all cases of social sniffing was directed at the tail. During encounters between temporarily separated group members, 25% of the sniffing was aimed at the tail, and a cow sniffed her calf at its tail in 36% of all cases.

Table 1 compares how often the 8 adult reindeer (4 males and 4 females) sniffed each other at the tail and at other body parts. The data were obtained during 20 hr of observation each in May, July, and September (first column), during observations of interactions between a cow and her calf, and during eight encounters between temporarily separated individuals and their group, observed for 30 min each. More details are given elsewhere (Müller-Schwarze et al., 1977b).

Excluding the interactions between mother and calf, and between reunited group members, adult males sniffed the body of other individuals more often (63 times in 60 hr) than females did (28 times). Of these, males sniffed the tail of other individuals 19 times, females did 31 times. Females were more often sniffed in general (66 times) than males (25) as well as at their tails (21 times, males 11 times), while females sniffed the calf's tail 4 times, and males only once. During the rut (September–November) sniffing of the tail did not increase, while the males' sniffing of the females' anogenital area and urine increased drastically (Müller-Schwarze et al., 1977b).

TABLE 1. FREQUENCIES OF SNIFFING VARIOUS BODY AREAS IN CAPTIVE REINDEER

	During 20 hr			Mother→Calf		During encounters of separated animals	Total	%
	May	July	Sept.	July	Sept.			
Tail	3	13	15	15	3	11	60	25.3
Nose	4	8	5	8	0	12	37	15.6
Anogenital area	3	1	19	7	0	5	35	14.8
"Sniff toward"	0	3	16	0	0	8	27	11.4
Body trunk	0	2	7	3	2	3	17	7.2
Urine	0	2	14	0	0	0	16	6.8
Rump	0	1	7	7	0	0	15	6.3
Tarsal gland	1	4	2	1	0	3	11	4.6
Other body areas	0	3	10	4	0	2	19	8.0
Totals	11	37	95	45	5	44	237	100

In our observations, the caudal gland played a role in four different behavioral contexts: social encounters of previously separated individuals, sexual behavior, maternal behavior, and alarm situations.

Social Encounters. As mentioned earlier, the most frequent olfactory response in social encounters is sniffing the tail of a conspecific. This occurs particularly frequently when two individuals meet again after separation. Of all body parts, the tail is sniffed most frequently both by the newcomer and the established group members.

Sexual Behavior. During the rut, a male collects a harem and keeps other males at a distance. At intervals, the harem bull will sniff his cows at their tails and the white hair on both sides of the anogenital area. The cows do not sniff the bull in this fashion. Other males stay too far away from the harem to permit any male–male sniffing. Overall, males then sniff females four times as often at their tails as females do males.

Maternal Behavior. A cow often sniffs her calf at the tail. This occurs when the calf approaches its mother with its tail wagging, after having been separated. In many cases nursing follows, and the cow permits her calf to nurse only after sniffing his tail. The calf rarely sniffs the tail of the mother;

FIG. 1. Position of tail in alarm situations, shown in a captured mature bull.

instead it sniffs most often her mane, tarsal gland, and when approaching the udder, the white long hair behind it. (Young calves even suck at the white mane hair). The body areas of the mother sniffed by the calf are limited by the calf's height.

Alarm Situations. During alarm, a reindeer will raise its tail and spread the long white hair on its ventral side (Figure 1). This way the exposed odor-carrying surface of the hair is maximized; the "odor flag is unfurled." We do not know the responses of conspecifics in this context. But it is likely that the tail gland discharges an alarm odor, especially since the genus *Rangifer* lacks the metatarsal gland which produces an alarm odor in the deer of the genus *Odocoileus*, for instance (Müller-Schwarze, 1971).

Histology

The caudal gland is the largest of all skin glands in *Rangifer*; it extends from base to tip of the tail both ventrally and laterally. Histologically, glandular modifications in the caudal gland region involve both the sebaceous and the apocrine sudoriferous units that are most widespread in mammalian skin glands. The apocrine sudoriferous units predominate by both their numbers and their amount of tissue. These are absent in the middorsal skin from basal to intermediate sites along the tail (Figure 2), but they are present, numerous, and large in the ventral caudal skin from tail base to tail tip and nearly equally well-developed bilaterally along the sides of the tail (Figure 3). The greatest thickness of the apocrine gland layer (about 2.0 mm) is seen at or near the tip of the tail ventrally.

The skin area of the thick apocrine gland layer corresponds fairly well with that of the thick white hairs. The thick, long white hair of the ventral side was also examined under the scanning electron microscope. The cuticular scales on the hair surface form cups that hold secretion, but the surface structure does not differ from that of hair of other body regions of reindeer. The relatively glandless middorsal skin is covered with finer brown (pigmented) hair. The hair follicles of both middorsal and ventral, as well as most of the tail, are equipped with equally thick bundles of arrector smooth muscles. The bellies of these muscles range from 100 to 300 μm in diameter and are sometimes morphologically complicated by subdivisions and ramifications leading to two or more attachments or heads. These observations suggest that behavioral erection of caudal hair is a generally distributed capacity, while the distribution of the apocrine glands on the tail is more limited.

Sebaceous gland units are also somewhat enlarged in the caudal gland region. Furthermore, there are cytological staining and structural differences in the sebaceous secretory cells of the caudal glands as compared with the

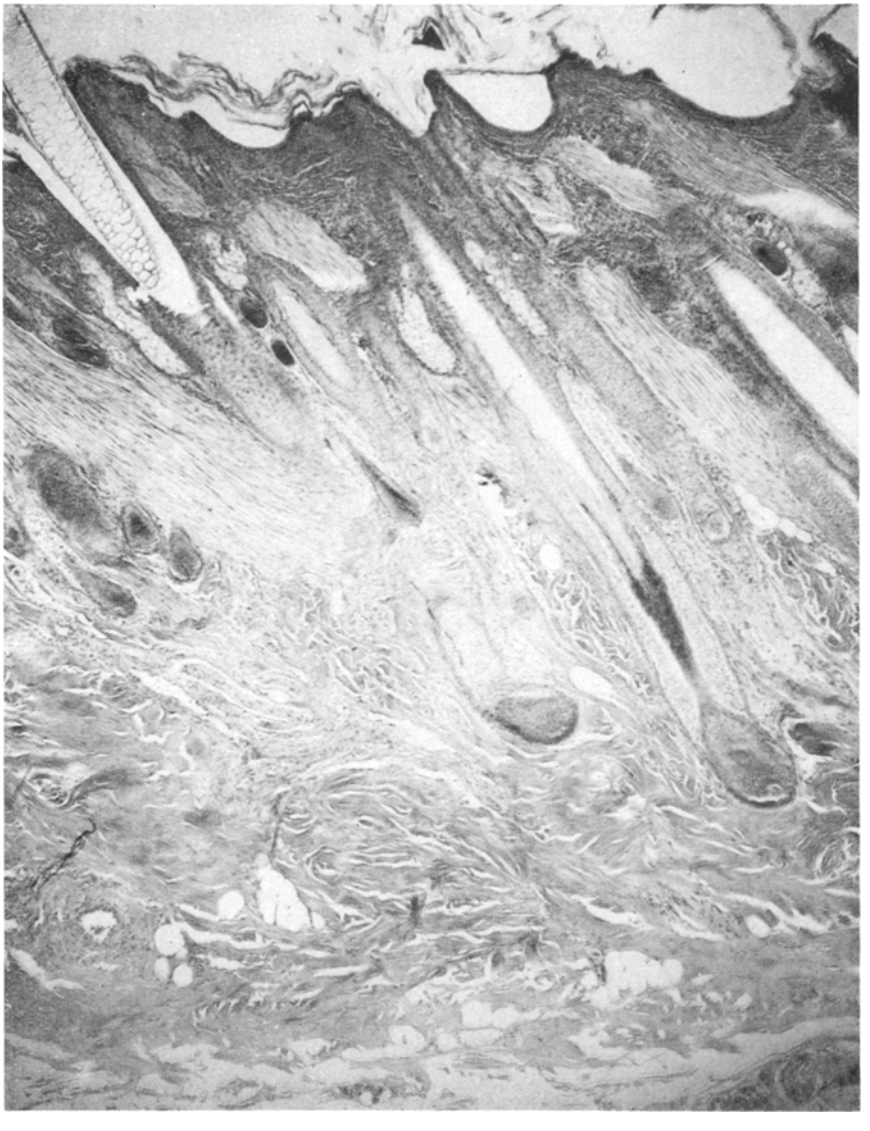

FIG. 2. Vertical section through the middorsal skin of the middle of the tail of an adult male reindeer showing the typical absence of apocrine sudoriferous glands.

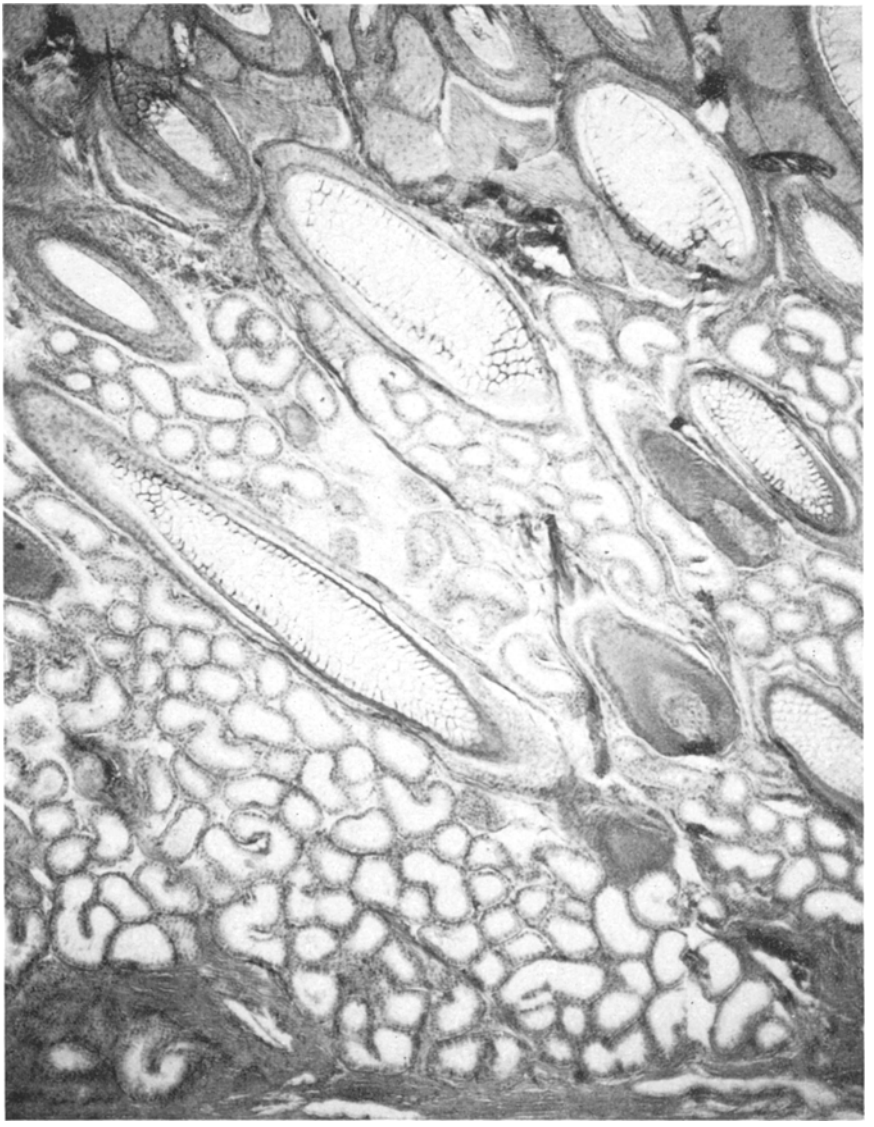

FIG. 3. Vertical section through the midventral skin of the middle of the tail of the same adult male reindeer as in Figure 2, showing the thick (1.4–1.7 mm) basal zone of coiled secretory apocrine sudoriferous glands. The follicles of the characteristic thick white hairs of the gland area are also shown.

more generally distributed sebaceous cells, such as those of the dorsal pigmented skin of the tail. Caudal gland sebaceous cells stain more darkly with eosin and have cytoplasm that is more finely granular and contains vacuoles that are extremely minute.

Chemistry

Odoriferous compounds of the yellowish secretion of the tail are clearly

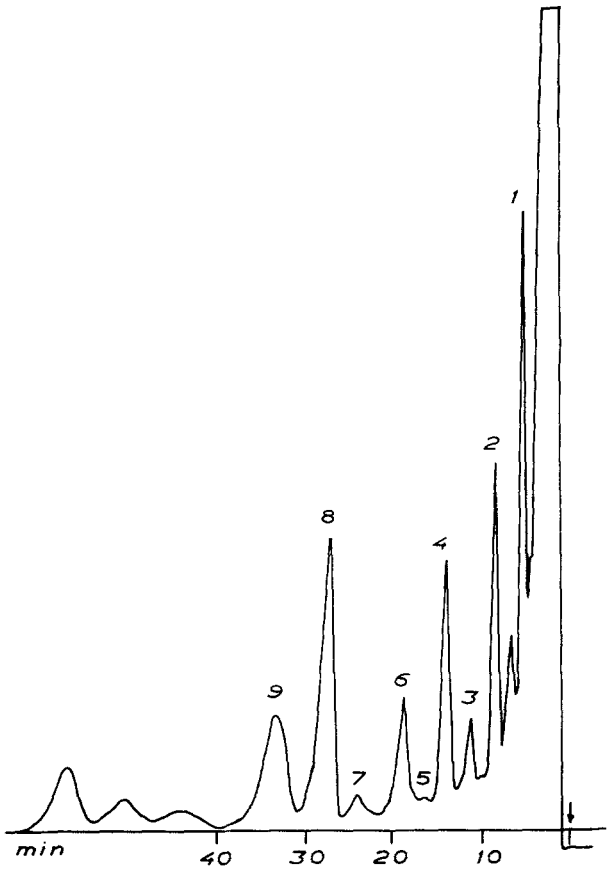

FIG. 4. Gas chromatogram of volatile components from the caudal gland of reindeer. The peaks represent: *n*-heptanal (1), *n*-octanal (2), acetic acid (3), *n*-nonanal (4), propionic acid (5), isobutyric acid (6), *n*-decanal (7), *n*-butyric acid (8), and an unresolved mixture of isovaleric and methylbutyric acid (9)

noticeable to the human nose. By GLC analysis of the volatile components we identified a series of saturated normal (C_7–C_{10}) aldehydes and a series of short-chain acids (Figure 4). The identity was confirmed with authentic samples and by GC–MS. The aldehydes had been found previously in the tarsal gland secretion of reindeer (*Rangifer tarandus* L.); however, the amounts in the tail-gland secretion far exceed those of the tarsal-gland secretion. The acids have also been found in interdigital-gland secretion (Brundin et al., 1977).

DISCUSSION

The current finding is an example of detailed behavioral observations leading to the discovery of a specialized anatomical structure. We are far from understanding all the information that may be provided by the caudal gland of a reindeer. However, the sniffing of the tail in social encounters, especially of the calf by the mother is known for other ungulates. Tschanz (1962) described olfactory recognition of lambs by their mothers in the mufflon (*Ovis musimon*) after sniffing their tails. Black-tailed deer (*Odocoileus hemionus columbianus*) females sniff their fawn's tails before they accept them as their own. In black-tailed deer, on many occasions we observed mothers sniffing and licking a strange fawn's head, shoulders, and back, and finally turning away or even attacking the fawn when reaching the tail area. In black-tailed deer vocal communication, on the other hand, does not serve in individual recognition: a bleating fawn will attract all mothers in the same and adjacent pens. For reindeer, however, Espmark (1971) showed that mothers can discriminate calves by their calls.

When comparing the functions of the various skin glands in reindeer with those in the better investigated black-tailed deer (Müller-Schwarze 1971), the most striking difference is the lack of a metatarsal gland in *Rangifer*. The tarsal gland of *Rangifer* does not have the central importance in social behavior typical for deer of the genus *Odocoileus*. It seems that the caudal gland in *Rangifer* combines functions of both the metatarsal and tarsal glands as found in *Odocoileus*: activation in alarm situations (as does the metatarsal gland) and recognition (as the tarsal gland).

In its histology, the caudal gland of reindeer with its preponderance of apocrine glands resembles the metatarsal gland of black-tailed deer (Quay and Müller-Schwarze, 1970) and the ischiadic gland of the pronghorn, *Antilocapra americana* (Moy, 1970). The glands in these two species produce airborne odors in situations of alarm. Therefore, a similar function is assumed for the caudal gland of reindeer. Glands used for scent marking, on the other hand, are usually dominated by sebaceous glands, as is the case in the sub-

auricular gland of the pronghorn (Moy, 1970), or the ventral gland of the Mongolian gerbil, *Meriones unguiculatus* (Glenn and Gray, 1965).

In reindeer, the tail brushes in a lateral motion over the white hair at both sides of the anogenital area ("rump patches"). Thus, the ventral tail hair distributes material from the caudal gland over an area that is several times greater than that of the gland surface. Thus, an "odor field" is created at the rear end of the animal. Furthermore, it is likely that in the females urinary components are picked up and incorporated into the "scent," in much the same fashion as urinary components are adsorbed by the lipids of the tarsal hair tuft in black-tailed deer (Müller-Schwarze, 1977a). Scanning electron microscope studies showed that in reindeer the white hair in the gland area does not significantly differ in its surface structure from other body hair. This is in contrast to black-tailed deer, where hair of tarsal glands, metatarsal glands, or the body trunk each show different cuticular patterns, with the hair of the tarsal gland being most specialized (Müller-Schwarze et al., 1977).

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ATTRACTION AND INHIBITION IN MOTH SPECIES RESPONDING TO SEX-ATTRACTANT LURES CONTAINING Z-11-HEXADECEN-1-YL ACETATE[1]

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Abstract—Sex attractants for several sympatric noctuid moths required *Z*-11-hexadecen-1-yl acetate and additional olefinic compounds (coattractants) for effective, species-specific operation. In nearly all cases at least one of the coattractant compounds for each species functioned as a strong inhibitor of one or more of the other species in the group. It was concluded that species specificity in sex attractants can be achieved through conspecific coattractants which are at the same time transspecific inhibitors.

Key Words—*Eurois occulta*, *Leucania commoides*, *Scotogramma trifolii*, *Crymodes devastator*, *Mamestra configurata*, sex pheromones, specificity, Lepidoptera, chemical communication.

INTRODUCTION

Z-11-Hexadecen-1-yl acetate (Z11–16:Ac) is a necessary component of sex attractants for male moths of several species, including the noctuids *Mamestra configurata* Walker (Chisholm et al., 1975), *Scotogramma trifolii* Rottemburg (Struble et al., 1975), *Eurois occulta* L. (Steck et al., 1976), *Peridroma saucia* Hübner (Struble et al., 1976), *Leucania commoides* Grote (Struble et al., unpublished), and *Crymodes devastator* Brace (Steck et al., 1977). The actual attractant mixtures described for each species involve from 2 to 4 chemical components. Species specificity in sex pheromones containing one or more common constituents has been attributed to the presence of different additional coattractant components, the coattractants each being effective for a single

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species. The use of different ratios of common components by related species has also been noted. A wealth of experimental data supports both of these mechanisms (see Roelofs and Cardé, 1974). At the same time, it is known (Sower et al., 1974) that some female moths are capable of releasing with their pheromone certain compounds which prevent males of other species from responding to the pheromone. Natural attractants and inhibitors found to date amongst lepidopterans have almost always had molecular structures very similar to each other, a simple but important fact seldom emphasized in descriptions of the systems. The vast majority of these substances are straight-chain olefins of even carbon number (C_{10}–C_{18}) with a terminal acetate, aldehyde, or alcohol functional group. The number of different monoolefins of these types known in sex pheromones is in fact small (less than 60) and comprises mostly compounds with C_{12}, C_{14}, and C_{16} carbon chains. The number of binary combinations possible from this number of components—1770—seemed insufficient to provide a complete array of specific attractants for the lepidoptera of our locality, for surveys of field attractancy of several hundred candidate binary lures had shown us that most combinations attracted no species. We therefore undertook a study of other possible chemical communication roles for some of these compounds, with emphasis on interspecific effects of sex attraction or inhibition.

The procedures generally employed in identification of sex pheromones in insects involve collection of the pheromone from females, isolation of the components via fractionation of the collected mixture, chemical identification of the attractant components, and finally confirmation of the laboratory work in field tests. In the laboratory, bioassays and/or antennal response measurements are usually employed during the isolation steps. This approach, sound for the purpose of finding conspecific attractants of a species, is not well suited to revealing interspecific chemical messengers. Discovery of naturally released compounds able to function as specific inhibitors for other species is not possible until the identity of the species being inhibited is first ascertained, which in turn requires at least some knowledge of the character of their sex attractants. Using synthetic sex attractants instead of isolated sex pheromones, we have obtained this information for a model group of noctuid moths all requiring *Z*-11-hexadecenyl acetate (*Z*11–16:Ac) for sex attraction. We describe here some experiments on interspecies inhibition within this group.

METHODS AND MATERIALS

Chemicals

The compounds used were synthesized in our laboratory by recognized methods (Warthen, 1968; Struble and Swailes, 1975) and purified isomerically

by large-scale argentation chromatography (Houx et al., 1974). Their purity was >99% as assayed by gas chromatography. For field trap use, lure mixtures were prepared using Dade micropipettes, by direct application of compounds to red rubber septa (Maitlen et al., 1976).

Field Trapping

Cone-orifice traps (Type B, Steck et al., 1976) were baited with 1.0 mg lure, unless stated otherwise, and placed horizontally on fence posts at a height of 1.0 m. Post separation within randomized blocks was 50 m for *E. occulta* and *S. trifolii*, 40 m for *L. commoides*. For the species in Tables 1 and 2 the 1976 trap sites were near Saskatoon, Saskatchewan (52°N, 106.5°W) in fields at research farms in which a variety of cereal grains and legume crops were grown during the season. Some trees and bushes (mixed coniferous and deciduous) were near the sites but all traps were placed where there was free sweep of wind. Blocks were 2–10 km apart. The mean max/min temperatures and wind velocity (at 0200 CST) in July 1976 were 25.2/12.1° and 14 kph; in August the values were 26.0/11.1° and 13 kph. There was no prevailing summer wind direction. Traps were inspected and rerandomized at intervals of 3–4 days. Some of the *L. commoides* field work was conducted similarly at Swift Current, Saskatchewan (50.5°N, 107°W), where a higher population of *L. commoides* was anticipated. Additive compounds were added to make up 10% of the test lures unless indicated otherwise. Test trapping periods and test replications for each species were: *Eurois occulta* (10 July–9 August, 1976; 3×), *Leucania commoides* (30 July–12 August, 1976; 3×), *Scotogramma trifolii* (11–30 August, 1976; 3×) *Crymodes devastator* (18–30 August, 1976; 2×), *Mamestra configurata* (July 1975; using field cages and laboratory-reared males, 2×).

RESULTS AND DISCUSSION

The attractants used as basic lures were arrived at through field testing of binary combinations of *Z*11–16:Ac and other olefins as candidate bait mixtures in traps, without specifying, at the outset, target species. Successful mixtures were then optimized for their evident target species in further field tests. These field surveys disclosed altogether more than a dozen lepidopterous species attracted to certain combinations. Several species were strongly attracted to combinations of *Z*11–16:Ac+*Z*9–14:Ac, including the bertha armyworm *Mamestra configurata* (to ratio 20:1), and the great dart *Eurois occulta* (to ratio 1:4). To ascertain the effects of additives upon the specificity of these basic-lure mixtures further surveys were carried out. The effects

observed suggested that additives in this type of experiment can be classified as *coattractants* (significant increase in captures over the basic lure), *inhibitors* (decrease in captures), and *nulls* (no significant difference from the basic lure). This procedure and terminology for additive tests can be extended to "basic lures" of any number of coattractant components, and indeed we now routinely test effects of additives on three-component attractants. Our three

TABLE 1. ALTERATION OF LURE ATTRACTANCY FOR THREE NOCTUID MOTHS BY 18 ADDITIVE COMPOUNDS

Additive	Total ♂♂ captured[a]		
	Eurois occulta[b]	*Leucania commoides*[c]	*Scotogramma trifolii*[d]
None	105 k	51 pqr	28 vwx
Acetates			
*E*11–16:Ac	100 k	57 opq	45 uv
*Z*9–14:Ac	Required	Required	6[e] x
*E*9–14:Ac	112 k	119 n	22 wx
*Z*11–14:Ac	167 k	77 nop	52 u
*E*11–14:Ac	108 k	41 pqrs	79 t
*Z*7–12:Ac	45 lm	2 s	10[e] x
*Z*9–12:Ac	130 k	29 qrs	24 vwx
Alcohols			
*Z*11–16:OH	8[f] m	101 no	20 wx
*E*11–16:OH	148 k	13 rs	26 vwx
*Z*9–14:OH	10 m	2 s	43 uv
*E*9–14:OH	108 k	111 n	20 wx
*Z*11–14:OH	105 k	26 qrs	23 vwx
*E*11–14:OH	107 k	66 opq	47 uv
16:OH	137 k	62 opq	37 uvw
Aldehydes			
*Z*11–16:Ald	4[g] m	46 pqr	16[e] wx
*Z*9–14:Ald	4[h] m	50 pqr	27 vwx
*Z*11–14:Ald	325 j	31 pqrs	20 wx
*E*11–14:Ald	128 k	62 opq	33 uvw

[a] Capture totals followed by the same letter are not significantly different at $P=0.05$ (Duncan's Bayesian LSD).
[b] Basic attractant was 1:4 of *Z*11–16:Ac+*Z*9–14:Ac.
[c] Basic attractant was 5:5:1 of *Z*11–16:Ac+*Z*9–14:Ac+Z11–16:OH.
[d] Basic attractant was 9:1 of *Z*11–16:Ac+*Z*11–16:OH.
[e] With 250 μg added *Z*9–14:Ac no *S. trifolii* were caught but some *L. commoides* were taken. With 500 μg added *Z*7–12:Ac or *Z*11–16:Ald no *S. trifolii* were captured.
[f] Principally *L. commoides* was attracted (109 males).
[g] Principally *Eurois astricta* Morr. was attracted in July (31 males).
[h] Principally *Euxoa acornis* Sm. was attracted in August (66 males).

classifications do not take into account the varied behavioral effects which may be associated with additives and which may bring about increased or decreased capture rates by field traps. These effects have been discussed by Cardé et al. (1975). Numbers of male moths captures by traps constituted our criterion of trap bait attractancy. One should also note that an additive compound may fall into different classifications according to its proportion in a lure. For example, males of the European corn borer which respond to one isomer of 11-tetradecenyl acetate are more strongly attracted if 3% of the opposite isomer is added to lures, but less strongly attracted if 50% of the opposite isomer is present (Klun et al., 1973). At 3% the additive is a co-attractant; at 50% an inhibitor. In general, additive effects are most meaningful when the additive content of test lures is kept low.

Results of a series of additive tests upon some two- or three-component attractants sharing *Z*11–16:Ac are presented in Table 1. The additive acetates chosen for testing included the E isomers of the components of the attractants, since such geometric isomers are known sometimes to affect the attractancy of a lure (Klun et al., 1973). The isomers of 11-tetradecenyl acetate, and *Z*-7- and *Z*-9-dodecenyl acetate, were also tested. The corresponding Z and E hexadecenols and tetradecenols were tested, and four closely related aldehydes were used.

Consider first the figures in Table 1 obtained with *Eurois occulta* as the target moth. A mixture of *Z*9–14:Ac+*Z*11–16:Ac (4:1) was the basic attractant. One additive (*Z*11–14:Ald) proved to be a coattractant, and four appeared as potent inhibitors (*Z*11–16:Ald, *Z*9–14:Ald, *Z*11–16:OH, and *Z*9–14:OH) with *Z*7–12:Ac somewhat inhibitory in addition. The remaining compounds were nulls with no effect upon the attractancy. The correspondence between inhibition toward *E. occulta* and attractancy for other species was remarkable. Traps containing the basic attractant along with *Z*11–16:Ald as the test additive captured only 4 *E. occulta* males but were unique in capturing 31 males of *Eurois astricta* Morrison, an uncommon prairie species. Near the end of the flight of *E. occulta*, the traps containing the basic lure plus *Z*9–14:Ald began to take males of *Euxoa acornis* Smith. In 1975, 110 of these males were taken in August–September using duplicate traps; in 1976, 66 were taken using 3 × replication. The same test traps attracted only 4 *E. occulta*. As might be expected, adding *Z*11–16:OH to the basic binary *E. occulta* lure resulted in the attraction of some *L. commoides* males, for their basic attractant comprises these three components. In fact 109 *L. commoides* were captured in the same traps which caught only 8 males of the target moth. With *Z*9–14:OH as additive 10 *E. occulta* were taken, but no other species was captured in the same traps.

Next consider only the data in Table 1 obtained with *Leucania commoides* as the target moth. The three-component mixture *Z*11–16:Ac+*Z*9–14:Ac+

Z11–16:OH (5:5:1) was the basic attractant. Three additive compounds proved beneficial to the basic attractant: E9–14:Ac, E9–14:OH, and Z11–16:OH. Two other compounds (Z7–12:Ac, and Z9–14:OH) were markedly inhibitory, and the remaining twelve additives were nulls. In these experiments, however, no extraneous species were observed in the traps except in the case of Z9–14:OH, where a few *S. trifolii* were attracted.

When *S. trifolii* was the target moth (Table 1) with Z11–16:Ac+Z11–16:OH (9:1) the basic attractant, E11–14:Ac and to a lesser degree Z11–14:Ac proved to be coattractants, Z9–14:Ac was inhibitory at levels above 10%, and all the other compounds were nulls. Inhibition of *S. trifolii* attractancy by Z9–14:Ac was paralleled by the increase in attractancy of these same lure mixtures for *L. commoides*. In separate additive trials using 250–500 μg of additive, Z7–12:Ac and Z11–16:Ald also proved highly inhibitory to the basic attractant for *S. trifolii*.

Considering all together the three moths in Table 1, there are ten instances of inhibition. In five of these the inhibition of the target species was accompanied by attraction of males of other species. Theories of attractant specificity through correct combinations (including ratios) of compounds do not suggest or require concurrent inhibition of other species, and conversely, the concept of interspecific inhibitors in natural sex attractants does not imply or demand that these inhibitors simultaneously function as conspecific coattractants in the producer species. However, our data indicate that such dual

TABLE 2. ALTERATIONS OF ATTRACTANT POWER FOR THREE MOTHS BY SIX ADDITIVES

Additive	Total ♂♂ captured[a]		
	Mamestra configurata[b]	*Crymodes devastator*[c]	*Scotogramma trifolii*[d]
None	21 hi	35 k	28 mn
E11–16:Ac	10 ij	34 k	45 m
Z9–14:Ac	Required	0	6 n[f]
E9–14:Ac	34 h	No test	22 mn
Z11–16:OH	1 j[e]	0	Required
E11–16:OH	15 hij	45 k	25 mn
Z11–16:Ald	7 ij	Required	16 n[f]

[a] Capture totals followed by the same letter are not significantly different at $P=0.05$ (Duncan's Bayesian LSD).
[b] Attractant was 19:1 of Z11–16:Ac+Z9–14:Ac.
[c] Attractant was 1:1:1 of Z11–16:Ac+Z11–16:Ald+Z7–12:Ac.
[d] Attractant was 9:1 of Z11–16:Ac+Z11–16:OH.
[e] In outdoor trials in 1976 this mixture attracted 9 *M. configurata*, 18 *S. trifolii*, and 2 *L. commoides* (4× replication, 110 μg lure).
[f] Higher concentrations of these additives caused complete inhibition.

attractant/inhibitor roles are common occurrences among sex-attractant compounds.

Table 2 shows some further examples of compounds of pheromone-like structure which act both as conspecific coattractants and as interspecific inhibitors in sex-attractant systems involving Z11–16:Ac. The target moths were the bertha armyworm *Mamestra configurata* (Walker), the glassy cutworm *Crymodes devastator* (Brace), and the clover cutworm *Scotogramma trifolii*. The coattractant of the last moth, Z11–16:OH, strongly inhibited attractants for the other two. Similarly, Z9–14:Ac, the coattractant for *M. configurata*, was an inhibitor of the attractants for both *C. devastator* and *S. trifolii*, although for the latter moth high proportions of Z9–14:Ac were required in the lure. Z11–16:Ald, the coattractant for *C. devastator*, inhibits *S. trifolii* under similar circumstances and may be somewhat inhibitory to *M. configurata* as well. These three moths, like the three of Table 1, are sympatric species and all entered traps at some time between 2 hr after sunset and 1 hr before sunrise (an interval of about 3–4 hr at our latitude in July). Traps baited as indicated in Table 2 were sex- and species-specific for males of the target moths.

A summary of the attractant–inhibitor relationships observed among the four moths, *E. occulta*, *S. trifolii*, *C. devastator*, and *L. commoides*, is presented in Figure 1. The figure shows inhibitory compounds which might, hypothetically, be produced as part of the sex pheromone of each moth, with arrows indicating chemical movements from the female of the producer species to the male of the inhibited species. For example, *Scotogramma*

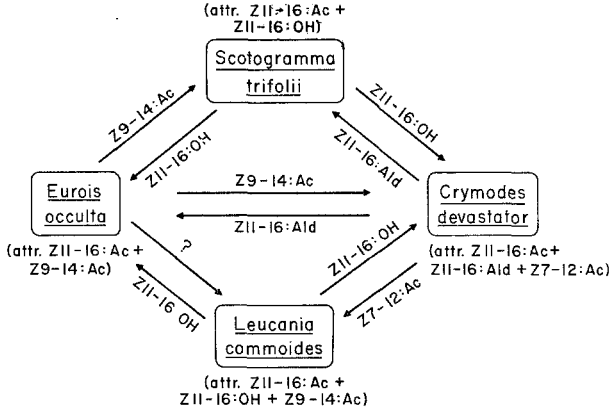

FIG. 1. Schematic representation of attractant–inhibitor relationships among four species requiring Z11–16:Ac as common attractant component.

trifolii females produce Z11–16:OH which is a compound inhibiting the attraction of male *C. devastator* and *E. occulta* to sex lures. Only in the case of *S. trifolii* has the listed attractant been shown to be essentially the natural sex pheromone (Underhill et al., 1976); in the three other cases the female sex pheromone may or may not approximate chemically the synthetic mixtures developed in our field lures. Figure 1 shows Z11–16:OH as an inhibitor which might be used by *L. commoides* against *C. devastator*; Z9–14:Ac might equally well perform this role. In addition, one inhibition effect could be described between *S. trifolii* and *L. commoides*, Z9–14:Ac being hypothetically used by the latter organism against the former. The schematic network of attraction–inhibition relationships could be expanded to take in further species. For example, the putative pheromone of *Eurois astricta*, if it consists of Z11–16:Ac+Z9–14:Ac+Z11–16:Ald (Table 1, footnotes), is provided with built-in inhibition for the co-occurring noctuids *E. occulta* (via Z11–16:Ald) and *C. devastator* (via Z9–14:Ac).

The number of noctuid species captured in our 1975 and 1976 experiments with Z11–16:Ac-based binary lures was substantial and included, besides the species mentioned above, *Cryptocala acadiensis* Bethune, *Sideridis rosea* (Harvey), *Feltia ducens* (Walker), *Polia atlantica* Grote, *Euxoa messoria* (Harris) and *E. lutulenta* Smith, *Faronta diffusa* (Walker), *Agroperina dubitans* (Walker) and *Schinia bina* Guenée (Underhill et al., unpublished data). In the few instances where we have screened additives with these species as targets, the preliminary results indicate again a widespread ability of co-attractants for one species to function as inhibitors of the attractant systems of others. The incidence of this phenomenon seems to be very much higher than what would be expected on the basis of random inhibition/attraction amongst the 60-odd monoolefinic C_{12}, C_{14}, and C_{16} compounds—acetates, alcohols, and aldehydes with unsaturation in odd-numbered positions of the carbon chain—with which we have been working, and amongst which are found nearly all the known monoolefinic components of lepidopterous pheromones. We are now conducting experiments designed to ascertain the generality of this phenomenon.

Two final remarks should be made in connection with the experiments described in this paper. First, the lures used were synthetic chemicals; we have dealt with sex attractants but not necessarily with sex pheromones in all cases. For *M. configurata* and *S. trifolii*, the natural pheromones have been shown (Chisholm et al., 1975; Underhill et al., 1976) to consist of the compounds used here. From evidence obtained from electroantennogram measurements of male antennal responses to synthetic compounds and to fractionated extracts of female abdominal tips, we believe that others of the synthetically developed sex attractants above also approximate the natural sex pheromone of the attracted species. Insofar as this is true, the foregoing

results have direct relevance to natural chemical communication systems. At present the results describe only what might be readily possible in pheromone systems.

Second, our findings suggest a cautionary remark about atmospheric permeation with pheromones as a technique for mating disruption in pest species (see Mitchell et al., 1974; Schwalbe et al., 1975). Although a permeation mixture may be carefully formulated for a target species, massive release of the component pheromones probably results in a more general disruption of several species whose chemical communication systems can utilize the released compounds as attractants. The extension of the technique to simultaneous disruption of more than one target pest (Mitchell, 1975) brings in the possibility of nonadditive confusion effects. For example, if concurrent disruption of *E. occulta* and *S. trifolii* were attempted using atmospheric permeation with the basic attractants for these moths (Table 1), *L. commoides* would be confused as well. Permeation methods may tend to appear more species-specific than they actually are because appropriate concurrent multispecies studies of the effects have been lacking.

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VARIATION IN CARDENOLIDE CONTENT OF THE LYGAEID BUGS, *Oncopeltus fasciatus* AND *Lygaeus kalmii kalmii* AND OF THEIR MILKWEED HOSTS (*Asclepias* spp.) IN CENTRAL CALIFORNIA

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Abstract—A colorimetric assay was used to quantify the amount of cardenolides in the lygaeid bugs *Oncopeltus fasciatus* and *Lygaeus kalmii kalmii* and their milkweed host plants (*Asclepias* spp.) in central California. The cardenolide content of individual insects, determined in microgram equivalents of digitoxin, varied from zero to over 300 μg per insect. Sources of variation of cardenolide content in the insects include interspecific and intraspecific differences in the content of the host plant species and also differences in the content of plant organs on which insects were feeding. This last source of variability may explain temporal variation in the cardenolide content of the insects. Adults of *O. fasciatus*, which migrate into California in the late spring and early summer, and adults of *L. k. kalmii*, which emerge from winter hibernacula in the early spring, contained small to immeasureable amounts of cardenolides. The colonization pattern of *O. fasciatus* on species of *Asclepias* in north central California suggests that this species does not maximize its opportunities to sequester large quantities of cardenolides from potential hosts. The emetic potential of lygaeids in California to vertebrate predators is briefly discussed.

Key Words—Variation in cardenolides, *Oncopeltus fasciatus*, *Lygaeus kalmii kalmii*, milkweeds, *Asclepias*, chemical defense.

INTRODUCTION

Many aposematic insects have been hypothesized to serve as models in

mimicry rings or complexes because they have the ability to sequester and store bitter-tasting or toxic chemicals from their host plants. These chemicals can act as deterrents to vertebrate predators, thus providing a form of defense which would not be available to the insects had they fed on a food source lacking these compounds (Rothschild, 1972).

One such class of chemicals is the cardenolides (cardiac glycosides), produced by several families of angiosperms; these heart poisons, which are highly toxic to most vertebrates (Thorp and Cobbin, 1967), also induce emesis in vertebrates (see Borison and Wang, 1953, for a review), and are very bitter (Brower and Glazier, 1975; Reichstein et al., 1968).

Temperate and subtropical milkweeds (Asclepiadaceae) are rich sources of cardenolides and support a discrete insect fauna. Several insects which specialize as herbivores of milkweeds have been shown to store large quantities of cardenolides in their tissues and glandular secretions, presumably for defensive purposes (von Euw et al., 1967, 1971; Duffey and Scudder, 1972; Brower et al., 1968; Rothschild et al., 1970; Abushama and Ahmed, 1976). Brower (1969) has shown that the monarch butterfly, *Danaus plexippus* L., varies in its potential to cause emesis in a vertebrate depending on the host plant of the larva, and more recently he reported that wild danaid butterflies vary considerably in their cardenolide content (Brower and Moffitt, 1974; Brower et al., 1975). The existence of a relationship between host-plant species, cardenolide content, and emetic potential of a milkweed-feeding insect has thus been well established.

As in the monarch butterfly, the ability of the milkweed bugs *Oncopeltus fasciatus* Dallas and *Lygaeus kalmii kalmii* Stål to sequester and store cardenolides from milkweed plants in the laboratory has been well documented (Duffey and Scudder, 1972, 1974). However, to date the cardenolide content and host–plant relationships of these lygaeid bugs in the field have not been examined. In this study we have investigated the gross cardenolide content of both *O. fasciatus* and *L. k. kalmii*, and their host plants (*Asclepias* spp.) in central California.

METHODS AND MATERIALS

Insects and Plants

Adult *O. fasciatus* and *L. k. kalmii* were collected on milkweed plants (*Asclepias* spp.) in central California between April and September, 1976. Field-caught insects were maintained in controlled environmental cabinets at 27°C, on a 13.5:10.5 LD light regime, and maintained on distilled water and husked sunflower seeds. Field-caught insects were used within two weeks of their capture: our unpublished results indicate that there is no

significant loss of cardenolides in insects feeding on sunflower seeds (which lack cardenolides) for this length of time, after having previously fed on a cardenolide-rich diet. This period on sunflower seeds allowed the insects to clear the gut, so that the cardenolide estimates we have reported are not influenced by gut contents.

Additional insects were reared in the laboratory on seeds of different species of *Asclepias*. Temperatures varied from 25° to 27°C, with light regimes from 13.5:10.5 LD to continuous light. Insects were analyzed for cardenolide content when they were 7-day-old adults, because sequestration of cardenolides also takes place in the adult stage (Duffey and Scudder, 1974).

Plant material was collected over the same time period at the same sites where insects were found. Mature leaves, flowering umbels, and seeds from mature pods were dried for 12 hr at 80°C in a forced-draft oven.

Extraction and Colorimetry

Different extraction procedures were used for insects, seeds, leaves and umbels, based on the methods described in Scudder and Duffey (1972) and Duffey and Scudder (1972). To maximize the extraction efficiency for quantitative analysis, plant material, especially dry seeds, require mechanical breakdown to better expose the cardenolide-containing tissues to the extracting solvents.

Insects, either live or dried, were placed individually in 2 ml of chloroform–methanol (2:1), allowed to stand for 24 hr, then removed from this solvent and placed in an additional 2 ml of chloroform–methanol (1:1). After a further 24 hr, the two extracts were pooled, evaporated to dryness, and dissolved in 1 ml of 95% ethanol. Half of this final solution was used for the colorimetric estimation of gross cardenolide content in each insect. More than 95% of an insect's cardenolide complement is extracted using this procedure: repeated extractions fail to produce more than 5% of the total quantity estimated.

Milkweed seeds were ground to a fine powder, and 0.2 g of this extracted with 4 ml of petroleum ether, shaken in a test tube, and allowed to settle for 6 hr. The hydrocarbon extract was discarded because our unpublished results showed that it lacked cardenolides. The residue was placed in 2 ml hot chloroform–methanol (10:1), shaken vigorously, allowed to stand for 12 hr, and the chloroform phase decanted. The residue was reextracted with 3 ml of 95% ethanol for 12 hr, and the liquor added to the chloroform extract, evaporated to dryness, and redissolved in 4 ml of ethanol: 0.5- or 1-ml aliquots of the pooled extract were used for colorimetric determinations.

Dried milkweed leaves or umbels were ground to a fine powder, 0.2 g

extracted once with 4 ml of hot 95% ethanol, and allowed to stand 12 hr before 0.5- and 1-ml aliquots were taken directly for cardenolide estimation.

The colorimetric assay used was that of Brower and Moffitt (1974) and Brower et al. (1975). Cardenolide estimations were based on a standard of digitoxin; all determinations are expressed as equivalents of digitoxin (Brower et al., 1975; Roeske et al., 1976). Hence our values are directly comparable to those reported for the monarch and other danaid butterflies (Brower and Glazier, 1975; Brower et al., 1975). Readings were taken on a Beckman model B spectrophotometer; accuracy of the assay was approximately $\pm 3\%$.

RESULTS

There is a wide variation in the cardenolide content of the milkweed bugs *O. fasciatus* and *L. k. kalmii* collected on *Asclepias* in the field in central California (Table 1). Some insects were found to either lack cardenolides or have levels of cardenolides below the colorimetric detection limit. Other insects were found to each contain up to 375 μg of cardenolide in their bodies.

Differences in cardenolide content between samples of lygaeid bugs taken on a particular host species indicated that interspecific differences in the cardenolide content of the host species cannot alone account for the variability in the insects, even between populations feeding on the same plant organ.

The data in Table 2 show analyses of the cardenolide content of milkweed bugs reared from egg to adult in the laboratory on seed from different species of *Asclepias*. *O. fasciatus* and *L. k. kalmii* sequester and store small quantities of cardenolides from *A. fascicularis*, moderate amounts from *A. erosa*, *A. cordifolia* and *A. eriocarpa*, and relatively large quantities from *A. californica greenei* and *A. vestita vestita*. There is a positive correlation between cardenolide content of laboratory-reared insects and the cardenolide content of the seeds of their respective host species (Table 2). The overall range in cardenolide content of the wild insects falls within the range of cardenolide content in laboratory-reared bugs.

The data in Table 3 suggest another source of variability in the insects: temporal and geographical intraspecific differences in the cardenolide content of milkweed seed. For example, the cardenolide content of seeds of *A. erosa*, collected in the same area, but in two different years, varies considerably, as does the content of seeds of *A. californica greenei*, collected in the same season from two geographically isolated populations.

However, the two samples of *A. fascicularis* collected in 1975 have similar

TABLE 1. GROSS CARDENOLIDE CONTENT OF WILD *O. fasciatus* AND *L. k. kalmii* COLLECTED ON *Asclepias* SPP. IN CENTRAL CALIFORNIA IN 1976

Milkweed species[a]	Collection data[b]			Cardenolide content[c] (μg per insect) Mean ± SE	*N*
Oncopeltus fasciatus					
A. californica greenei Woodson	Fresno	May	pod	5.5 ± 5.5	6
A. eriocarpa Benth.	Fresno	May	umbel	20 ± 8.8	9
	Fresno	July	umbel	107 ± 16	9
	Fresno	August	pod	82 ± 8.3	9
	Fresno	August	stem	15 ± 7.0	6
A. erosa Torrey	Tulare	July	pod	159 ± 26	9
A. fascicularis Dcne.	Napa	June	umbel	37 ± 7.7	9
	Fresno	July	pod	61 ± 10	9
	Tulare	July	pod	13 ± 8.3	9
	Yolo	August	pod	11 ± 2.8	9
	Solano	August	pod	18 ± 5.4	9
A. vestita vestita Hook & Arn.	Fresno	May	umbel	17 ± 14	6
	Fresno	July	pod	195 ± 32	9
Lygaeus kalmii kalmii					
A. californica greenei	Contra Costa	May	umbel	5.6 ± 4.3	9
A. cordifolia Jepson	Placer	June	pod	3.3 ± 1.7	3
	Napa	June	umbel	nil	2
A. eriocarpa	San Luis Obispo	June	umbel	nil	2
	Yolo	June	umbel	17 ± 8.4	6
	Fresno	July	umbel	152 ± 13	3
	Fresno	July	umbel	162 ± 22	4
	Yolo	August	umbel	93 ± 23	7
	Fresno	August	stem	116 ± 23	9
A. erosa	Tulare	July	umbel	74 ± 23	9
	Tulare	August	pod	205 ± 31	8
A. fascicularis	Yolo	August	pod	5.7 ± 1.4	6
A. v. vestita	San Luis Obispo[d]	June	pod	51 ± 23	6
	Fresno	July	pod	256 ± 35	9

[a] Voucher specimens of flowers of the milkweed species are in the possession of M.B.I.
[b] County in California, month of collection and part of plant on which insects were predominantly feeding.
[c] *N* = no. of insects assayed from site, SE = standard error of the mean.
[d] Plants at this locality were taxonomically between *A. v. vestita* and *A. v. parishii* Woodson.

TABLE 2. GROSS CARDENOLIDE CONTENT OF *O. fasciatus* AND *L. k. kalmii* REARED IN THE LABORATORY ON MILKWEED SEED

Milkweed species	Collection data[a]		Seed[b]	*O. fasciatus*[c] Mean±SE	*N*	*L. k. kalmii* Mean±SE	*N*
A. californica greenei	Contra Costa	1976	4.3	251±29	9	419±92	6
A. cordifolia	Placer	1974	1.3	125±19	9	78±14	9
A. eriocarpa	Fresno	1975	3.4	149±13	9	219±42	9
A. erosa	Tulare	1974	1.9	151±23	9	268±58	9
A. fascicularis	Napa	1975	0.4	34±5.1	9	50±19	9
A. vestita vestita	Fresno	1976	7.1	281±20	18	765±72	9

[a] Californian collection data for milkweed seed fed to insects.
[b] Mg per g dry seed; data from Table 3.
[c] μg equivalents of digitoxin per insect: *N* = no. of insects in each sample, SE = standard error of the mean.

cardenolide content, whereas the 1976 sample from adjacent Yolo County contains a somewhat lower amount of cardenolide than the former two.

For lygaeids feeding on *Asclepias* in central California, only two of the host species may be considered to be different from the others, in an ecological sense. These two species are *A. fascicularis* (ranging from 0.25 to 0.44 mg/g of seed), and *A. v. vestita* (7.1 mg/g for the single sample analyzed). Cardenolide contents of the six other species examined fall between 1.0 and 4.3 mg/g.

Variation also exists in the cardenolide content found in the different organs of a single host-plant species (Table 4). However, a knowledge of the organ on which the insects feed cannot necessarily be used to predict the cardenolide content of the insects, as there is no set relationship between the relative amounts of cardenolide present and the plant organs, at least in the few species examined here. Umbels contained the greatest concentrations of cardenolides in two of the species examined (*A. fascicularis* and *A. erosa*), while the umbels contained the lowest concentrations of cardenolides in two other species examined (*A. eriocarpa* and *A. v. vestita*).

Finally, there is evidence of temporal variation in the cardenolide content of the lygaeids (Table 5). Adults of *O. fasciatus* collected in Fresno County in May from three broad-leaf milkweed species (*A. californica greenei*, *A. eriocarpa*, and *A. v. vestita*) all contained small quantities of cardenolides; adult insects collected on *A. eriocarpa* and *A. v. vestita* later in the season from the same localities had much higher levels of cardenolides in their bodies. On the other hand, this trend did not exist among adults of *O. fasciatus* collected on *A. fascicularis* from June through August.

TABLE 3. GROSS CARDENOLIDE CONTENT OF SEED OF *Asclepias* SPP. FROM CALIFORNIA

Milkweed species	Collection data[a]		Cardenolide content[b] Mean ± SE	N[c]
A. californica greenei	Contra Costa	1976	4.3 ± 0.40	5
	Fresno	1976	3.1 ± 0.08	3
A. cordifolia	Napa	1976	3.1 ± 0.15	3
	Placer	1974	1.3 ± 0.05	2
A. eriocarpa	Fresno	1976	2.4 ± 0.07	5
	Yolo	1976	1.3	1
	Fresno	1975	3.4 ± 0.57	3
A. erosa	Tulare	1976	2.4 ± 0.22	3
	Tulare	1974	1.9 ± 0.14	5
A. fascicularis	Yolo	1976	0.25 ± 0.03	3
	Napa	1975	0.44 ± 0.04	5
	Solano	1975	0.44 ± 0.04	3
A. solanoana Woodson	Tehama	1973	1.2 ± 0.04	3
A. speciosa Torrey	Solano	1976	2.7 ± 0.15	3
	B.C.[d]	1975	1.8 ± 0.03	2
A. vestita vestita	Fresno	1976	7.1 ± 0.29	5

[a] Californian collection data for milkweed seeds.
[b] Mg equivalents of digitoxin per g of dried seed.
[c] For each collection locality, seeds from several plants were pooled and 1.0 g of seed ground to a powder; sample size N is the number of 0.2-g aliquots taken from the 1-g sample.
[d] Cache Creek, British Columbia, Canada.

Therefore, if temporal variation in the cardenolide content of the insects is the result of some aspect of the phenologies of their respective hosts (e.g., availability of pods and seed), this situation is limited to the cardenolide-rich broad-leaf species of milkweed in California.

The colonizing adults of *O. fasciatus*, which migrate into California every year from southern sites of overwintering, all contained relatively low levels of cardenolides (Table 5). Thin-layer chromatographic analyses (unpublished data) of the cardenolide profiles of these insects suggest that their cardenolides were probably accumulated from the host plant on which the insects were collected, rather than from a host on which they overwintered. If this is the case, these immigrants may have lacked cardenolides until they colonized the milkweeds on which they were found.

TABLE 4. GROSS CARDENOLIDE CONTENT OF PLANT SAMPLES (*Asclepias*) FROM CENTRAL CALIFORNIA

Milkweed species	Locality	Cardenolide content[a]		
		Leaf	Umbel	Seed[b]
A. californica greenei	Contra Costa			4.3
	Fresno	0.27		
A. eriocarpa	Fresno	3.7	5.5	3.4
	Fresno			2.4
	Yolo		1.7	1.3
A. erosa	Tulare	3.0	0.89	2.4
	Tulare			1.9
A. fascicularis	Yolo	0.22	0.73	0.25
	Fresno		1.0	
	Napa			0.44
A. v. vestita	Fresno	6.8	2.6	7.1

[a] Mg equivalents of digitoxin per g of dried tissue; data for leaf and umbel represents single estimations.
[b] Data from Table 3.

TABLE 5. TEMPORAL VARIATION IN GROSS CARDENOLIDE CONTENT OF FIELD CAUGHT *O. fasciatus* FROM CENTRAL CALIFORNIA

Milkweed species	Cardenolide content[a]		
	May–June[b]	July–August[b]	Lab reared[c]
A. californica greenei	5.5		251
A. eriocarpa	20	107, 82	149
A. erosa		159	151
A. fascicularis	37	61, 13, 11, 18	34
A. v. vestita	17	195	281

[a] Mean in μg equivalents of digitoxin per insect.
[b] Data from Table 1.
[c] Reared from egg to adult exclusively on seed from respective milkweed species; data from Table 2.

DISCUSSION

Larvae of the ground-dwelling lygaeid *L. k. kalmii* develop successfully on diets other than milkweed seed, although this latter food source enhances

development (R.B. Root, personal communication). In contrast, larvae of the European lygaeid *L. equestris* L. develop faster on seeds of nonasclepiadaceous plants compared to a diet of seeds of *Cynanchum vinetoxicum*, the sole asclepiadaceous host in the northern end of this insect's range (Kugelberg, 1973).

L. k. kalmii colonizes most of the Californian species of *Asclepias* in the spring, the insect remaining at low densities until seed pods develop on the plants later in the season. Because these bugs have the ability to develop on plants other than milkweed, a proportion of the insect population may not have had the opportunity to sequester and store cardenolides.

On the other hand, the host plant specificity of *O. fasciatus* in California is restricted to members of the genus *Asclepias*. We have seen this lygaeid in California on five species of milkweed (*A. californica greenei*, *A. eriocarpa*, *A. erosa*, *A. fascicularis*, and *A. v. vestita*), and our laboratory-rearing study suggests that at least two other western species (*A. cordifolia* and *A. speciosa*) will also support larval development and adult reproduction.

Determination of the cardenolide content of laboratory seed-reared *O. fasciatus* and their conspecifics collected in the field in midsummer on the same broad-leaf milkweed species (i.e., those species we have referred to with the exception of *A. fascicularis*) shows that *O. fasciatus* can usually sequester more that 100 μg of cardenolide per insect from these hosts. However, the laboratory seed-reared insects often contain more cardenolide than insects collected in the field in midsummer on the same host, suggesting that bugs in the natural environment may not feed exclusively on seed, even when it is available. Alternately, the thick pod wall of several species of *Asclepias* may prevent lygaeids from feeding on the seeds until the earliest maturing pods begin to dehisce (Ralph, 1976).

In central California, *O. fasciatus* can immigrate several weeks before milkweed seed pods have begun to develop on some host-plant species. Before pods and seeds are available, *O. fasciatus* feeds almost exclusively on developing flower buds and open flowers (K. Evans, personal communication; personal observation). Our study has shown that for at least four of the native species of *Asclepias* in California, the umbels (buds and flowers) contain relatively large quantities of cardenolides, yet in most areas, the bugs do not sequester proportionally large quantities of cardenolides from this food source.

Although the umbels may contain high levels of cardenolides, Ralph (1976) has recently reported that *O. fasciatus* feeding on open *A. syriaca* L. flowers does not usually probe and ingest material from the ovary, but instead appears to feed on the nectar, which may well lack cardenolides entirely. Thus, the availability of seed of the host plant may be critical in order for *O. fasciatus* to sequester significant amounts of cardenolides.

In contrast, the higher relative cardenolide content of *L. k. kalmii* feeding on umbels and stems of *Asclepias* may indicate that this species is more capable of growth and reproduction on vegetative parts of the milkweed plant than is *O. fasciatus*.

In the Napa Valley and adjacent Yolo and Solano counties in central California, the species diversity index of *Asclepias* varies through the season, yet only *A. fascicularis* is colonized annually by immigrating *O. fasciatus*. It is on this host plant that large populations build up over the summer months (K. Evans, unpublished results). This study has shown that individuals of *O. fasciatus* on *A. fascicularis* contain relatively small amounts of cardenolides in their bodies, reflective of the cardenolide content of this host plant. Therefore, our findings suggest that *O. fasciatus* in this part of its geographical range does not maximize its opportunities to sequester large quantities of cardenolides for it does not colonize the species of *Asclepias* which are richer in cardenolide content.

We have shown that the lygaeid bugs *O. fasciatus* and *L. k. kalmii* in central California contain varied quantities of cardenolides. The role of cardenolides in the milkweed-feeding lygaeids remains largely in question. We must again emphasize that only when the natural predators of these insects are known will we be able to test the effectiveness of cardenolides as a potential chemical defense agent in these insects. Because it is not known what level of cardenolide content in these insects is required to cause an emetic or distressful response in a predator, the full implication of this variation cannot yet be established.

However, in examining the emetic response of the blue jay (*Cyanocitta cristata*) to the monarch butterfly (*D. plexippus*) reared on the neotropical milkweed *A. curassavica*, Brower et al. (1975) reported that one emetic dose (ED_{50}) required between 68 and 84 μg equivalents of digitoxin for an 85-g bird. Additional calculations from the data of Brower and Glazier (1975) indicate that for the abdomen of such a butterfly, a single ED_{50} consists of 44–54 μg of cardenolide, whereas a single ED_{50} from the cardenolides stored in the wings of the same insect requires 232–238 μg.

It has been established that owing to physiological barriers in vertebrates, polar cardenolides tend to have lower emetic potencies than do nonpolar ones, when administered via the oral route (see Duffey, 1976, for a complete discussion).

Preliminary studies (Parsons, 1965; Reichstein et al., 1968) have shown that the principal cardenolides stored by the monarch reared on *A. curassavica* are the relatively nonpolar chemicals calotropin and calactin, which would account for the relatively high emetic potential of the whole butterfly (68–84 $\mu g/ED_{50}$) compared to that of the wings (232–238 $\mu g/ED_{50}$).

It is conceivable that the lower emeticity of the cardenolides in the wings

of the monarch butterflies may be owing to the presence of a larger proportion of polar cardenolides in these organs in comparison to the cardenolide compliment of the emetically potent abdomen. Thus the monarch may partition its cardenolides in a fashion similar to *O. fasciatus* which preferentially sequesters more polar compounds in the dorsolateral space fluid, while more lipophilic cardenolides occur in the fat body (Duffey, unpublished results).

Our examination of whole-body extracts of field-collected *O. fasciatus* and *L. k. kalmii* shows that almost all of the cardenolides stored in these insects are more polar than calotropin or calactin. Should these polar cardenolides derived by lygaeids from the Californian species of *Asclepias* be found to have somewhat similar pharmacological properties to the cardenolide compliment in the wings of the monarch butterfly reared on *A. curassavica*, then it would seem possible that few, if any of the individual lygaeids collected in this study contained enough cardenolide of the required polarity to elicit an emetic response from a predator with a sensitivity similar to the blue jay. Roughly 50% of the field-caught milkweed bugs that we examined were found to contain less than 50 μg of cardenolide per insect.

Even if the adults of *O. fasciatus* and *L. k. kalmii* with greater than 100 μg per insect are found to have some chemical protection from predators, our data suggest that the effectiveness of cardenolide sequestration as a chemical defense strategy may vary over the geographical range of these species and may vary in the course of a season at a particular location.

Various investigations have shown that concentrations of secondary plant compounds vary both inter- and intraspecifically in plants, and it has been suggested that such differences could give rise to variation in the content of plant-derived toxins stored by insects (Aplin and Rothschild, 1971; Duffey and Scudder, 1972). We have established that this situation exists among adults of *O. fasciatus* and *L. k. kalmii* feeding on *Asclepias* in central California.

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PRODUCTION OF α-FARNESENE, AN ATTRACTANT AND OVIPOSITION STIMULANT FOR CODLING MOTH, BY DEVELOPING FRUIT OF TEN VARIETIES OF APPLE

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Abstract—α-Farnesene levels in the outer coating of developing fruit of ten varieties of apple were monitored at fortnightly intervals from fruit set to harvest. The compound was present in all varieties and the total amount on individual fruit increased with increasing fruit size. However, varieties differed in the levels of α-farnesene/unit area of apple skin, with two peaks evident in some varieties during the season. The relationship between varietal and seasonal variation in α-farnesene production and the ecology and behavior of codling moth adults and larvae is discussed.

Key Words—*Laspeyresia pomonella*, codling moth, apple, α-farnesene, oviposition stimulant, larval attractant, behavioral ecology.

INTRODUCTION

The sesquiterpene α-farnesene (3,7,11-trimethyldodeca-1,3,6,10-tetraene) is present in the natural outer coating of apples. Some years ago, the compound aroused considerable interest as it was believed to be implicated in the appearance of superficial scald, a disorder of stored apples (Murray et al., 1964; Huelin and Murray, 1966; Meigh and Filmer, 1967; Huelin and Coggiola, 1968). We have found that α-farnesene affects the behavior of codling moth (*Laspeyresia pomonella*) larvae and adults. (*E,E*)- and (*Z,E*)-α-farnesene, the two isomers which occur naturally (Anet, 1970), are olfactory attractants

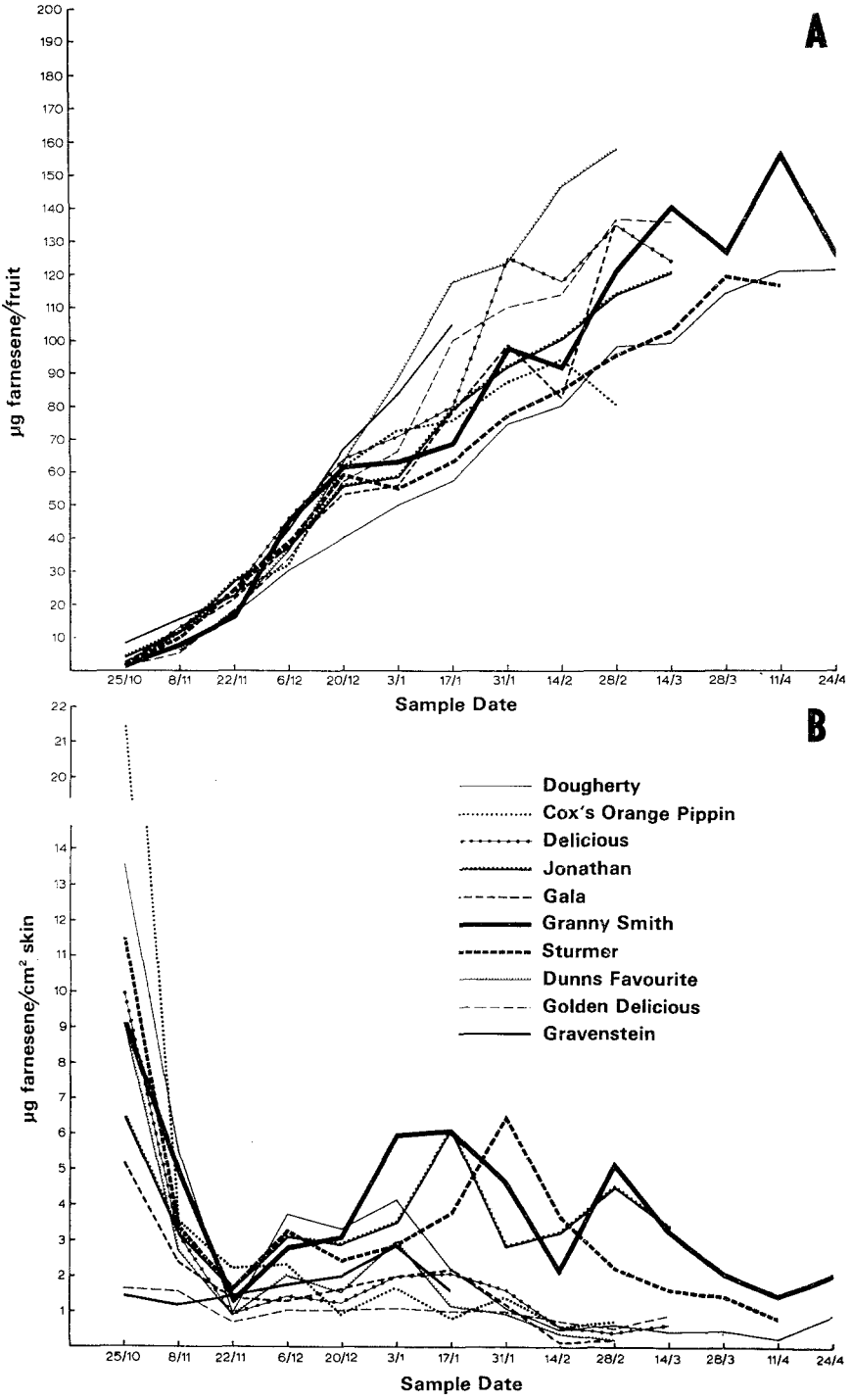
for newly hatched codling moth larvae and oviposition stimulants for gravid female moths (Sutherland, 1972; Sutherland and Hutchins, 1972, 1973; Wearing and Hutchins, 1973). Since then, Russ (1976) has shown that apples are more attractive to neonate codling larvae than pears and that in each case the attractiveness of the fruit varies with fruit development. Russ tentatively concludes that different stages of the fruit have different amounts of the attractant. In order to determine whether α-farnesene might have a role in influencing the extent of codling moth damage to apples, it was important to study the production of α-farnesene by developing fruit and to determine whether it was related to the life cycle of the pest.

METHODS AND MATERIALS

Samples of developing fruit were collected at random from single marked trees of each of ten varieties of apple from October to April 1972–1973 and 1973–1974. The trees were located in the D.S.I.R. research orchard at Appleby, Nelson, New Zealand. Fruits were collected at fortnightly intervals from fruit set. Care was taken to ensure that each sample comprised fruit picked throughout the tree. On the first sampling occasion, a 20-g weighed sample of set fruits was taken. On subsequent occasions, as the fruit matured, 20, 10, and eventually 5 individual fruit were collected. Samples were taken during the afternoon and were weighed and extracted with solvent within 6 hr. Very small fruit of each variety were completely immersed in an excess of redistilled chloroform for 1 min. Fruit exceeding 2 cm diameter were dipped for 1 min into three successive chloroform baths. These were combined and concentrated by rotary evaporation under reduced pressure at $<35°C$. Because no other hydrocarbons with conjugated double bonds are present in the outer coating of apples (Huelin and Coggiola, 1968; Murray, 1969), α-farnesene can be readily detected and quantitatively assayed by UV absorption (Huelin and Coggiola, 1968), and we followed their method.

α-Farnesene influences the behavior of female codling moths, which are stimulated to lay eggs by the odor of whole fruits. For this reason we measured overall levels of the chemical in apples. But α-farnesene also acts as a short-range oviposition stimulant for gravid moths and as an attractant for newly hatched larvae. In this regard, the amount per unit area of skin is likely to be of greatest importance. Apple surface areas were estimated by a method based on the surface of revolution of measured profiles (Galbreath, 1976).

FIG. 1. α-Farnesene levels on fruit of ten varieties of apple measured at fortnightly intervals from fruit set to harvest: (A) per fruit; (B) per cm^2 skin.


A
µg farnesene/fruit
200
190
180
170
160
150
140
130
120
110
100
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80
70
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50
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30
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10
25/10
8/11
22/11
6/12
20/12
3/1
17/1
31/1
14/2
28/2
14/3
28/3
11/4
24/4
Sample Date
B
µg farnesene/cm² skin
22
21
20
14
13
12
11
10
9
8
7
6
5
4
3
2
1
Dougherty
Cox's Orange Pippin
Delicious
Jonathan
Gala
Granny Smith
Sturmer
Dunns Favourite
Golden Delicious
Gravenstein
25/10
8/11
22/11
6/12
20/12
3/1
17/1
31/1
14/2
28/2
14/3
28/3
11/4
24/4
Sample Date

RESULTS

Samples from all varieties showed a steady increase in the α-farnesene content per apple as the season progressed with a leveling off approaching harvest (Figure 1A). This increase reflected the increasing size of the fruit, and this factor accounted for much of the difference between levels in different varieties. By comparing Figures 1A and B, for example, it can be seen that the three varieties with consistently high farnesene levels per apple, Dunns Favourite, Gravenstein, and Golden Delicious, were those which produced the largest fruit but did not have high production of α-farnesene per unit area. Nevertheless, in all cases, there was at least a 20-fold increase in the total α-farnesene level during fruit maturation and therefore a concomitant increase in the amount of the chemical present in each tree.

The pattern of α-farnesene levels per unit area of apple skin was more complicated (Figure 1B). A general pattern emerged in which high levels were initially found in newly set fruit. These levels rapidly diminished over the next 4 weeks, followed by a gradual build-up and second peak in January, after which a second decline was evident. But not all varieties followed this pattern equally. Subsequent to the low levels recorded in November, two main groupings of varieties were apparent: those with a high peak in January (Granny Smith, Jonathan, and Sturmer) and those with considerably lower and less sustained second peaks (Dougherty, Dunns, Gravenstein, Gala, and Delicious). Cox's Orange had an exceptionally high level of α-farnesene in the first fruit sample, but no apparent second peak, and the levels for Golden Delicious were low throughout. The low level of farnesene in Gravenstein on October 25 was almost certainly due to the longer period since flowering for this early variety. Only 19 Gravenstein fruit were required on this date to make the 20-g sample compared with from 30 to over 100 of the other varieties.

DISCUSSION

In Eurasia, where the association between *L. pomonella* and its apple hosts (*Malus* spp.) evolved, the life history of the moth varies with latitude and temperature. At higher latitudes the moth is univoltine, whereas at lower latitudes two or more generations are completed annually (Shel'deshova, 1967; Russ, 1966). A similar variation in voltinism with latitude occurs in the southern hemisphere (Geier, 1963; Wearing, 1974). Furthermore, as Shel'deshova (1967) showed, the life history of the codling moth at 44°S in New Zealand parallels that at 45°N in the North Caucasus. It appears, therefore, that the relationship between the pest and the phenology of its host in the two regions is very similar.

In the north of New Zealand overwintering larvae pupate and adults emerge from mid-October through November. The second generation of moths reaches a peak during the month of January and some third generation moths may emerge in February/March. In Nelson, New Zealand, moths emerge from late October onwards and peak population and oviposition is reached in December/January (Wood, 1965). Comparison of these events with Figure 1B suggests that the times of maximum moth flight, oviposition, and larval hatching occur at the two peaks of production of α-farnesene per unit surface area of fruit.

The first peak during and immediately after flowering does not result in large quantities of α-farnesene per fruit. It has been found that females emerging at this time lay only about 10% of their eggs on the young fruitlets (Wearing, unpublished data). Nevertheless, Hattingh (1942) reported that codling moths ovipositing on pear trees during October preferred flower clusters in the most advanced stages of development (full bloom to fruit set) rather than the younger stages, and Nel (1941) stated that early in the season, those varieties that are blossoming and setting fruit are "preferred" for oviposition to those in which the fruit is further advanced. It is possible that this is at least partially in response to the early production of α-farnesene during and immediately following flowering. In any event, the steep odor gradients of the attractant early in the season may be critical for short-range fruit location by neonate larvae which hatch mainly within 10 cm of the fruit (Geier, 1963).

The second peak of α-farnesene production in December/January (Figure 1B) coincides with the periods of maximum flight, oviposition, and larval hatching in both northern and southern areas of New Zealand. The total α-farnesene level on the fruit steadily increases during fruit growth and maturation (Figure 1A) and is therefore much higher at the time of the second peak in production than early in the season. There seems little doubt that the presence of increasing quantities of an effective oviposition stimulant on the fruit is important. Wearing et al. (1973) showed that both moth flight activity and oviposition are stimulated by the odor of whole apples, and several observers have recorded a greater proportion of eggs being laid directly on pome fruits as maturation proceeds (Pettey, 1926; Hall, 1928; Hattingh, 1943).

Notwithstanding the increased proportion of eggs laid directly on the fruit at this time, about 55% of newly hatched larvae must still locate the fruit after hatching on leaves and twigs nearby (Wearing et al., 1973), so steep gradients of α-farnesene close to the fruit surface would continue to be important for larval orientation. Laboratory bioassays showed that larvae were indeed attracted to immature whole fruit at this stage in the season (Sutherland, unpublished data).

Whether or not codling moths are attracted by the odor of trees with developing fruit, or whether they locate trees by undirected flight is unknown, but the contribution of α-farnesene to tree odor would certainly increase as the season progresses. Chugunin (1931) noted greatest codling moth infestation in apple trees with the largest crowns and the most fruit, particularly in the second generation.

The assessment of the susceptibility of apple variations to codling moth attack in the field is fraught with difficulties because of the many variables affecting the final crop infestation by this pest. On the basis of the data given here, it is tempting to correlate varietal character, in this case the production of a chemical implicated in host plant location, with published reports on varietal susceptibility to codling moth, but attempts to do this have yielded many contradictions (Cutright and Morrison, 1935). However, our data clearly showed that fruit size was the main determinant of α-farnesene content per fruit, and in this respect both Chugunin (1931) and Cutright and Morrison (1935) concluded that varieties of apples and pears producing the larger fruits were more susceptible to codling moth attack.

Field evidence therefore supports the view that the seasonal changes in α-farnesene production per apple may influence oviposition behavior of the moth, particularly the distribution of eggs. However, the significance of the correlation between the timing of seasonal peaks of α-farnesene production per unit area of fruit surface and moth emergence and larval hatching is more difficult to substantiate with field observations. This relationship must be regarded as tenuous in view of the variability both of the codling moth life cycle and the pattern of α-farnesene production between apple varieties. Confirmation of the relationship would depend on a close study of the life history of bivoltine codling moth and α-farnesene production on one apple variety or species on a site where the insect and host plant have been long associated.

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EFFECTS OF *Hymenaea courbaril* LEAF RESIN ON THE GENERALIST HERBIVORE *Spodoptera exigua* (BEET ARMYWORM)

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Abstract—The hypothesis that leaf resin of *Hymenaea courbaril* (Leguminosae) functions as a defence against herbivory was tested in a series of experiments with the generalist herbivore, beet armyworm (*Spodoptera exigua* Hübn). Pure leaf resin obtained by steam distillation was incorporated into an artificial diet at 0.0%, 0.16%, 0.50%, and 1.60% (dry weight) in one experiment and 0.0%, 1.0%, and 3.2% (dry weight) in a second experiment; *S. exigua* larvae were reared on these diets. In four palatability experiments the amount of leaf area eaten was compared between pairs of different leaf material. Mortality due to viral infection of larvae in the first feeding experiment showed a dose-response to leaf resin concentration. In the second experiment larvae showed a dose-response in the reduction of pupal weight and delay of time to pupation. These are interpreted as a dose-related stress on *S. exigua* which occurs with increased resin concentration in the diet. In the palatability tests, *S. exigua* strongly preferred untreated to resin-treated bean leaf disks, bean disks to *H. courbaril* immature leaf disks, *H. courbaril* immature leaf to *H. courbaril* mature leaf, and bean leaf disks to *H. courbaril* newly emerged leaf disks. From these results, we conclude that *H. courbaril* leaf resins possess toxic and feeding-deterrent properties which make them effective as an herbivore defense.

Key Words—*Hymenaea courbaril*, Leguminosae, leaf resin, sesquiterpenes, plant defense, herbivore, *Spodoptera exigua*, feeding deterrent, plant toxin, allomones.

INTRODUCTION

Resin production in plants has been studied primarily in commercially valuable temperate-zone conifers (von Rudloff, 1975). A survey of resins

through geologic time, however, has indicated the importance of tropical angiosperms as resin producers and the possible significant role of tropical environments in the evolution of resin-producing plants (Langenheim, 1964, 1969a, b, 1975). In order to examine the role of tropical conditions in this evolutionary process, a long-range study was initiated with the tropical leguminous tree, *Hymenaea* (Langenheim, 1967). This genus was chosen for study, in part, because it was a member of the Leguminosae, one of the most important resin-producing groups, and because leguminous trees are prominent components of tropical ecosystems (Langenheim, 1972, 1973). *Hymenaea* is a genus comprised of 14 species with 13 occurring in the New World and one in Africa (Lee and Langenheim, 1975). One of the New World species, *H. courbaril*, spans the latitudinal range of the tropics and occurs in all major tropical lowland ecosystem types. In this genus, three distinct resin systems occur in different organs of the tree: trunk, fruit, and leaves (Langenheim, 1967, 1969). Recent work in this study has focused on the leaf resin system, because the chemistry is comparatively simple and because it is possible to do experimental work with this system in seedlings. Also, selective pressures would be expected to be intense for seedlings. Leaf resin in *Hymenaea* consists of 17 different sesquiterpene hydrocarbons, and it is secreted into schizogenous pockets distributed throughout the leaf (Martin et al., 1972). A high degree of intra- and interspecific quantitative variation occurs in the leaf resin composition (Martin et al., 1974, 1976; Langenheim et al., 1977), and the phenotypic plasticity of quantitative composition is extremely low (Stubblebine et al., 1976). These terpenes are metabolically and energetically very expensive compounds to produce; however, many terpenoids also have toxic or repellent effects on herbivores and pathogenic fungi (Levin, 1976).

From these considerations, it is reasonable to hypothesize that the terpenoid resin systems in *Hymenaea* evolved as defenses in response to selection by herbivores. Such a hypothesis would be supported by the observation that herbivory in tropical regions appears to be more intense than in temperate areas (Levin, 1975) as well as the correlation between the evolution of resin production and tropical environments (Langenheim, 1969, 1975). A similar logic applies to variation in resin composition. It is more difficult to envision physical environmental factors acting as selective agents than it is to use models in which herbivores act as selective agents. Again, this would be especially true for many tropical areas where insect herbivores are particularly diverse (Janzen, 1973a,b; Janzen and Schoener, 1968; Elton, 1973).

If herbivores are to act as strong selective forces in maintaining the diversity of quantitative compositions of *Hymenaea*, there are two major conditions which must be fulfilled: (1) the resin must deter the feeding of some herbivores which would otherwise consume large quantities of *Hymenaea*

leaves, and (2) different quantitative composition of leaf resins must have differential deterrent effects on some of these herbivores. If both of these conditions are true for at least a few potentially important herbivores of *Hymenaea*, then geographic and temporal variation in herbivore populations could select for diversity in quantitative composition of *Hymenaea* leaf resin. This selective process would be greatly facilititated by the low phenolytic plasticity of quantitative leaf resin composition.

Because the first of these conditions is independent of the second, but the second one is dependent on the first, we have initiated our studies by an examination of the first condition with a well-studied laboratory herbivore. We chose a widespread super generalist, the beet armyworm *Spodoptera exigua* Hübn. (Lepidoptera: Noctuidae), which is known to be successful on numerous diverse and taxonomically unrelated crops, including the monoterpenoid-producing genera *Citrus* and *Mentha* (Atkins, 1960; Singh and Chibber, 1971). Also, because this species is widely used in laboratory studies, rearing procedures and diets had been developed previously (Shorey and Hale, 1965), and excellent background literature on this organism exists. This is important, because it often requires years of trial and error to develop suitable rearing diets and procedures for many insects. Because this herbivore has a cosmopolitan distribution as well as an extremely diverse host range (including essential-oil producers and several legumes), any deterrent effect of the *Hymenaea* leaf resin shown for beet armyworm would also tend to indicate an effect for other generalist herbivores which might attack *Hymenaea* under natural conditions.

To test this first condition on beet armyworm we have performed two different types of experiments. One type is a no-choice feeding design in which larvae are raised on artificial diet containing known amounts of purified *H. courbaril* leaf resin. The second type is a preference design in which the palatabilities of various test materials are measured relative to each other.

METHODS AND MATERIALS

The major sesquiterpenes of *Hymenaea* leaf resin have been identified by spectroscopy (Martin et al., 1972), and subsequently methods were developed to assay these major constituents by GLC retention time. The composition of the resin of *H. courbaril* used in the experiments is shown in Table 1. Resin was extracted from two large samples of fresh leaves of *H. courbaril* collected from saplings grown in the greenhouse at the University of California, Santa Cruz (UCSC), from seeds collected from Villa Colón, Costa Rica (JHL 5057). The resin was extracted by steam distillation in several batches using a Likens-Nickerson apparatus (Maarse and Kepner, 1970). This

TABLE 1. QUANTITATIVE COMPOSITIONS OF PURIFIED *H. coubaril* LEAF RESIN USED IN FEEDING EXPERIMENTS AND PALATABILITY EXPERIMENT 1. ($R_c \equiv$ GLC RETENTION TIME RELATIVE TO CARYOPHYLLENE)

Compound	R_c	Feeding experiment 1 and palatability experiment 1	Feeding experiment 2
α-Cubebene	0.70	trace	1.3
α-Copaene	0.76	0.8	5.7
Unknown	0.85	6.3	4.7
β-Copaene	0.95	5.4	6.4
Caryophyllene	1.00	39.5	34.7
α- and β-Humulene	1.22	8.5	5.6
γ-Muurolene	1.25	1.8	3.8
β-Selinene	1.36	4.6	10.5
α-Selinene	1.41	24.2	17.9
δ-Cadinene	1.53	4.2	6.0

method is superior to cold extraction methods, because distillation cleanly separates the resin from the leaf pigments and the large amounts of leaf waxes which are unavoidably extracted by cold methods.

Fresh leaves were ground in a Waring blender with distilled water and added to the 1-liter sample flask of the distillation apparatus. The distillation was initiated with 500 ml of aqueous leaf mash in the sample flask and 50 ml of high purity *n*-pentane in the collection flask. The clear pentane extract was removed from the collection flask and dried by filtering over anhydrous Na_2SO_4. It was then concentrated using a microvigreau condenser and a water bath at 50°C. Extractions from each large sample were pooled into two accumulation flasks each containing a small amount of pyrogallol to absorb O_2, concentrated again, and stored in the freezer under N_2. After all the extractions for each sample were completed, the remaining pentane solvent was removed by rotoevaporation under high vacuum. The purified resin was then stored in the freezer with pyrogallol and under N_2.

Leaf resin composition of each large sample was monitored by gas–liquid chromatography (GLC) during three stages of the extraction procedures. Leaves subsampled from the main leaf sample were extracted under pentane in a mortar on ice. The mortar contained small amounts of Standard Ottawa Sand and granular anhydrous Na_2SO_4. The crude extract was filtered Na_2SO_4 and concentrated under a slow stream of high purity N_2. This extract was analyzed by GLC and compared with analyses of the distilled extract which were made before and after the pentane was removed by rotoevapora-

tion. Differences between these three analyses were very small and well within experimental error of the analytical methods.

GLC analyses were performed on a Perkin-Elmer 880 gas chromatograph with a flame ionization detector (FID). The GLC was fitted for on column injection with a 24 ft × 1/8 in (OD) aluminium column packed with 2% Carbowax 20M on Gas Chrom Q 100/120 mesh. Oven temperature was 130°C, and both injector and detector temperatures were maintained at 40°C above the oven temperature. Carrier gas (N_2) flow rate was 25 ml/min. Integration of GLC peaks was achieved by an electronic integrator designed and constructed at UCSC. Calculations of GLC data were performed on a PDP 11-45 computer. Results with packed aluminium columns have been compared to analyses run on a glass capillary column using a standard *Hymenaea* leaf resin sample. This comparison showed a greater efficiency in separation on the glass capillary column; however, there is no evidence from this that any compounds were being altered by GLC analysis with packed aluminium columns.

Adult beet armyworm moths, *S. exigua* Hübn., were collected at night in late August 1975 with a UV lamp in cotton fields near Corcoran, California. These adults were used to start a colony of beet armyworm which was maintained for one year at UCSC. Moths were mated in a 16 × 16 × 16-in. well-ventilated cage covered with glass. The cage was kept in a growth chamber with a photoperiod of 14 hr and relative humidity between 50 and 60%. Temperature for most of the year was kept between 24 and 26°C until it was found that a constant temperature of 20° produced healthier larvae.

Moths in the cage were fed a 5% sucrose solution and crumpled wax paper was provided for hiding as well as a surface on which females could lay eggs. Egg masses were cut out of wax paper and several placed in one 8-oz Dixie Cup (Dixie Container #358, treated) containing a 1-cm layer of modified Shorey diet. These hatching cups were covered with a clear plastic lid (Dixie Lids #3068), the edges of which were sealed with a #62 Eberhard Faber rubber band. A 1-cm-diameter hole was punched in the lid with a cork borer and covered with organdy fabric taped to the lid with masking tape. This was found to be necessary in order to allow adequate gas exchange. When larvae were at least 0.5 cm long, they were transferred to rearing cups containing diet. These were the same design as the hatching cups except that gas exchange was provided by 1-mm holes punched near the edge of the lid. A maximum of 8 larvae were transferred to each rearing cup to reduce cannibalism. These cups were placed in the growth chamber with the mating cage and left undisturbed until the larvae had burrowed under the remaining diet, pupated, and emerged as moths. Moths were transferred to the cage within 24 hr after emerging.

The diet used was a modification of the Shorey bean diet (Shorey and

TABLE 2. SHOREY BEAN DIET (MODIFIED FROM SHOREY ET AL., 1965) USED IN FEEDING EXPERIMENTS AND IN MAINTENANCE OF *S. exigua* COLONY

Ingredient	Amount
Boiled soaked beans	145 g
Distilled H_2O	300 ml
Corn oil	2–4 ml
Dry yeast	32 g
Wheat germ	25 g
Me-*p*-OH-Benzoate	2 g
Sorbic acid	1 g
Ascorbic acid	4 g
Casein	30 g
Agar	12 g
Distilled H_2O	300 ml

Hale, 1965), and the ingredients are shown in Table 2. Beet armyworm is susceptible to nuclear polyhedrosis viruses (Chautani and Rehnborg, 1971) which cause the larvae to liquefy. As soon as acute symptoms were noticed, the cup containing the infected larvae was discarded. Virus continued to be a problem until changes were made in the diet and its preparation and a lower temperature (20°C) was used in the growth chamber. The changes in the diet were as follows: the amount of corn oil was increased from 2 to 4 ml, wheat germ was purchased on the same day that diet was prepared, dry yeast was also purchased more frequently, and diet was always made on the day it was to be used. Thus diet for hatching cups and rearing cups were made on different days. It was not until these changes were made in a coordinated manner between the first and second feeding experiments that all symptoms of virus disappeared. Before initiating the second experiment, egg masses were obtained from the University of California at Davis and added to the colony without the reappearance of virus symptoms.

Feeding Experiments with Artificial Diet

Experiment 1. S. exigua egg masses were collected 2 days apart and hatched on a modification of Shorey's diet (Table 2). When larvae were between 0.5 cm and 1 cm long they were placed in experimental rearing cups.

One larva was placed in each cup to prevent cannibalism and to facilitate daily observations of individual larvae. A total of 55 cups was divided into four groups. Each group contained diet with different concentrations of *H. courbaril* resin: 13 with no resin (controls), 15 with 0.16% resin, 13 with 0.50% resin, and 14 with 1.60% resin. All larvae in cups with resin diets and four of the larvae in cups with control diet came from one batch of egg masses obtained on the first day of collecting from the laboratory colony. The other nine larvae in the control cups came from egg masses collected 2 days later. All larvae from both batches of egg masses showed acute signs of viral infection where mortality among larvae not used in the experiment was much higher in the second group (more than 90%) than in the first group. The source of this virus is undoubtedly the moths which were collected in the field originally and such virus can be passed from one generation to the next through the eggs. Viruses are known to occur in several lepidopteran larvae in a latent condition and to cause acute symptoms only when the larvae are subjected to stress conditions such as high temperature, high humidity, or toxic chemicals in the diet (Steinhaus, 1956). The difference in the virus-related mortality between these two batches is probably due to slight differences in the environmental conditions under which they were hatched. All

TABLE 3. EFFECT OF *H. courbaril* LEAF RESIN CONCENTRATION ON *S. exigua* PUPAL WEIGHT IN TWO FEEDING EXPERIMENTS. MEAN PUPAL WEIGHT IN 3.2%-RESIN DIET DIFFERS SIGNIFICANTLY FROM CONTROL ($P<0.05$)

	Resin concentration			
A. Experiment 1	Control	0.16%	0.50%	1.60%
Mean pupal weight (mg)	103.0 (101.8)	98.1	103.3	105.0
Standard deviation	13.6 (11.4)	17.6	18.7	1.13
N	3 (4)	11	9	2

	Resin concentration		
B. Experiment 2	Control	1.0%	3.2%
Mean pupal weight (mg)	145.3	138.0	128.3
Standard deviation	16.1	14.7	18.5
N	12	12	12

experimental cups were placed in the growth chamber at 23°C with a 14-hr photoperiod. Surviving larvae which had not yet burrowed under the diet to pupate were weighed every third day and pupae were removed from the diet and weighed. Survival was measured by the number of moths which had emerged 4 weeks after the last viable larvae had pupated. Previous experience had shown this to be an adequate time to allow all viable moths to emerge.

The data for the control in this experiment were treated in two ways. One set of calculations was based on all 13 larvae in this group and another was based on the 4 larvae derived from the first collection of eggs. Although it may weaken the effect of the control, the 4 control larvae from the first collection of egg masses were much more representative of other larvae in the colony which were not acutely infected with virus.

Experiment 2. This feeding experiment was the same design as experiment 1 except that all larvae were obtained from one collection of egg masses and were free of viral symptoms; the resin concentrations in the diet were 0%, 1.0%, and 3.2% and the composition of this resin was considered to be sufficiently similar to that used in the first feeding experiment that this was not a large factor; there were 15 cups in the control and 3.2% concentration and 16 cups in the 1% concentration; the growth chamber temperature was 20°C, and larvae were weighed daily.

Palatability Experiments

Experiment 1. This experiment tested the palatability of bush bean leaves (*Phaseolus vulgaris* var. *humilis*) treated with *H. courbaril* leaf resin relative to the palatability of untreated leaves of the same bean plant. Disks 1 cm in diameter were punched from mature bean leaves with a cork borer. Control sandwiches were made by pressing the undersides of two disks together which were held together by trichomes on the underside of the leaves. Test sandwiches were made by painting a small amount of *H. courbaril* leaf resin on the underside of one leaf disk and pressing it against the underside of an unpainted disk. Two control and two test sandwiches were placed in each of 8 empty Dixie cups. Sandwiches were mounted vertically on the cup walls with insect mounting pins. They were positioned equidistant from each other, and controls were alternated with test sandwiches. A fourth instar larva which had been starved for 4 hr was placed in each of the 8 cups. Cups were covered with ventilated lids, and larvae were allowed to feed for 8 hr. The experiment was initiated within 20 min after the first leaves were harvested. Leaf disk remains were then removed and copied on an IBM copier. Leaf disk copies were cut out and weighed to obtain estimates of the percent leaf area eaten.

Experiment 2. In this experiment the relative palatabilities of young, nearly expanded *Hymenaea* leaves and untreated bean leaves were tested. Leaf disks of 1.2-cm diameter were used individually rather than in sandwiches. Two disks of *H. courbaril* and two of bean were mounted in each of 10 empty cups in a scheme and procedure similar to experiment 1. One 4-hr starved *S. exigua* larvae was placed in each cup and allowed to feed for 8 hr. Percent leaf area eaten was then calculated as in experiment 1.

Experiment 3. Relative palatability of the same young *H. courbaril* leaf used in experiment 2 was compared with a fully mature but not senescent leaf of the same plant. Design was identical to experiment 2 except that feeding period was extended to 24 hr.

Experiment 4. Bean leaves were tested relative to the youngest *H. courbaril* leaves obtainable. The *Hymenaea* leaves were newly emerged from the bud and were softer and more pliable than the bean leaves. Design was the same as in experiment 2.

RESULTS

Feeding Experiments

In the first experiment there was no significant effect of *H. courbaril* resin concentration on the growth of surviving *S. exigua* larvae nor on final pupal weight (Figure 1, Table 3A); however, there was a striking effect on the survival (Figure 2). The survival curves in Figure 2 show that the increase in mortality at high resin concentrations is primarily due to the increase in acute cases of virus. The highest resin concentration in this experiment is within the range of resin concentration found in mature leaves of *H. courbaril* (0.4–2.2% dry weight).

In the second feeding experiment there was no significant effect of resin concentration on larval growth during the period between the initiation of the experiment and the time when the first larvae began to pupate (Figure 3); however, with increased resin concentration there was a small increase in the mean number of days to pupation, as well as a reduction in mean pupal weight (Table 3B). There was no effect of resin concentration on mortality in this experiment (20% in control, 25% in 1.0% resin diet, and 20% in 3.2% diet), and no symptoms of virus were present.

A general result which was quantified in the feeding experiments and also observed during the maintenance of the colony is that there is much more variation in larval weight than in pupal weight. Oftentimes a pupa would weigh half of its maximum weight as a larva (compare Table 3 with Figures 1 and 3).

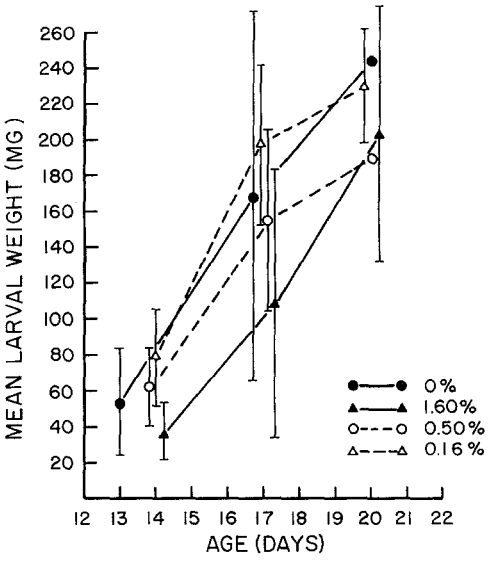

FIG. 1. Mean larval weight of surviving *S. exigua* larvae in the first feeding experiment during the period just before the first larvae begin to pupate. Percentages indicate the amount of resin incorporated into the artificial diets on a dry weight basis. One standard deviation is shown above and below each mean.

TABLE 4. MEAN PERCENT OF LEAF MATERIAL EATEN IN PALATABILITY EXPERIMENTS[a]

	Experiment							
	1		2		3		4	
Feeding period	8 hours		8 hours		24 hours		8 hours	
Plant material	Resin painted bean	Bean	Young *Hymenaea*	Bean	Mature *Hymenaea*	Young *Hymenaea*	Very young *Hymenaea*	Bean
Mean (%)	6.1	47.8	3.9	56.2	0.0	2.9	0.0	22.6
SD	5.4	19.8	7.7	25.3	0.0	2.4	0.0	18.0
N	8	8	10	10	10	10	10	10
t	5.369		5.786		3.851		3.986	
df	14		18		18		18	
$t_{.995}$	2.98		2.88		2.88		2.88	

[a] SD = standard deviation, *N* = sample size. *t* = *t* statistic, *df* = degrees of freedom, $t_{.995}$ = critical value of *t* for significance at the 0.01 level in a two-tailed test.

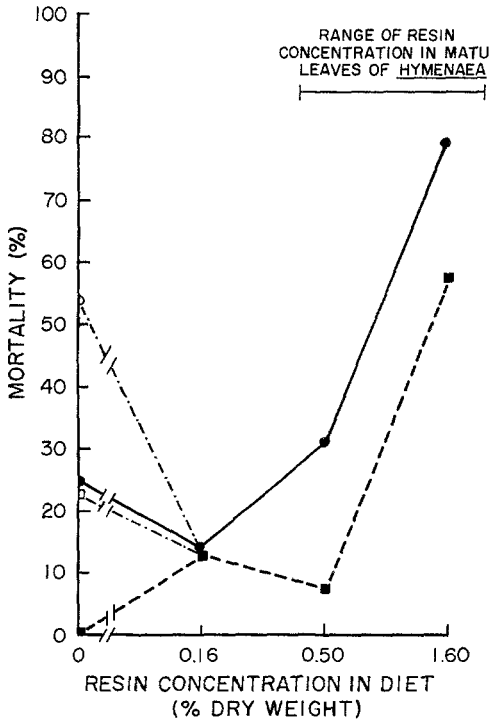

FIG. 2. Mortality of *S. exigua* as a function of *H. courbaril* leaf resin concentration in feeding experiment 1: ●———●, total mortality; ■– – –■, mortality due to virus; ○ - ·- ○ and □ - · -□, calculations based on 13 larvae in the control.

Palatability Experiments

Results of the three palatability tests are shown in Table 4. In the first test, the untreated bean leaves were greatly preferred over the leaves treated with resin. Here the only difference between the leaf materials was the presence or absence of *H. courbaril* leaf resin. The larvae had not been previously conditioned on a diet containing resin and therefore were in a state more capable of responding to possible deterrent and toxic effects of the resin than if they had been conditioned. If they had been conditioned before the experiment, a lack of any deterrent effect would not have distinguished between the lack of toxic or deterrent properties of the resin on the one hand and efficient detoxification on the other. In the latter case, any deterrence related to toxic properties which actually exists might not be revealed in the experiment. The results of this experiment constitute the most direct evidence

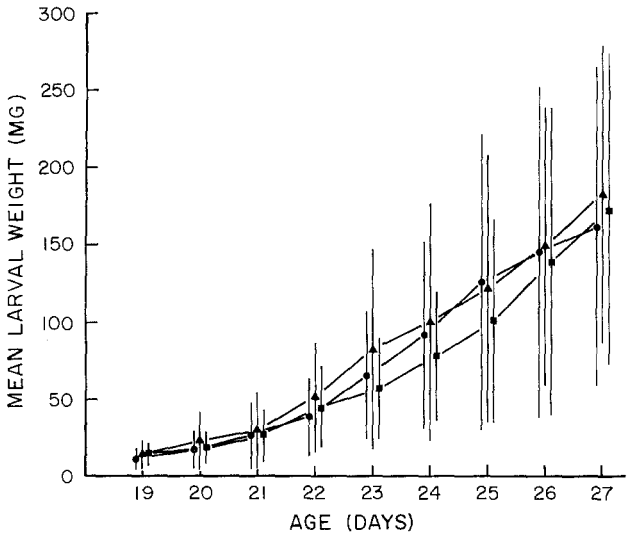

FIG. 3. Growth of *S. exigua* in feeding experiment 2 during the period just before the first larvae began to pupate. One standard deviation is shown above and below each mean. ● = 0.0%; ▲ = 1.0%; ■ = 3.2%.

that the leaf resin has a strong deterrent effect on the feeding behavior of *S. exigua*. The second experiment shows that beet armyworm larvae prefer bean leaves to young nearly expanded *H. courbaril* leaves. This is consistent with the deterrent effect of the resin shown in experiment 1; however, the cause of the preference is not as clear in this experiment as the first. Numerous different leaf characteristics exist between *P. vulgaris* and young *H. courbaril* leaves other than presence or absence of resin. A notable difference is that leaves from greenhouse-grown saplings of *H. courbaril* are somewhat more tough and fibrous than the bean leaves, and this may have been a contributing factor to the observed preference. A third test showed that (1) young nearly expanded *H. courbaril* leaves are preferred to mature ones and (2) mature *H. courbaril* leaves are very resistant to attack by the beet armyworm. One of the major differences between young and mature leaves in *Hymenaea* is their toughness, and this again may be a major contributing factor in the resistance of mature leaves.

The fourth experiment showed that bean leaves are clearly preferred to newly emerged *H. courbaril* leaves. This preference cannot be attributed to avoidance of tough, fibrous leaves because it was the more fibrous leaf material that was preferred.

DISCUSSION

The increased susceptibility to virus in the first feeding experiment, reduction in pupal weight, and lengthening of time to pupation at increased concentration of *Hymenaea courbaril* leaf resin in the second experiment all suggest that the beet armyworm is stressed when on a diet containing the resin. This stress is evident at resin concentrations within the normal range for mature leaves of *Hymenaea* but well below the range found in very young unexpanded *Hymenaea* leaves. The absence of any effect on growth rate, especially in the second feeding experiment, suggests that the deterrent effects which were evident in the palatability experiments were probably ineffective in reducing the feeding of beet armyworms under no-choice conditions. To verify this it would be necessary to measure the amount of diet eaten as well as utilization efficiencies. Feeding experiments 1 and 2 do not directly distinguish between possible toxic effects following digestion and digestion-inhibiting effects of the resin. However, the concentrations occurring in leaves are low relative to concentrations of digestion inhibitors in many plants, and also were effective in the experiments. This, as well as the nearly identical growth curves for different resin concentrations under virus-free conditions, suggest that the primary effect is probably a toxic one. Additionally, the properties of *Hymenaea* leaf resin constituents are precisely those postulated for plant toxins functioning as antiherbivore defenses (Rhoades and Cates, 1976). The sesquiterpene hydrocarbons which comprise the leaf resin are of low molecular weight and highly lipophilic, allowing easy passage through cell membranes, and they are active in relatively small amounts (less than 2% dry weight). Terpenes are energetically rather expensive to produce when compared to most carbohydrates; however, they are relatively cheap from a nutrient standpoint, unlike the nitrogen-containing alkaloids. Nutrient frugality, rather than photosynthate conservation, is likely to be most important in humid tropical areas where conditions for photosynthesis are generally excellent and nutrients often may be limiting. With respect to target specificity, however, terpenes do not fit the observed and predicted patterns for plant toxins. Scora (personal communication) has found the essential oils of *Citrus* to be autotoxic when compartmentation is disrupted, but this aspect has not been studied in *Hymenaea* as yet. Nevertheless, the mono- and sesquiterpenes, as well as oxygenated terpenes, fit the pattern of plant toxins better than that of digestion inhibitors (Rhoades and Cates, 1976).

The palatability experiments generally show that (1) *Hymenaea* leaves are of low palatability relative to bean leaves and (2) the resin of *Hymenaea* leaves is a major contributing factor to this low palatability. It is reasonable to assume that herbivores will evolve a distaste for plants which contain effective

toxins. Therefore, it is not surprising to find the same compounds have both toxic and deterrent effects.

In these experiments we have tested the hypothesis that *Hymenaea* leaf resins reduce herbivory by acting as toxins, digestion inhibitors, or feeding deterrents to potential herbivores. The results show clear deterrent properties of this resin and suggest toxic properties as well. Further experiments are in progress to determine whether or not different quantitative compositions have differential deterrent effects on various natural herbivores of *Hymenaea.*

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CHEMISTRY OF THE DEFENSIVE SECRETION OF THE CADDISFLY *Pycnopsyche scabripennis* (TRICHOPTERA: LIMNEPHILIDAE)

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Abstract—From a pair of exocrine glands located on the fifth abdominal sternite, the caddisfly *Pycnopsyche scabripennis* secretes a defensive exudate which contains *p*-cresol, indole, and skatole. This secretion effectively repels invertebrate predators such as ants. The probable significance of this secretion in the biology of these caddisflies is discussed.

Key words—*Pycnopsyche scabripennis*, caddisfly, defensive secretion, exocrine gland, indole, *p*-cresol, skatole.

INTRODUCTION

The various responses of insects to threatening stimuli can be collectively described as "defensive behaviors" and form the basis of each species' "defensive strategy." Furthermore, when many species of insects—like caddisflies—change from one developmental stage to the next, so do their defensive strategies. Caddisflies (Trichoptera) are aquatic in their early developmental stages and many construct portable cases which not only aid in respiration (Hynes, 1970) but also serve as protective shelters. These cases are constructed of either pieces of plant or inorganic materials and, being cryptic in appearance, probably elude the attention of some potential predators. Adult caddisflies, on the other hand, are terrestrial and much more exposed to a variety of predatory animals.

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When adults of the genus *Pycnopsyche* (Limnephilidae) emerge during the late summer and early autumn, they can be attracted to black lights. We have observed that one of these large, golden-brown caddisfly species, *P. scabripennis*, when disturbed, emits a foul skatolic odor somewhat similar to that emitted by some green lacewings (*Chrysopa* spp., Neuroptera) (Blum et al., 1973). Herein we report that this trichopterous secretion is dominated by indole and also contains traces of skatole and *p*-cresol. In this paper we discuss the chemistry, glandular origin, and function of this secretion as a defensive exudate.

METHODS AND MATERIALS

Collection of Animals

Adults of *P. scabripennis* were collected in the late summer and fall of 1973–1975 at the Coweeta Hydrologic Station, Macon Co., North Carolina, and the Wahalla National Fish Hatchery, Oconee Co., South Carolina. At each collection site, a large white sheet was hung behind the black light and as adult caddisflies landed on the sheet, each was removed and placed into an individual plastic vial. The *Pycnopsyche* adults were then chilled in an ice chest and transported to the laboratory where they were stored at 4°C for periods up to two weeks.

Localization of the Source of Secretion

Male and female specimens of *P. scabripennis* were cleared in KOH and examined for the presence of glandular elements. These were then excised, mounted on slides, and photographed.

The external surfaces of the glandular areas were prepared for scanning electron microscopy (SEM) by subjecting intact adult abdomens to the critical-point drying procedures described by Anderson (1951) using 100% ethanol as the initial fluid and liquid CO_2 as the transition fluid. The abdomens were then mounted on aluminum stubs and examined by SEM.

Collection of the Secretion

Extracts were prepared by either dissecting glands from adults and placing them in *n*-pentane or dipping the whole abdomens in *n*-pentane. Results of gas–liquid chromatographic (GLC) analyses of both extracts were identical. Males and females were analyzed separately.

Gas–Liquid Chromatographic–Mass Spectral Analysis

Caddisfly extracts were initially run on a 10% Carbowax 20 M column,

1.8 m × 0.63 cm OD, programmed from 60–200°C/5° min. Mass spectral analyses were made on an LKB-9000 GLC–MS unit utilizing a 1.8-m 10% SP-1000 glass column programmed from 60–240°C/10° min.

Bioassay Procedures

The palatability of *P. scabripennis* adults was tested by placing caddisflies in plastic containers containing either a dusky salamander (*Desmognathus* sp.) or a toad (*Bufo americanus*) and leaving them overnight in an environmental cabinet. This was repeated at least twice for each predator.

In a second series of experiments, mature gerbils (*Meriones shawi*) and lizards (*Scelophorus woodi*) were regularly fed adult *Tenebrio molitor* beetles or cockroaches which had been coated with 10 μl of one of the compounds (10 μg/μl solution) identified in the defensive secretion of *P. scabripennis*. This procedure was repeated for each compound as well as for a mixture of the compounds.

Further tests were performed by placing dewinged caddisflies in the foraging arena of a laboratory colony of the ant *Formica subsericea*, and observing ant–caddisfly interactions.

The deterrent value of the exocrine products was also investigated by mixing 10 μl of a solution of either *p*-cresol, indole, or skatole (10 μg/μl acetone) with 0.1 ml honey and placing these solutions on the foraging platform of a colony of fire ants, *Solenopsis invicta*. The deterrent value of the *P. scabripennis* compounds vs. controls (10 μl acetone) was analyzed in terms of the time required by the ants to consume the honey droplets.

RESULTS AND DISCUSSION

Chemistry of the Secretion

GLC analyses of excised glands of *P. scabripennis* adults demonstrated the presence of one major compound, which consituted over 98% of the observed volatiles, and two minor constituents. Extracts of females and males exhibited no appreciable differences. The mass spectrum of the compound which first eluted from the column exhibited a molecular ion at *m/e* 108. The identification of this compound as *p*-cresol was based on the congruency of its mass spectrum and GLC retention times with those of this phenolic.

The mass spectrum of the second volatile, the major component in the extract, gave a molecular ion at *m/e* 117 and was identical to that of indole. The GLC retention times of the trichopteran volatile and indole were completely congruent.

The third volatile compound had a molecular ion at *m/e* 131. A normalized spectrum of this compound indicated it to be a methyl-substituted indole. Comparison of it with all methylindole isomers demonstrated that only 3-methylindole (skatole) had the same mass spectrum.

The abdominal secretion of *P. scabripennis* is somewhat unusual in that the major constituent, indole, has not been previously isolated from an arthropod exocrine source. Presumably formed from tryptophan, indole is, however, known to be a common excretory product in many groups of animals. Like indole, skatole is presumably formed from tryptophan, and it similarly does not appear to be a common exocrine product of arthropods. Blum et al. (1973) demonstrated the presence of skatole and tridecene in the prothoracic glandular exudate of the green lacewing, *Chrysopa oculata* (Neuroptera), where it is believed to be utilized defensively. Skatole has also been tentatively identified in ants (Formicidae) both from the poison glands of soldiers of *Pheidole fallax* and as a sex-specific compound in the mandibular glands of *Lasius* and *Acanthomyops* males (Law et al., 1965).

The presence of *p*-cresol in the abdominal secretion of *P. scabripennis* is another example of the widespread use of phenolics as defensive allomones. For example, *p*-cresol dominates the defensive secretion of the millipede, *Abacion magnum* (Eisner et al., 1963), and this phenolic, along with *p*-ethylphenol, fortifies the ventral glandular exudate of the American cockroach, *Periplaneta americana* (Takahashi and Kitamura, 1972).

Both indole and skatole are foul-smelling and rather persistent compounds, remaining on a treated object for a relatively long period of time. We found that they are detectable on the fingers many minutes after handling an adult *P. scabripennis*. Similarly, Betten (1934) noted that some species of caddisflies in the genus *Stenophylax* emitted compounds with a foul, persistent odor. In a later revision of *Pycnopsyche*, Betten (1950) includes species which were originally placed in the genus *Stenophylax*.

Glandular Structure and Exudate Dispersion

Examination of the abdomens of males and females demonstrated the presence of a dorsolateral structure on the fifth abdominal sternite at the release site of the glandular exudate (Figures 1 and 3).

In males of *P. scabripennis* the region of the gland is visible externally as a curved projection terminating in a point at the apex (Figures 1 and 2). The outer distal surface of this projection is curved, with short peglike setae (Figure 2). In females, the external release site is a slightly raised area (Figure 3) without the projecting tip found in males. However, females possess a series of peglike setae at this site. This peglike region, which is found in both males

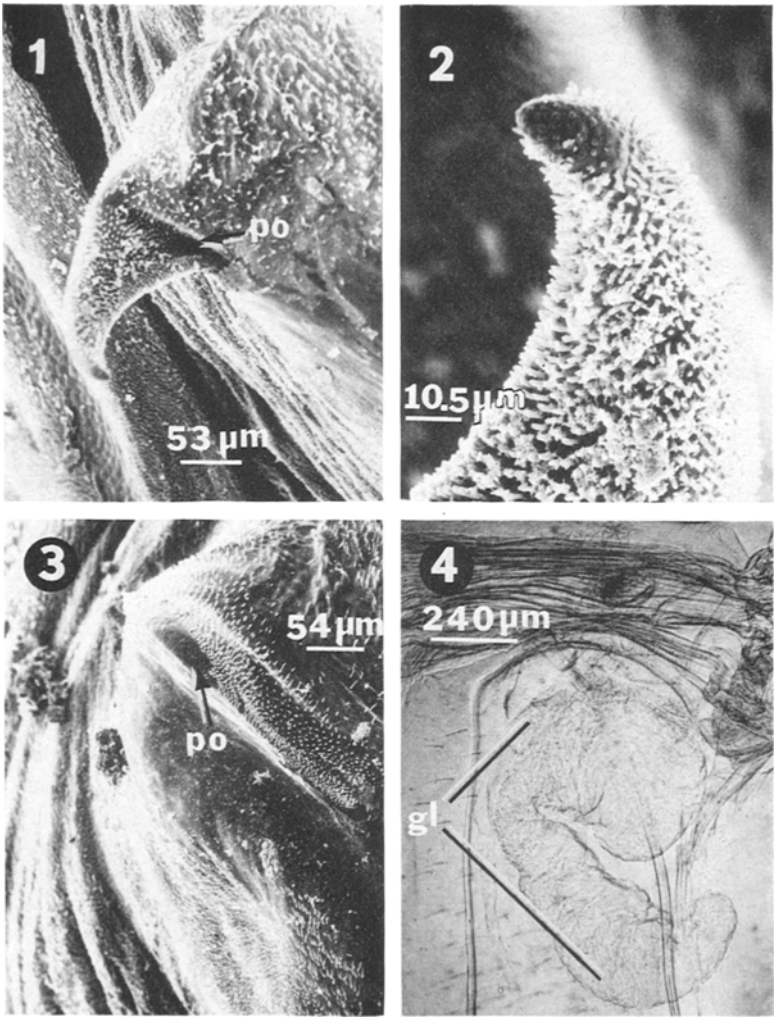

FIG. 1. SEM micrograph of *P. scabripennis* adult: Male, showing curved projection in the glandular region and presumed glandular opening (po).

FIG. 2. SEM micrograph of *P. scabripennis* adult: close-up of the curved projection in the males.

FIG. 3. SEM micrograph of *P. scabripennis* adult: female glandular region and presumed opening (po).

FIG. 4. KOH-cleared mount of a male *P. scabripennis* showing the exocrine gland (gl).

and females, may be instrumental in releasing and spreading the secretion at the orifice of the glands.

The glandular reservoir of the KOH-cleared specimens was readily visible as a C-shaped, saclike structure about 1.8 mm in length (Figure 4, gl). There is no apparent connection between the paired glands on either side of the abdomen. Each gland has a constricted duct that leads to the presumed opening (Figures 1 and 3, po) on the raised external area.

Analysis of leg and body movement by cinematography demonstrated that adults of *P. scabripennis* have an interesting adaptation for dispersing the glandular exudate. The joint of the hind femur and tibia is moved over the glandular orifice, possibly stimulating the release of the exudate and further dispersing the volatile secretion. It appears that the hind legs not only make contact with the glandular region, but spread the secretion over the surface of the abdomen as well. Furthermore, if a *P. scabripennis* adult takes flight after being disturbed, it can effectively disperse its odoriferous exudate by means of the air flow over the evaporative surface caused by movement of the wings.

In the past, various authors have noted the presence of what appeared to be a glandular orifice on the fifth abdominal segment of a number of species of caddisflies. This structure, or one similar, appears to be a basic caddisfly structure variously modified in different taxa (Ross, 1956). In males of a species of South American *Barypenthus* (Odontoceridae), a large dorsal exocrine gland opens between the fifth and sixth abdominal tergites; the function of the gland is unknown (Barth, 1963a,b).

Significantly, *Pycnopsyche* is the only known genus in the Limnephilidae to go through extensive speciation in the eastern United States (Flint, 1960). We have found several other species of *Pycnopsyche* which produce defensive secretions and are currently analyzing the chemistry of these exudates. The possibility exists that all these eastern North American trichopterans represent a Müllerian or Batesian mimetic complex based on the presence or absence of defensive glands.

Bioassays

P. scabripennis adults were readily eaten by both toads and salamanders, the indole-rich abdominal secretions proving ineffective in repelling these vertebrate predators.

The experiments designed to evaluate the possible value of the secretory components as gustatory or olfactory repellents to vertebrates indicated that the compounds in this exudate possessed little deterrent "punch" for the candidate predators. The gerbils readily chewed beetles which were coated with the secretion and consumed them in toto. Lizards also swallowed all the

treated cockroaches, no unusual behavior being exhibited by these vertebrates before or after feeding on the caddisflies.

In contrast, dewinged adults of *P. scabripennis* readily repelled workers of the ant *Formica subsericea.* The first ant to make contact with the caddisfly would invariably try to discharge its formic acid. However, immediately after the first encounter, a strong indolic odor would be detected. The ants in the vicinity became more excited but only feebly attacked the caddisfly. After 10 or 20 min, the caddisfly would succumb to the ant attacks, a result probably of very little significance, since in nature a molested trichopteran could simply fly from the scene of the encounter.

The value of the compounds as ant repellents was further documented by the experiments with the treated baits. When standard honey mixtures were compared to those containing either *p*-cresol, indole, or skatole, the controls followed by indole and *p*-cresol mixtures were consumed immediately by the fire ant workers. Little attention was paid to the skatolic mixture for the hour duration of the experiment. Thus, the compounds in the abdominal secretions of *P. scabripennis* adults appear to be at least partially effective in repelling ants.

We have observed that *P. scabripennis* adults which approached the black light and landed on the adjacent sheet were not characterized by an indolic odor. Conversely, if an adult collided with the black light and then landed on the sheet, the indolic odor was easily detectable. These observations are consistent with those expected for the discharge of a defensive secretion. Sticky traps baited with each compound or with their mixtures did not attract any caddisfles, which apparently rules out the possibility that these constituents function as either attractants or aggregation pheromones.

The ability to fly when molested must be considered one of their evasive maneuvers, but this defensive strategy may increase the chances of the caddisflies being attacked by avian predators working the vegetation for food. Effectively repelling foraging ants, these trichopterans may be able to retain their secluded perches without taking wing, thus reducing the possibility of detection by diurnal birds. In addition, the odoriferous indolic secretion of *Pycnopsyche* species may have great selective value in terms of nocturnal predators. Bats should be able to easily distinguish the flight patterns of these large trichopterans and may learn to discriminate against them as food items.

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BARK-BEETLE PHEROMONES
Enhancement of *Dendroctonus frontalis* (Coleoptera: Scolytidae) Aggregation Pheromone by Yeast Metabolites in Laboratory Bioassays[1]

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Abstract—The two major yeasts isolated from the southern pine beetle, *Dendroctonus frontalis* Zimmerman, are *Hansenula holstii* Wickerham and *Pichia pinus* (Holst) Phaff; a third yeast, *P. bovis* van Uden et do Carmo-Sousa, has been isolated far less frequently. The main volatile metabolites produced by these yeasts are isoamyl alcohol, isoamyl acetate, 2-phenylethanol, and 2-phenylethyl acetate. We have found that certain of these compounds, particularly the esters, can greatly, enhance the attractiveness of a mixture of frontalin: *trans*-verbenol: turpentine (1:1:12), at certain limiting concentrations, to walking beetles in a laboratory bioassay.

Key Words—Pheromones, bark beetles, *Dendroctonus frontalis*, southern pine beetle, bioassays, microbial metabolites, isoamyl alcohol, isoamyl acetate, 2-phenylethanol, 2-phenylethyl acetate.

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INTRODUCTION

We have proposed that microorganisms associated with certain bark beetles may be responsible in part for the ultimate blend of behavioral chemicals that these insects encounter and respond to in their natural habitat (Brand et al., 1975, 1976). This idea concerning bark beetles was by no means new, as Person wrote in 1931 "... fermenting inner bark was more attractive than any of the other substances tested. This suggested the possibility that the attractiveness of attacked trees might be due to some fermentation organism, such as a yeast, associated with the beetle" (Person, 1931). After the successful initial attack by a few beetles Person concluded "... a second, stronger attraction is started by the yeast introduced by the attacking beetles, finding the inner bark a favorable medium for its growth." Person's reference was to a closely related species, *D. brevicomis* Le Conte, attacking ponderosa pine.

In principle we agree with the statements made by Person almost 50 years ago. However, our conviction is not universally shared as it had been either concluded or suggested that the role of yeasts in the secondary attraction of bark beetles is questionable (Anderson, 1948; Wood and Vité, 1961; Vité and Gara, 1962; Francke-Grosmann, 1963; Graham, 1967). It should also be pointed out that we have not yet been able to unequivocally demonstrate the involvement of microorganisms in the production of compounds utilized by bark beetles as attractants during their orientation to attacked trees. A good review of selection and colonization of ponderosa pine by bark beetles has been written by Wood (1972).

In this paper we present evidence that certain metabolites of yeasts associated with the southern pine beetle, *Dendroctonus frontalis*, can enhance the response of walking beetles to a mixture of frontalin, *trans*-verbenol, and loblolly turpentine, in laboratory bioassays.

METHODS AND MATERIALS

Isolation and Culturing of Yeasts

Yeasts were isolated on malt agar plates (pH 3.7) and peptone:yeast extract:glucose [1%:0.4%:1%; PYG; in Dworkin–Foster salts medium (Dworkin and Foster, 1958)] agar plates from whole beetles, excised whole guts, and various locations in galleries in loblolly pine. All cultures were grown at 22° and identified by the criteria of Lodder (1970).

The yeasts were grown in 100 ml PYG (1%:0.4%:2%; in Dworkin–Foster salts medium) shake cultures at 22° prior to extraction and assay of volatiles. A phloem medium for assaying production of volatiles was made by homogenizing fresh phloem in water and filtering the homogenate through

cheese cloth. Approximately 10 g of fresh phloem yielded 100 ml of filtrate, and this filtrate was used as a culture broth after autoclaving.

Identification of Volatile Metabolites

The yeast cultures were extracted with ether at various time intervals, the extracts dried over Na_2SO_4, and concentrated under a stream of nitrogen. The major volatile components in the concentrated extract were separated gas chromatographically on Carbowax 20 M temperature programmed from 60° to 200° at 10° per minute. Mass spectra of the major volatile metabolites were obtained either by the direct insertion technique of each compound adsorbed on powdered graphite, or by GC–MS (SP-1000 column) in a Finnigan 3600 instrument. Compounds were adsorbed directly on graphite as they eluted from the effluent-split gas chromatographic column. Extracts of sterile PYG medium did not contain detectable amounts of volatile substances.

Laboratory Bioassays

The bioassay procedure, which is described in detail by Payne et al. (1976), was employed for all bioassays. In this assay a group of beetles of one sex were placed on an arena. Test compounds in *n*-pentane were delivered by means of a 10-μl syringe driven by a timer motor into an air stream directed across the bioassay arena. Positively responding beetles walked upwind to the release portal.

The standard attractant mixture was frontalin: *trans*-verbenol: turpentine (1:1:12) diluted with *n*-pentane (Payne et al., 1976). This mixture will be referred to as the triplicate standard. *n*-Pentane was used as a control. The attractancy of the triplicate standard was assayed both on its own and together with each of the following compounds or mixtures respectively: isoamyl alcohol (1), 2-phenylethanol (2), isoamyl acetate (3), 2-phenylethyl acetate (4), isoamyl alcohol and 2-phenylethanol (5), isoamyl acetate and 2-phenylethyl acetate (6), isoamyl alcohol, 2-phenylethanol, isoamyl acetate, and 2-phenylethyl acetate (7).

The triplicate standard was assayed at the following rates: 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} $\mu g\ \mu l^{-1}\ min^{-1}$. The various compounds and mixtures were assayed together with the triplicate standard, each compound or mixture being at a rate 5 times greater than the triplicate standard, i.e., triplicate standard (10^{-5} $\mu g^{-1}\ min^{-1}$) and (1) (5×10^{-5} $\mu g\ /l^{-1}\ min^{-1}$)

RESULTS

Identity of Yeasts

The vast majority of isolated yeast colonies were either *Hansenula*

holstii Wickerham or *Pichia pinus* (Holst) Phaff in agreement with the results of Shifrine and Phaff (1956), but occasionally we obtained isolates of *Pichia bovis* van Uden et do Carmo-Sousa.

Identification of Compounds

When ether extracts of the three yeast cultures were analyzed gas chromatographically, a number of volatile compounds were found to be present. The mass spectrum of each major compound, trapped on powdered graphite, was obtained by the direct insertion technique. In addition, mass spectra of both the major and minor components were obtained by coupled GC–MS. From these data, and by comparison with data obtained from authentic standards, the four major compounds were identified as isoamyl alcohol, 2-phenylethanol, isoamyl acetate, and 2-phenylethyl acetate.

P. bovis, in liquid culture, produced isoamyl alcohol and 2-phenylethanol after approximately 30 hr, and the amount of these two alcohols reached a maximum at about 100 hr, thereafter decreasing in amount. At approximately 70 hr significant amounts of isoamyl acetate and 2-phenylethyl acetate were produced and the amounts of these two esters equaled the amounts of the two alcohols at about 100 hr. When cultures were extracted at a time greater than 150 hr, these compounds disappeared and valeric acid was found to be the predominant compound. Both *H. holstii* and *P. pinus* produced isoamyl alcohol and 2-phenylethanol after approximately 30 hr in liquid cultures, but they did not produce the acetates under these conditions. In addition, cultures extracted up to 250 hr continued to contain 2-phenylethanol as the major volatile component, together with valeric acid.

All three yeast species also produced methanol (with the exception of *H. holstii*), ethanol, *n*-propanol, and isobutanol in their earlier stages of growth. Extracts of cultures of each of these three species on the sterile phloem medium indicated that *H. holstii* and *P. bovis* produced only 2-phenylethanol as a major component under these conditions. The other alcohols and acetates, however, were detected if the phloem medium was supplemented with glucose.

Bioassays

The major volatile compounds produced by the three yeasts were bioassayed in a laboratory bioassay against walking beetles, and the results are presented in Tables 1 to 4. Table 1 shows the response of males and females to the triplicate standard at a rate of 10^{-3} μg μl^{-1} min^{-1} as well as their response to the triplicate standard plus each of the various compounds or combinations of compounds. While minor differences exist, it is obvious that

TABLE 1. RESPONSE OF *D. frontalis* MALES AND FEMALES IN A WALKING BIOASSAY TO FRONTALIN: *trans*-VERBENOL:TURPENTINE (TS; 1:1:12) DELIVERED AT A RATE OF 10^{-3} μg ul^{-1} min^{-1} AND TO TS PLUS CERTAIN YEAST METABOLITES, EACH METABOLITE OR MIXTURE AT A RATE OF 5×10^{-3} μg μl^{-1} min^{-1}

Compound[a]	% Response[b]			
	♂	$SE_{\bar{x}}$	♀	$SE_{\bar{x}}$
Control	17	±4	13	±4
TS	90	±4	62	±9
TS+(1)	87	±7	80	±7
TS+(2)	80	±7	60	±7
TS+(3)	90	±5	77	±9
TS+(4)	93	±4	57	±7
TS+(5)	93	±4	63	±8
TS+(6)	100		67	±9
TS+(7)	97	±3	67	±9

[a] Control = *n*-pentane delivered at a rate of 1 μl min^{-1}; (1) isoamyl alcohol, (2) 2-phenylethanol, (3) isoamyl acetate, (4) 2-phenylethyl acetate, (5) isoamyl alcohol+2-phenylethanol, (6) isoamyl acetate+2-phenylethyl acetate, (7) isoamyl alcohol + 2-phenylethanol + isoamyl acetate + 2-phenylethyl acetate; each compound or mixture at a rate of 5×10^{-3} μg μl^{-1} min^{-1}.

[b] 30 Beetles/value except the control which was 60 beetles/value.

no real inhibition of the high response to the triplicate standard was caused by any of the compounds or mixtures. The low response of females to the triplicate standard plus 2-phenylethanol [Table 2, test TS+(2)] is noteworthy.

The results in Table 3, however, are the most interesting. At a rate of 10^{-5} μg μl^{-1} min^{-1} the response of both males and females to the triplicate standard was not much greater than the control. The addition of either 2-phenylethyl acetate [TS+(4)] or isoamyl acetate [TS+(3)] at 5×10^{-5} μg μl^{-1} min^{-1} increases the response of male beetles to 87%. The combination of isoamyl alcohol and 2-phenylethanol, or their acetates, or all four compounds, also produced a greatly increased response from the males. The response of females, while lower overall, is of a similar pattern, 2-phenylethanol [TS+(2)] again appearing as a possible exception. None of the yeast metabolites elicited significant responses from the beetles when assayed alone at a rate of 10^{-4} μg μl^{-1} min^{-1}.

TABLE 2. RESPONSE OF *D. frontalis* MALES AND FEMALES IN A WALKING BIOASSAY TO FRONTALIN: *trans*-VERBENOL:TURPENTINE (TS; 1:1:12) DELIVERED AT A RATE OF 10^{-4} μg μl^{-1} min^{-1} AND TO TS PLUS CERTAIN YEAST METABOLITES, EACH METABOLITE OR MIXTURE AT A RATE OF 5×10^{-4} μg μl^{-1} min^{-1}

Compound[a]	% Response[b]			
	♂	$SE_{\bar{x}}$	♀	$SE_{\bar{x}}$
Control	3	±2	5	±3
TS	73	±8	53	±5
TS+(1)	83	±6	73	±8
TS+(2)	60	±10	20	±7
TS+(3)	90	±5	80	±7
TS+(4)	83	±7	63	±8
TS+(5)	83	±9	50	±9
TS+(6)	83	±7	80	±7
TS±(7)	90	±5	47	±7

[a,b] See Table 1. 10^{-4} μg μl^{-1} min^{-1} delivery rate for TS and 5×10^{-4} μg μl^{-1} min^{-1} for other compounds or mixtures.

TABLE 3. RESPONSE OF *D. frontalis* MALES AND FEMALES IN A WALKING BIOASSAY TO FRONTALIN: *trans*-VERBENOL:TURPENTINE (TS; 1:1:12) DELIVERED AT A RATE OF 10^{-5} μg μl^{-1} min^{-1} AND TO TS PLUS CERTAIN YEAST METABOLITES, EACH METABOLITE OR MIXTURE AT A RATE OF 5×10^{-5} μg μl^{-1} min^{-1}

Compound[a]	% Response[b]			
	♂	$SE_{\bar{x}}$	♀	$SE_{\bar{x}}$
Control	7	±3	10	±4
TS	27	±8	23	±7
TS+(1)	50	±12	50	±7
TS+(2)	47	±9	27	±10
TS+(3)	87	±5	70	±9
TS+(4)	87	±7	63	±3
TS+(5)	73	±8	43	±9
TS+(6)	67	±10	47	±9
TS+(7)	57	±9	43	±9

[a,b] See Table 1. 10^{-5} μg μl^{-1} min^{-1} delivery rate for TS and 5×10^{-5} μg μl^{-1} min^{-1} for other compounds or mixtures.

TABLE 4. RESPONSE OF *D. frontalis* MALES AND FEMALES IN A WALKING BIOASSAY TO FRONTALIN: *trans*-VERBENOL:TURPENTINE (TS; 1:1:12) DELIVERED AT A RATE OF 10^{-6} $\mu g\ \mu l^{-1}\ min^{-1}$ AND TO TS PLUS CERTAIN YEAST METABOLITES, EACH METABOLITE OR MIXTURE AT A RATE OF 5×10^{-6} $\mu g\ \mu l^{-1}\ min^{-1}$

Compound[a]	% Response[b]			
	♂	SE_x	♀	SE_x
Control	18	± 5	10	±4
TS	23	±11	17	±6
TS+(1)	23	±11	23	±9
TS+(2)	20	±10	10	±5
TS+(3)	43	±9	20	±10
TS+(4)	43	±10	17	±7
TS+(5)	40	±8	17	±7
TS+(6)	30	±12	23	±5
TS+(7)	23	±10	13	±5

[a,b] See Table 1. 10^{-6} $\mu g\ \mu l^{-1}\ min^{-1}$ delivery rate for TS and 5×10^{-6} $\mu g\ \mu l^{-1}\ min^{-1}$ for other compounds or mixtures.

DISCUSSION

Our isolation of *H. holstii* and *P. pinus* is not surprising as they were shown by Shifrine and Phaff (1956) to be the two main yeasts associated with the bark beetles *Ips* and *Dendroctonus*. Shifrine and Phaff used the synonym *Candida silvicola* for *H. holstii* (Lodder, 1970, p. 279) and *Saccharomyces pini* for *P. pinus* (Lodder, 1970, p. 515). We isolated *P. bovis* far less frequently than the above two. The association of yeasts with bark beetles has also been described by Holst (1936), Whitney (1971), and Gouger (1972). We should also point out that both *H. holstii* and *P. pinus* are found in the mycangium of female *D. frontalis* (Barras and Perry, 1972) and that their introduction in the galleries is not dependent on chance transport on the exoskeleton. This adds to our conviction that our results indicate these yeasts have an important function in the bark beetle–host ecosystem.

As yeasts produce numerous volatile products during growth, we analyzed these compounds from extracts of cultures of these three yeasts. Both *H. holstii* and *P. pinus* produced three main compounds, namely ethanol, isoamyl alcohol, and 2-phenylethanol. *H. holstii* usually grew more rapidly than *P. pinus* and produced larger quantities of these three alcohols. In

addition to these compounds, *P. bovis* also produced appreciable quantities of isoamyl acetate and 2-phenylethyl acetate.

All of these identified compounds are known to be produced by certain yeasts during the fermentation of glucose (Suomalainen, 1969), so their production in this case was not unexpected. As we have found that 2-phenylethanol was the only one of these substances (with the possible exception of ethanol) produced by *H. holstii* and *P. bovis* in detectable quantities on a phloem medium, the growth medium influences the quantity and ratios of these metabolites. Their production may be dependent on the amount of glucose or carbohydrate available, as the addition of 2% glucose to the phloem medium resulted in their production. In the laboratory bioassays that we conducted, we did not find any behavioural response to these yeast metabolites (ethanol was not tested). However, ethanol is a common end product of fermentation by yeasts, and the ambrosia beetles *Trypodendron lineatum* Olivier (Moeck, 1970, 1971) and *Gnathotrichus sulcatus* Le Conte (Cade et al., 1970; Moeck, 1971) are attracted to it. Its production by anaerobic metabolism in potential hosts has been suggested (Graham, 1968).

We consider that these various yeasts growing in the galleries during the early stages of attack by *D. frontalis*, and during the initial mass attack phase, would have an ample supply of carbohydrate available and would therefore produce these various compounds. However, at this stage in these investigations we do not know whether these yeasts growing in situ produce the various volatile compounds identified above or whether these compounds are produced in time to actually interact with other known attractants, e.g., frontalin, and thereby influence the behavior of beetles toward trees under attack. These problems are complex and are being investigated further.

If we assume that these volatile substances are produced in the galleries of a tree under active attack, we consider it very likely that they would be perceived by attacking bettles and could influence their behavior. The presence of 2-phenylethanol in the hind guts of emergent *D. brevicomis* males and feeding *Ips paraconfusus* males has recently been reported (Renwick et al., 1976). In field bioassays, the response of *D. brevicomis* was not affected by the addition of 2-phenylethanol to its known attractant, whereas the response of *I. paraconfusus* to male-infested log sections was greatly enhanced by the addition of 2-phenylethanol (Renwick et al., 1976). No effect of this compound on the behavior of *D. frontalis* has been reported.

It was for these reasons that we bioassayed the various yeast metabolites together with the triplicate standard against *D. frontalis* males and females in the laboratory. We chose a concentration range of the triplicate standard such that the attractiveness of the mixture ranged from very high to very low. With this range of response either inhibition or enhancement of attraction by any of the yeast metabolites should be evident. The results obtained indicate that

the slight response to low concentrations of the triplicate standard can be greatly improved by the addition of low concentrations of certain yeast metabolites, e.g. isoamyl acetate and 2-phenylethyl acetate. It appears, therefore, that certain yeast metabolites enhance the attractive qualities of the triplicate standard to walking beetles in a laboratory bioassay and that the delivery concentration may be an important variable in the response elicited. In addition, inhibitory substances may be produced, and in this context the results obtained from the addition of 2-phenylethanol [TS+(2)] to the triplicate standard seem particularly worthy of further investigation. We appreciate the possibility that the results of walking bioassays in the laboratory may not be directly applicable to the field (Vité et al., 1963), and we are attempting to obtain data on the effect of these metabolites under field conditions. This study is continuing, and we predict that in order to describe fully the complex of behavioral chemicals to which the southern pine beetle displays its attraction, aggregation, and dispersal behavior, volatile microbial products will have to be considered as part of the system.

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EFFECTS OF SOIL MOISTURE STRESS ON MONOTERPENES IN LOBLOLLY PINE

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Abstract—Monoterpene composition of xylem oleoresin in pole-size loblolly pine (*Pinus taeda* L.) was different between moisture-stressed and -unstressed trees during two test years. α-Pinene concentration increased in stressed trees whereas β-pinene, myrcene, and limonene decreased. Variations in camphene concentration with soil moisture stress were inconclusive. It increased with moisture stress in one test year and decreased in stressed trees during the other test year.

Key Words—Monoterpene, soil moisture, *Pinus taeda*, moisture stress.

INTRODUCTION

Monoterpenes in conifers have received considerable interest in recent years. They have been used in taxonomic studies of plants (Turner and Flake, 1974), for identification of hybrids (Hanover and Wilkinson, 1969), and in evaluating the genetic variations in certain pine species (Rockwood, 1973). Recently, studies have demonstrated relationships between these terpenes and insect resistance (Squillace, 1976).

A number of studies have reported that environment has little, if any, effect on monoterpene composition in coniferous species. For example, Hanover (1966), using genotypically identical western white pine growing in three diverse experiments, showed negligible differences in monoterpene levels. However, Hilton (1968) found a large percentage of the variation in monoterpene levels of 23 geographic seed sources of eastern white pine to be dependent upon environmental influences. Blight and McDonald (1964), using pairs of trees from clones of *Pinus attenuata* × *radiata*, found that the

terpene concentration between pairs usually was similar, but in some cases differences existed, suggesting that the environment could be affecting the concentration. Considering the importance of environmental factors upon many physiological processes in plants, it is logical to assume that certain conditions, possibly soil moisture, may influence monoterpene composition and/or concentration.

The purpose of this study was to determine the changes in monoterpene concentrations of loblolly pine (*Pinus taeda* L.) growing under various soil moisture conditions throughout a growing season. This paper presents the results of tests conducted during 1968 and 1970.

METHODS AND MATERIALS

Trees used in the 1968 and 1970 tests were growing in a loblolly pine plantation in southern Illinois that was established in 1946 from seed of unknown origin. The soil within the plantation is uniformly a Grantsburg silt loam (ochreptic typic fragiudalf), which is characterized by a moderately well-developed fragipan located 76 cm below the surface. This layer impedes both root penetration and the downward movement of water. Field capacity is 31% and wilting percent is 10% for the soil above the fragipan layer as determined at 1/3 and 15 bars, respectively (Richards, 1949).

1968 Test

Trees in this test were growing in three 0.02-hectare plots and averaged 12 m in height and 19 cm in diameter at 1.3 m above ground. Moisture was excluded from the plots from the fall of 1967 until July 1, 1968, by means of plastic sheeting that covered the surface of each plot. The sheeting extended downward through the soil fragipan at the periphery of each plot, prohibiting lateral moisture movement along the fragipan. This long drought period brought the soil to near the wilting point. After July 1, 1968, soil moisture was maintained near field capacity by irrigating under the sheeting.

Each plot contained two access tubes that extended 2 m into the soil. Into these tubes, a nuclear probe was inserted three times per week during the study period to monitor soil moisture levels. Irrigation water, free of chemical contamination, was obtained from a pond especially constructed for the study. A metered quantity of water was supplied to each plot depending upon the soil moisture content at that time and the calibrated flow of the irrigation system.

Oleoresin samples were obtained from each of seven randomly selected trees on each of the three plots beginning April 30, 1968, and on these same

trees around the stem at intervals of two weeks thereafter until September 23, 1968. Oleoresin samples were collected at 0.3 m above ground by making V-notch cuts that extended through the bark to the cambium. The oleoresin that exuded from a cut was placed in a small vial; the air in the vial was replaced with nitrogen and the sample was frozen shortly after collection. Hodges and Lorio (1975) used a similar procedure in their study.

All oleoresin was dissolved in pentane and analyzed with a Hewlett-Packard model 5750 gas chromatograph equipped with dual hydrogen–air ionization detectors. Aliquots of the dilutions were analyzed for monoterpenes using stainless-steel columns with 20% Carbowax 20 M liquid phase on 60–80 mesh, acid-washed Chromosorb W solid support. Operating conditions were: injection port, 200°C; detector, 225°C; column, 110°C; and He flow, 17 cm^3/min. Carbowax was replaced by 5%, *b,b'*-oxidipropionitrile in the liquid phase to check the peak separation. Since the check revealed no new peaks, Carbowax 20 M was used throughout the study. Individual monoterpenes were identified by comparing relative retention times for the unknown with those of known compounds injected into the column under the same operating conditions.

Each monoterpene was expressed as a percentage of the total monoterpene concentration. Each percentage was calculated as the ratio of the mechanical integrator trace of the area under an appropriate peak to the total area under all peaks. Analysis of variance was computed on the mean of each monoterpene according to soil moisture treatment and sampling date.

1970 Test

One plot from the 1968 test was used together with two new plots established in the same plantation. At the beginning of the test, trees were 12–15 m in height and 18–21 cm in diameter at 1.3 m above ground. Seven trees were randomly selected for sampling in each of the three 0.02-hectare plots. Soil moisture was monitored as described for the 1968 test; however, the following soil moisture regimes were applied as treatments: (plot A) maintained near the wilting point (W.P.) with a plastic covering that not only prevented water movement downward into the soil but also laterally into the soil from adjacent plots (this was the plot also used in the 1968 study); (plot B) maintained near field capacity (FC) by supplemental irrigation; and (plot C) maintained by current rainfall. The last two plots had been subjected to these treatments during the previous four years. In contrast to the 1968 test, plot A was maintained near the wilting point throughout the growing season.

Beginning May 15, 1970, and approximately every two weeks thereafter, oleoresin samples were collected from each sample tree at 1.3 m above ground

on 11 dates during the growing season. The collection and storage of oleoresin samples and the laboratory analyses followed the same procedures used for the 1968 study. Statistical analysis consisted of determining the analysis of variance of each monoterpene mean according to treatment by sampling date.

RESULTS

1968 Test

Five monoterpenes found in the oleoresin of loblolly pine as they eluted from the gas chromatograph column were α-pinene, camphene, β-pinene, myrcene, and limonene (Table 1). α-Pinene was present in greatest concentrations followed by β-pinene, while camphene, myrcene, and limonene were consistently low in concentrations. The range in concentrations of a monoterpene between trees during each sampling period was large. α-Pinene

TABLE 1. MONOTERPENES CONCENTRATIONS OF LOBLOLLY PINE OLEORESIN THROUGHOUT 1968 GROWING SEASON, VALUES ARE MEAN OF 21 TREES SAMPLED

Treatment and elapse sampling period	Percent[a]				
	α-Pinene	Camphene	β-Pinene	Myrcene	Limonene
Low soil moisture (days)					
0	89.4	1.3	6.5	1.8	1.0
15	89.8	1.3	6.1	1.8	1.0
27	90.0	1.3	6.0	1.8	0.9
42	90.4	1.2	6.0	1.6	0.8
57	88.8	1.4	6.9	1.8	1.1
Average[b]	89.8	1.3	6.3	1.7	0.9
High soil moisture (days)					
76	87.4	1.2	7.5	2.7	1.2
90	84.6	1.3	9.7	2.8	1.6
104	81.9	1.9	8.6	5.1	2.5
120	81.4	2.0	9.1	4.9	2.6
134	81.1	1.9	10.0	4.6	2.4
148	79.2	2.0	10.9	5.3	2.6
Average[b]	82.6	1.7	9.4	4.2	2.1

[a] Significant differences (5% level) for all monoterpenes throughout that part of the season under high soil moisture conditions; β-pinene excluded.
[b] Significant differences (5% level) between averages of low and high soil moisture levels.

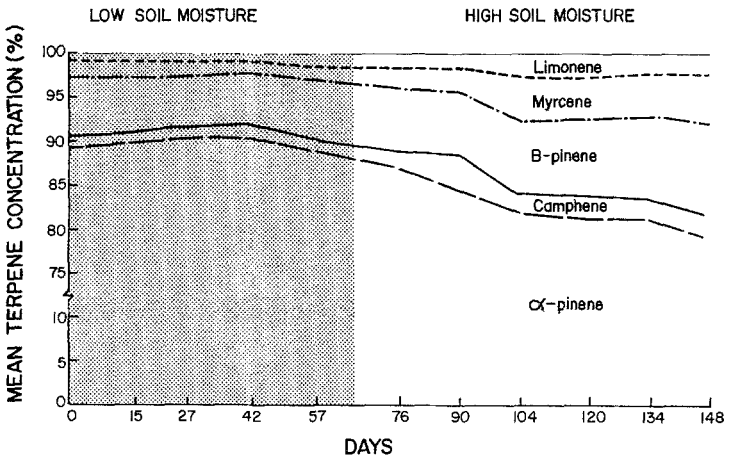

FIG. 1. Terpene concentrations under droughty conditions (low soil moisture level) and field capacity condition (high soil moisture level) according to time and treatments during the 1968 study. Concentrations are additive for each date

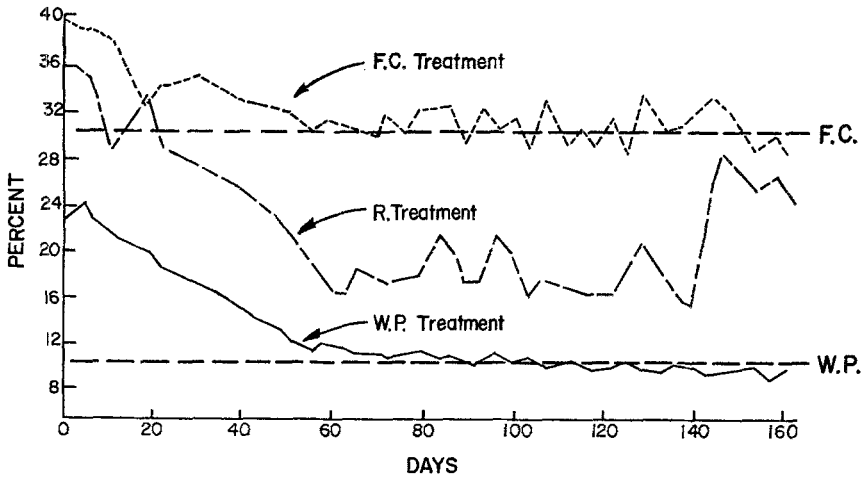

FIG. 2. Average soil moisture levels during the 1970 study according to treatments. F.C. indicates field capacity of soil, R. refers to rainfall, and W.P. indicates wilting point of soil.

ranged from 40% to 96%, and β-pinene ranged from 1% to 46%, with the other monoterpenes varying to a lesser degree.

There was no change in monoterpene composition over time under low soil moisture conditions; but with the exception of β-pinene, there was a

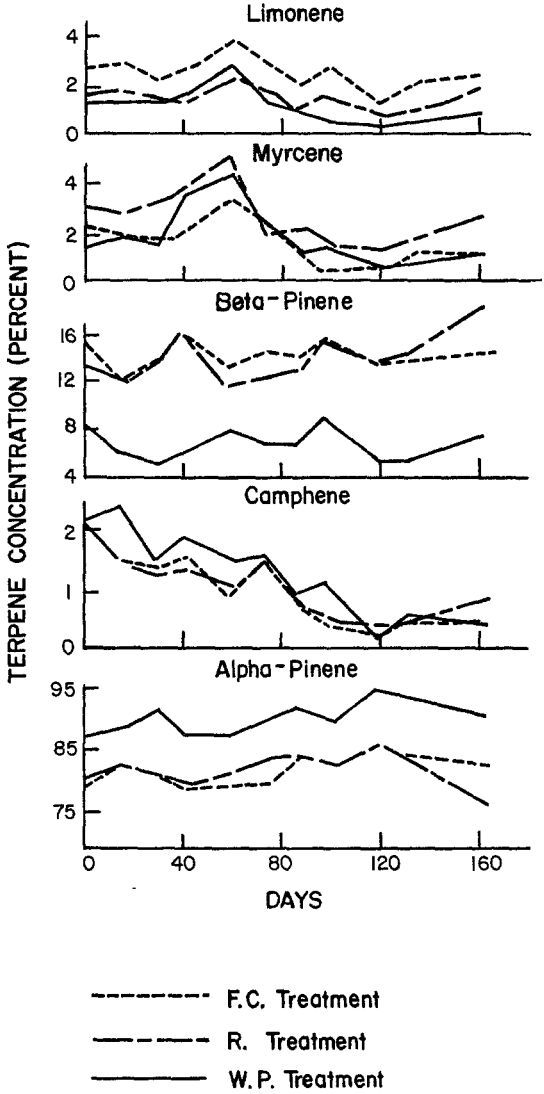

FIG. 3. Average terpene concentrations during the 1970 growing season. F.C. indicates field capacity of soil, R. refers to rainfall, and W.P. indicates wilting point of soil.

significant (5% level) change over time under high soil moisture conditions (Table 1, Figure 1).

The average concentration of α-pinene was higher in the oleoresin of

TABLE 2. AVERAGE MONOTERPENE CONCENTRATIONS OF LOBLOLLY PINE OLEORESIN DURING 1970 STUDY PERIOD, EACH VALUE REPRESENTS 77 SAMPLES

Monoterpene	Percent[a]		
	At wilting point	Soil moisture maintained near field capacity	By rainfall
α-Pinene	89.5	80.9	81.3
Camphene	1.3[b]	1.0	1.0
β-Pinene	6.3	14.0	13.7
Myrcene	1.8	1.6	2.6
Limonene	1.1	2.5	1.4

[a] Means in same horizontal line not underlined are different at the 1% level; camphene excluded.
[b] Percentages for wilting-point treatment different from field-capacity and rainfall treatment at 5% level.

those trees growing at low soil moisture content than trees growing at high soil moisture level. Conversely, all other monoterpenes were lower at the low soil moisture level.

1970 Test

Soil moisture contents for the field-capacity and rainfall plots exceeded field capacity at the time the first oleoresin samples were collected on May 15, 1970 (Figure 2). Moisture dropped rapidly in both plots during the first 20 days. After this period, moisture in the field-capacity plot stabilized around 31% whereas moisture in the rainfall plot decreased to about 18% after 50 days and then fluctuated, depending upon current rainfall. Soil moisture of the wilting-point plot was 22% when the first oleoresin samples were taken, but declined at a rapid rate during the following 55 days until it almost reached the wilting percentage. A further decrease in soil moisture was observed in this plot after 80 days, and it remained slightly below wilting percentage for the remainder of the study.

The average concentration for each monoterpene throughout the 1970 test is shown in Table 2 and Figure 3. There was considerable variation in concentrations of each monoterpene between trees in a treatment. Concentrations of each monoterpene fluctuated during the study period, but the only seasonal difference found was for camphene (significant at 1% level, $r = 0.69$). The concentrations of α-pinene, camphene, and β-pinene in the test were

affected only by extreme soil moisture stress, whereas the slight soil moisture stress on the rainfall plots influenced only the concentration of limonene. No pattern was detected between concentration of myrcene at different soil moisture levels. At the beginning of the test there were significant differences (5% level) between concentrations of monoterpenes found in the wilting-point treatment and monoterpenes found in the other two treatments except for camphene (Figure 3).

DISCUSSION

The wide range in concentrations of monoterpenes found in both tests is not surprising as a number of other workers have reported similar results for loblolly pine, as well as for other tree species (Hanover, 1966; Smith, 1967; Hilton, 1968; Rockwood, 1973).

Results from the 1968 test by themselves do not clearly indicate if the changes in concentration of monoterpenes over time (Figure 1) are due to soil moisture level differences alone. Phenological processes, a factor not adequately accounted for, might produce the same results irrespective of soil moisture differences. Some authors (Hilton, 1968) have indicated there is insignificant season variation in the monoterpene composition of xylem and cortical oleoresin in pines, whereas others (Rockwood, 1973) have reported contradictory findings. The fact that α-pinene concentrations begin to decrease and β-pinene concentrations begin to increase about two weeks prior to the time the soil was watered to field capacity in the 1968 test strongly suggest phenology to be a very important factor. These results tend to support Rockwood (1973), who reported similar results for these terpenes in loblolly pine. However, results from the 1970 test show that camphene was the only monoterpene in which concentrations changed during the season (Figure 3), and these results were from trees in the same plantation used in the 1968 test. The evidence seems to be more conclusive that soil moisture has a greater effect on monoterpene concentration in loblolly pine than phenological processes. The above conclusion agrees with Hodges and Lorio (1975), who found that moisture stress over a 3-year period results in a change in the α- and β-pinene composition of the xylem oleoresin of mature loblolly pine.

Methods used in expressing monoterpene composition can in some cases influence interpretations of the results (Squillace, 1976). The most accurate method is to use an internal standard and express each monoterpene as a percent by weight of the oleoresin sample. But trends and relative values are valid, however, when concentrations are expressed as percentages of the total monoterpenes concentration, providing the response factor (RF) of the monoterpene and standard are similar as they were in this study (Table 3).

TABLE 3. RESPONSE FACTORS (RF) BETWEEN INTERNAL STANDARD CUMENE AND DETECTED MONOTERPENES

Monoterpene	Response factor
α-Pinene	0.93
Camphene	0.83
β-Pinene	0.93
Myrcene	0.32
Limonene	0.86

The probable reason for the differences in concentrations of α-pinene, β-pinene, and limonene at the beginning of the 1970 test (Figure 3) is preconditioning. Trees used in this test had been subjected to these or similar treatments during a 4-year period prior to the beginning of the 1970 test.

CONCLUSIONS

This study demonstrates that soil moisture content can play an important role in influencing the monoterpene composition of loblolly pine, which may in turn influence other factors in the tree's environment. The direction of change in concentrations of monoterpenes attributed to internal stress in pine trees by various workers is not always consistent. It appears likely that the direction of concentration change is influenced not only by moisture stress in the tree but also by other factors such as tree age, competition, physiographic locations, genetics, and unknown environmental factors both within the tree as well as outside.

As has been pointed out in numerous reports, trees under moisture stress seem to be more susceptible to pine-bark beetles than nonstressed trees (Thatcher, 1960; Dixon & Osgood, 1961; Shrimpton, 1973). This susceptibility has been attributed to a decrease in the oleoresin exudation pressure and flow in southern pines (Lorio and Hodges, 1968), but it is also conceivable that chemical changes in the oleoresin of stressed trees influence beetle attack. At least a mixture of ethanol plus α-pinene has been shown to attract species of *Trypodendron* in West Germany and western Canada (Nijholt and Schönherr, 1976).

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MECHANISM OF FEEDING DISCRIMINATION BETWEEN MATURED AND JUVENILE FOLIAGE BY TWO SPECIES OF PINE SAWFLIES

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Abstract—Two species of pine sawflies, *Neodiprion rugifrons* Midd. and *N. swainei* Midd., feed only on matured foliage of jack pine, *Pinus banksiana* Lamb., and leave juvenile or current-season foliage intact. This unique form of adaptation was studied from the viewpoint of the chemical ecology of this insect–host plant relationship. It was first determined that the differential larval feeding behavior reflects the presence of feeding deterrents. Two major biologically active substances were isolated and identified as 13-keto-8(14)-podocarpen-18-oic acid and dehydroabietic acid. These account for 63.5% and 24.6% of the total deterrency, respectively. The content of the former substance in current-year foliage decreases, as the foliage begins to mature, to the levels that become acceptable to *N. swainei* by August (60 days old) and to the second generation *N. rugifrons* by September (90 days old). The timing of their acceptance of juvenile foliage indicates the high levels of adaptation by these insects to allow oviposition for the on-coming adults and acceptable needles for the next generation of larvae. The level of dehydroabietic acid, on the other hand, does not change appreciably during the same time period: this indicates that the component does not play a significant role in the mechanism of differentiating juvenile from matured foliage by the sawfly larvae.

Key Words—Feeding differentiation, pine sawflies, 13-keto-8(14)-podocarpen-18-oic acid, dehydroabietic acid, antifeedants, jack pine, adaptation of monophagous insects.

INTRODUCTION

Neodiprion sawflies, one of several genera belonging to the family *Diprionidae*

(Hymenoptera) tend to feed on foliage one or more years old rather than on current-year (juvenile) foliage of the family *Pinaceae* (Ross, 1955; Smith, 1974). Most of these sawflies occur in North America and cause serious widespread damage in the valuable coniferous forest (Benjamin and All, 1972). They have a long coevolutionary history that adapts them well to their specific host species (Knerer and Atwood, 1973).

Among over 30 species that feed on pine are two monophagous sawflies on jack pine (*Pinus banksiana* Lambert); *Neodiprion rugifrons* Middleton and *N. swainei* Midd. actively feed on matured pine foliage, while leaving current-season (juvenile) foliage intact early in the growing season (All et al., 1975. Wilkinson et al., 1966; Becker and Benjamin, 1964). Matured foliage is consumed by the first-generation *N. rugifrons* larvae in June and July, and if a second generation develops late in the growing season (September), the larvae begin to accept the current-season foliage. Similarly, *N. swainei* may feed on current-season foliage at the beginning of August and then only after the removal of old foliage by early-feeding larvae.

Recently, it has been shown that this preferential feeding behavior may occur because of the presence of feeding deterrents or antifeedants, and not because of the lack of specific feeding attractants or stimulants in juvenile foliage (All and Benjamin, 1975a–c; All et al., 1975).

The purpose of this study is to identify the chemicals involved in the mechanism of larval differentiation of juvenile from matured foliage, and thereby to understand the ecological meaning of this unique coevolution of the insect–host plant relationship.

METHODS AND MATERIALS

Source of Pine Sawfly Larvae. First-generation *N. swainei* larvae and first- and second-generation *N. rugifrons* larvae were field collected on jack pines at Arena and Mondovi, Wisconsin, respectively, and used in the laboratory bioassays unless otherwise noted in the text. Also, third- to fifth-generation *N. rugifrons* were used in the bioassays. These were from first or second instar larvae collected in the field and reared in incubators at 18 hr photoperiod and 24°C. Cocooned larvae were placed individually in gelatin capsules and held at the same conditions as the larvae. Adults emerged approximately 3 weeks later; two or three each of males and females were confined in small cages on fresh jack-pine foliage one or more years old. Adults mated and eggs were laid within two days; larvae were reared in the manner described above (Brelje, 1970).

Bioassay (Choice Tests). A 7–10-cm twig of mature jack-pine foliage was rinsed with distilled water and separated in the middle by removing

needles to leave 10 pairs of needles at both ends. Individual needles on one end were topically treated by pipetting several drops of the solution of new needle extract at the needle base and allowing it to drip down to the needle tip; needles on the other end were treated in the same manner with the same solvent as used on treated end. Approximately 0.2 ml of solution was needed to coat test needles. The twig was allowed to dry at room temperature for more than 30 min before bioassay testing.

Chemically treated test twigs were placed into $6 \times 7 \times 21$-cm plastic containers. Plain green index cards were laid on the floor of the container. Ten larvae (instar 3–5) were placed on needles on each end of the twig and their behavior was observed. These observations continued until most larvae (more than 80%) started feeding on one or both ends. The number of larvae on both ends were counted at least every 30 min. The ratio of the number of larvae on the untreated end vs. total larvae suggested the strength of the biological activity. The test fraction was recognized to have deterrent activity when more than 70% of the total number of larvae moved away from the treated end and settled on the untreated end.

Bioassay under Laboratory Conditions. The fresh branches having six twigs of mature foliage were placed in a 500-ml paper cup filled with water to carry out this bioassay. A 10-ml aliquot (a few drops of ethanol and the rest of water) of compound A (1 mg/ml concentration) was sprayed over both larvae (20, third instar *N. swainei*) and the entire branches. In the case of *N. rugifrons*, a 5-ml aliquot of compound A with same concentration (1 mg/ml) was sprayed in the same manner. In all tests larvae were placed on the mature foliage adjacent to the new foliage.

Bioassay under Field Conditions. The field tests were conducted during the middle of July 1975 at Arena (Iowa Co.), Wisconsin. Two isolated young trees (approx. 2 m high) were selected in the natural jack-pine stand. Four to five colonies (16–43 larvae/colony) were established randomly on the old foliage. Areas approx. 1 m^2 under both trees were cleared off by removing grasses. After larvae (*N. swainei*, 2–3 instar) had established feeding on mature foliage for 24 hr, a 30-ml (5 ml EtOH and 25 ml H_2O) aliquot of substance A (2 mg/ml concentration) was evenly sprayed over the entire tree. The same amounts of water and ethanol also were sprayed on another tree for control.

Column, Thin-Layer, and Gas Chromatography. For column chromatography of both *n*-hexane and ethyl acetate extracts, columns (4×60 cm) of silicic acid (450 g, Fisher Scientific Co.) were used. For TLC silica gel HF_{254} (Brinkmann Instruments, Inc.) was used throughout. The plates coated with 0.25-mm thickness were used for analysis and those with 0.75-mm thickness utilized for preparative purposes. All TLC plates were activated at 125°C for 4 hr. The gas chromatography was carried out at 193°C and 222°C with

the helium flow rate of 30 ml/min by using F & M 5370 model with a FID detector.

Spectroscopy. Infrared spectra were recorded on a Beckman IR-33 spectrometer and calibrated with the bands of a polystyrene film. Proton magnetic resonance (PMR) spectra were recorded either on a Bruker HX-90E (90 MHz) or Hitachi R-24 (60 MHz) spectrometer. Ultraviolet spectra were determined with a Varian 635 spectrophotometer, and mass spectra with a Finnigan 1015 model.

RESULTS

Extraction and Isolation of Antifeedants from Juvenile Jack-Pine Foliage

Fresh juvenile jack-pine needles (2.880 kg) under 1 cm long were collected from the University of Wisconsin Arboretum on June 10, 1975. They were extracted three times with methanol (3 × 18 liters) by first immersing them for 6 days and then washing them twice with equal amounts of methanol, filtered and concentrated in vacuo (45°C) to give the aqueous concentrates

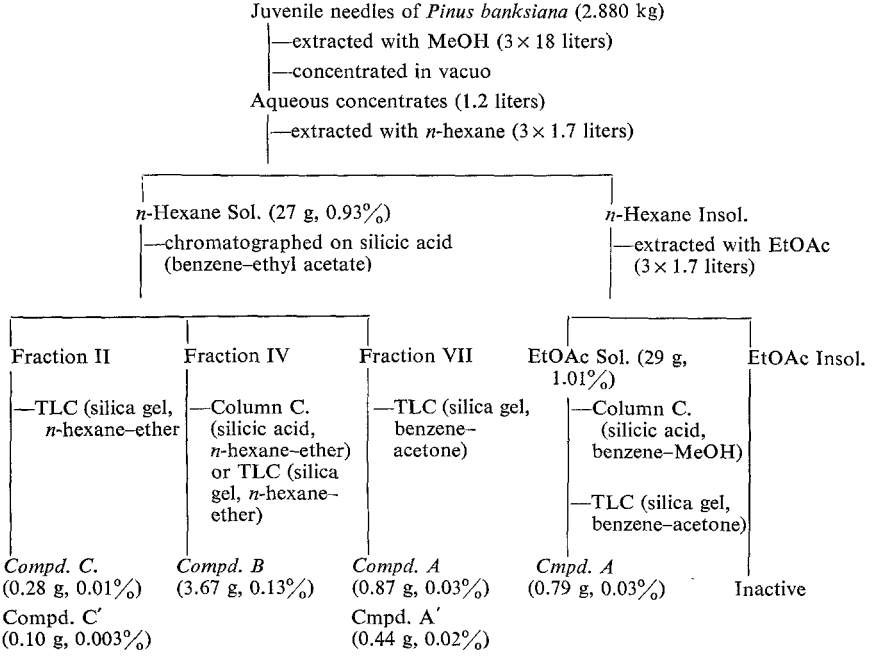

FIG. 1. Extraction and isolation procedure of antifeedants. The yields are expressed in % fresh needle weight equivalent.

TABLE 1. ELUTION PATTERN OF ANTIFEEDANTS IN *n*-HEXANE AND ETHYL ACETATE EXTRACT FROM SILICIC ACID COLUMN[a]

Fract. No.[b]	Vol. eluted	Active principle	Dilution of the concentrate (no. of exp.)			
			N. rugifrons[d]		*N. swainei*	
n-Hexane extract			10-fold (2)	40-fold (3)	5-fold (3)	20-fold (4)
N-I	500		−	−	−	−
II	250	C, C′	+	+	+	−
III	250		−	−	−	−
IV	250	B	++	++	++	+
V	250		−	−	−	−
VI	250		+	+	−	−
VII	250	A, A′	++	++	++	+
VIII	250		−	−	−	−
IX	250		+	+	−	−
Ethyl acetate extract[c]			10-fold (2)[e]	20-fold (2)[e]	10-fold (2)	20-fold (2)
E-I	500	C and/or C′	−	−	+	+
II	500		−	−	−	−
III	500		−	−	−	−
IV	500	A	+	−	++	+
V	500		−	−	−	−
VI	500		−	−	−	−

[a] All the elutes were first concentrated through evaporation to 20 ml and dilution series were prepared by using fresh *n*-hexane or ethyl acetate.
[b] I: benzene; II, III: benzene–EtOAc (10:1), IV, V: same mixture (4:1); VI, VII: same mixture (2:1); and VIII, IX: EtOAc.
[c] I: benzene–MeOH (10:1), II: same mixture (8:1), III: (4:1), IV: (2:1), V: (1:1), VI: MeOH.
[d] Fourth generation of *N. rugifrons* reared in the laboratory was used for assays.
[e] Fifth generation of *N. rugifrons* reared in laboratory was used for assays.

(1.2 liters) (Figure 1). The concentrate, which contained suspended solids and emulsion, was further extracted three times each with *n*-hexane (3 × 1.7 litres) and ethyl acetate (3 × 1.7 liters), successively. The yields of *n*-hexane and ethyl acetate extracts were 0.94% and 1.01% of original foliage by weight, respectively. Preliminary bioassay tests conducted in 1974 for methanol extract, which was successively extracted with *n*-hexane and ethyl acetate, showed that the biological activity occurred in methanol, *n*-hexane, and ethyl acetate extracts but not in remaining water phase.

Twenty grams of *n*-hexane extract was chromatographed on silicic acid (450 g) and eluted with several solvent mixtures as shown in Table 1. Each fraction was concentrated to 20 ml and bioassayed. Twenty grams of ethyl

TABLE 2. TLC FRACTIONATION OF FRACTION N-II OF *n*-HEXANE EXTRACT

Active principle (R_f)	Concentration (mg/ml)	Activity (no. of exp.)	
		N. rugifrons	*N. swainei*
C′ (0.55–0.65)	5.0	− (4)	++ (7)
	2.0	− (5)	
	1.5	− (2)	
C (0.45–0.55)	14.0	+ (4)	(+) (8)
	5.6	+ (8)	
	1.9	+ (3)	
	1.1	(+)(4)	

TABLE 3. COLUMN CHROMATOGRAPHY OF FRACTION N-IV OF *n*-HEXANE EXTRACT

Fraction[a]	Active principle	Vol. eluted (ml)	Yield (%)	Activity[b]	No. of exp.
N-IV-1		500	0.1	−	5
2		200	17.8	−	5
3	B	200	35.0	+	5
4	B	200	24.4	+	5
5		200	1.0	−	5
6		200	0.9	−	5
7		200	2.1	−	5
8		200	3.5	−	5
Total recovery			84.0		

[a] IV-1: *n*-hexane–ether 10:1, IV-2–8: same mixture 5:1.
[b] Fourth generation of *N. rugifrons* was used for assays.

acetate extract also was chromatographed on silicic acid (450 g) (Table 1). Each fraction was concentrated to 20 ml and bioassayed. Three fractions, N-II, N-IV, and N-VII, of *n*-hexane extract and two fractions, E-I and E-IV, of ethyl acetate extract showed biological activity. When tested with fifth-generation *N. rugifrons*, only fraction N-VII showed strong activity at a 40-fold dilution of the above concentrate (3 replicates). Fractions N-II, N-IV, N-VII, and E-IV were further separated on column and/or thin-layer chromatography.

TABLE 4. TLC OF FRACTION N-VII OF *n*-HEXANE EXTRACT

R_f [a]	Active principle	Yield[b] (%)	Dilution (no. of exp.)[c] 1-fold	2-fold	4-fold	8-fold	16-fold
0.6–0.7		1.7	− (4)				
0.5–0.6		3.1	− (4)				
0.4–0.5		17.3	− (4)				
0.3–0.4	A	30.0	++ (4)	++ (2)	+ (2)	+ (2)	− (2)
0.2–0.3	A′	13.3	++ (4)	++ (2)	−(2)		
0.1–0.2		13.2	+ (4)	− (2)			
Total recovery		74.6					

[a] Solvent system used is benzene–acetone (2:1).
[b] Each yield is shown as % of the amounts of fraction N-VII originally spotted on TLC.
[c] Fourth generation of *N. rugifrons* was used for the bioassay.

Fraction N-II was chromatographed on TLC (HF_{254}) developed with *n*-hexane–ether (3:1), and then was sectioned into two parts (Table 2). The upper fraction (R_f 0.55–0.65) was designated C′ as the active principle, since it showed activity against *N. swainei* at a concentration of 5 mg/ml, but not against *N. rugifrons*. On the other hand, the lower fraction (R_f 0.45–0.55) designated as C, was strongly active against *N. rugifrons*. Neither fraction, each consisting of several compounds, was further examined for chemical identities as their overall contribution for deterrency was not great.

Fraction N-IV (4 g) was chromatographed on silicic acid (200 g) and eluted with the mixture of *n*-hexane–ether (Table 3). Each eluate was concentrated to 25 ml and bioassayed. Fractions 3 and 4, with activity, were evaporated by a stream of nitrogen, dissolved in *n*-hexane, and allowed to stand overnight at 5°C to yield the crystalline compound (B); this later was found to be identical to the authentic reference sample of dehydroabietic acid (Figure 5, III). The compound in these two biologically active fractions, 3 and 4, was designated as B and is the active principle (Table 3).

A part (200 mg) of fraction N-VII of *n*-hexane extract was chromatographed on TLC (silica gel HF_{254}) developed with benzene–acetone (2:1) as the mobile phase. The plate was sectioned into six parts and each part was scraped off and extracted with 10 ml of ethanol and bioassayed (Table 4). The fraction having R_f value of 0.3–0.4 showed strong absorption under a UV lamp and extremely strong biological activity. The fraction consisted of a pure-compound designated as A. From the column fraction E-IV of the ethyl acetate extract, the same compound (A), which was not sufficiently extracted with *n*-hexane, was recovered. It was the active principle of the ethyl acetate

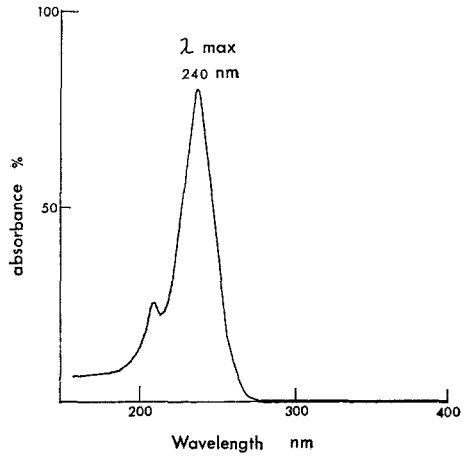

FIG. 2. UV spectrum of compound A methyl ester.

extract. The total yield of compound A (Ia) and B (dehydroabietic acid, III) was 0.055 and 0.128% of original amount of juvenile needles, respectively.

Identification of Compound A

Compound A was detected as a blue spot under UV lamp on a thin-layer plate of silica gel HF_{254}. The R_f value was 0.35 with the solvent system

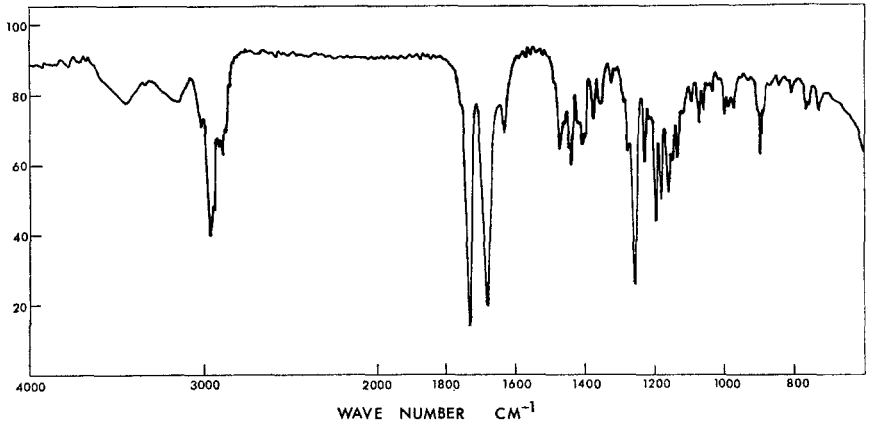

FIG. 3. IR spectrum of compound A methyl ester.

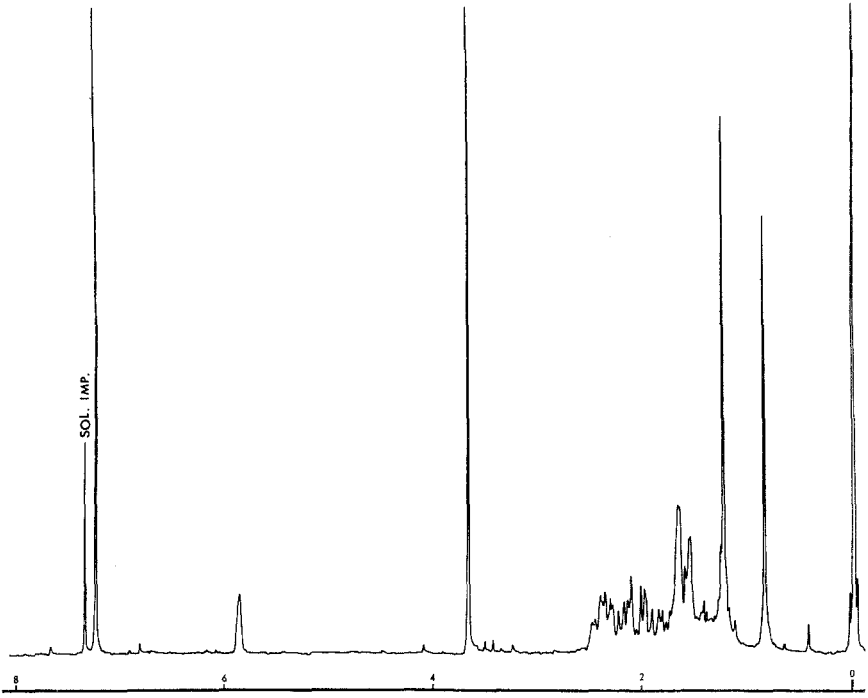

FIG. 4. PMR spectrum of compound A methyl ester.

benzene–acetone (2:1), and 0.25 with $CHCl_3$–MeOH (9:1). The UV absorption (in EtOH) at 242 nm ($\varepsilon = 1.2 \times 10^4$) of compound A, and at 240 nm ($\varepsilon = 8.3 \times 10^3$, Figure 2) of the methyl ester suggested the presence of an α, β-unsaturated ketone moiety. This also was confirmed by the appearance of bands at 1725 and 1675 cm^{-1} in the IR spectrum and a broad singlet at 5.92 ppm (1H, s) in the PMR spectrum of compound A. The broad IR absorption band at 3600–3400 cm^{-1}, which disappeared on methylation with diazomethane, indicated the presence of a carboxylic acid moiety (Figure 3). The methyl ester showed a carboxylic methyl protons at 3.681 ppm (3H, s) and also protons for two tertiary methyls at 1.231 and 0.849 ppm in its PMR (Figure 4). The mass spectrum of the methyl ester showed a molecular ion peak at 290 *m/e* ($C_{18}H_{26}O_3$) and also major fragment peaks at 259 (M-OCH_3) and 231 *m/e* (M-$COOCH_3$). From these data the chemical structure of compound A is proposed as 13-keto-8(14)-podocarpen-18-oic acid (Ia, Figure 5). The instrumental data of compound A (Ia) and its methyl ester (Ib) are shown in Table 5.

The proposed structure of the methyl ester (Ib) was further confirmed by comparing the chromatographic and spectroscopic data of the standard

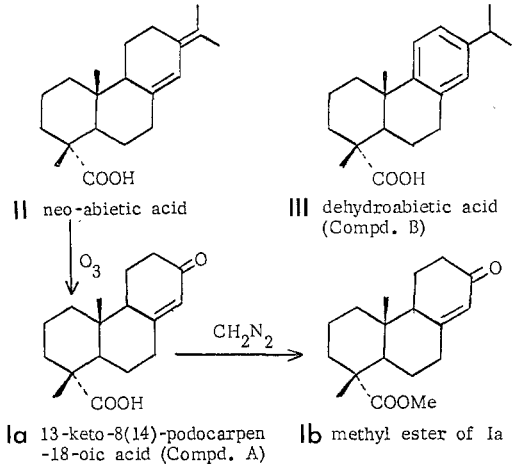

FIG. 5. Partial synthesis and methylation of compound A

sample (supplied by Dr. D.F. Zinkel). It showed the same R_f values (0.70 on silica gel HF_{254} eluted with $CHCl_3$–MeOH 9:1, 0.35 with benzene–acetone 4:1, and 0.20 with benzene–EtOAc 7:1) and the same retention times (45 and 48 min on SE 30/EGIP at 193°C and on EGSS-Y at 222°C, respectively) as the methyl ester of compound A. Moreover, IR, PMR and mass data all were identical. The identical IR and PMR data were supplied by Dr. S.W. Pelletier.

Partial Ozonolysis of Neoabietic Acid (Figure 5, II)

To synthesize compound A, neoabietic acid (II, 2.0 g) was treated with ozonized oxygen in ethyl acetate (25 ml) at −70° for 30 min (Burgstahler and

TABLE 5. INSTRUMENTAL DATA OF COMPOUND A (Ia) AND ITS METHYL ESTER (Ib)

Spectrum	Unit	Ia	Ib
UV	EtOH λ_{max} (mm)	242 ($\varepsilon = 1.2 \times 10^4$)	240 ($\varepsilon = 8.3 \times 10^3$)
IR	υ_{max} (cm^{-1})	3600–3400 (broad), 1725, 1675, 1465, 1265	1735, 1680, 1255, 895
PMR	δ (ppm $CDCl_3$)	0.86 (3H, s), 1.21 (3H, s) 5.92 (1H, s)	0.85 (3H, s), 1.23 (3H, s) 3.68 (3H, s), 5.87 (1H, s)
Mass	(*m/e*)		290 (M^+), 259, 231, 181, 121, 110

TABLE 6. FEEDING RESPONSE OF *N. rugifrons* AND *N. swainei* ON MATURE FOLIAGE TREATED WITH SYNTHETIC COMPOUND A

Concentration (mg/ml)	Activity %[a] (no. of exp.)	
	N. rugifrons	*N. swainei*
10.0	++ 100.0 (1)	
5.0	++ 100.0 (1)	++ 85.8 (6)
1.0	++ 89.2 (5)	+ 75.0 (9)
0.5	+ 74.0 (10)	− 63.5 (7)
0.2	(+) 66.7 (3)	− 58.3 (3)
0.1	− 27.5 (2)	

[a] Ratio of the number of larvae on untreated end vs. total number larvae.

Worden, 1964). The progress reactions were checked every 5 min by microscale TLC (silica gel HF_{254}, $CHCl_3$–benzene 10:1) to prevent excess reactions. The reaction mixture was allowed to stand at room temperature for 30 min, and then treated with 2% KI (25 ml) and a few drops of methanol. The solvent phase was washed with sodium thiosulfate followed with H_2O, dried over sodium sulfate, and evaporated to give a yellow-colored oil. The mixture was chromatographed on a silicic acid (100 g) column eluted with $CHCl_3$–MeOH (20:1) by collecting a 20-ml eluate. Fraction 5 was condensed and purified on

TABLE 7. ANTIFEEDING ACTIVITY OF COMPOUND B (DEHYDROABIETIC ACID)

Concentration (mg)	Activity (no. of exp.)	
	N. rugifrons[a]	*N. swainei*
4.0	++ (4)	
3.0	++ (7)	++ (4)
1.5	− (4)	+ (4)
1.0		− (4)
0.75	− (4)	
0.38	− (2)	

[a] Fourth generation of larvae reared in the laboratory was used for the bioassay.

TABLE 8. FEEDING RESPONSE OF *N. rugifrons* AND *N. swainei* TO COMPOUND A IN LABORATORY CONDITIONS

Hr elapsed	*N. rugifrons* (20)	*N. swainei* (20)	Control (20)
1.0		0[a]	0[b]
2.3	0[a]	4[a]	8
4.0	0	>10	>10
5.5	0	>10	>10
8.0	0	>10	>10
22.0	>10	>10	>10

[a] The colony was observed to be broken up.
[b] Figures indicate the numbers of larvae feeding on mature jack-pine foliage.

TLC (benzene–acetone 3:1) to yield 230 mg of pure ketoacid (Ia), which was identical with compound A (Figure 5). The synthesized compound A has been used for bioassay tests both in laboratory and field conditions.

Biological Activity of Antifeedants. In bioassay choice tests, *N. rugifrons* exhibited antifeeding activity at a concentration of 0.75 mg/ml (corresponding to eight-fold diluted fraction of R_f 0.4–0.3 in Table 4) of isolated compound A. The minimum concentration (0.2–0.5 mg/ml) of synthetic compound A to deter the larvae was practically indistinguishable with that (0.38–0.75 mg/ml) of isolated natural product (Table 6). When comparing the response of *N.*

TABLE 9. FEEDING RESPONSE OF *N. swainei* TO COMPOUND A IN FIELD CONDITIONS

Hr elapsed	No. of colony feeding	
	Treated	Control
3	0/5[a]	3/4[b]
6	0/5	3/4
24	3/5	4/4
51	5/5	4/4

[a] All colonies were observed to be broken up.
[b] The figure 3/4 shows 3 out of 4 colonies of larvae fed on mature foliage.

rugifrons and *N. swainei* to compound A, the former was more sensitive than the latter (Table 6). This situation, however, was reversed with compound B (III, Table 7).

Under laboratory conditions, the colonizing activity of *N. rugifrons* was disturbed for up to 3 hr and larval feeding ceased for up to 8 hr by compound A (Table 8). In the case of *N. swainei* the overall response was similar to that of *rugifrons* except that the compound A was less disturbing to *swainei* than to *rugifrons* based upon minimum effective concentrations of A (1 mg/ml) against *N. swainei*. This also agrees well with the results of choice tests (Table 6). Under field conditions (Table 9), all colonies on sprayed branches were disturbed for at least 3 hr and the larvae did not feed during the initial 6 hr. After 24 hr, approximately half the larvae were found on the new foliage, but they did not feed. By contrast, larvae on the control tree fed normally, and colony-forming behavior was normal within 3 hr of the time of treatment.

In the above fractionation series, four fractions designated as A, A′, B, and C caused varying degrees of antifeeding activity against *N. rugifrons*. Two of them (A and B) were isolated in pure form while others were not. The question here is how many compounds (or fractions) among these four active principles really affect the larvae as the antifeedant in nature, since at large enough doses, many normal components in the mature foliage could act as antifeedants.

To study this problem, the relative contribution was first evaluated by calculating an activity index; this was obtained by dividing the total yields by the activity (minimum effective concentration) of each fraction. It is evident that dipterpenoid acids, A and B, possess antifeeding principles responsible together for 88% (63.5 and 24.6%, respectively) of antifeeding properties of the juvenile foliage against *N. rugifrons* (Table 10).

Seasonal Variation of the Levels of Compounds A and B. To investigate the seasonal variation of the yields of compound A in jack-pine foliage, 100 g

TABLE 10. RELATIVE CONTRIBUTION OF ANTIFEEDING COMPOUNDS IN JUVENILE JACK-PINE FOLIAGE TO *N. rugifrons*

Active principles	Yields (%)	Activity (mg/ml)	Yields/Activity ($\times 10^3$)	Contribution (%)
A	0.055	0.5	110.0	63.5
A′[a]	0.015	1.3	11.5	6.6
B	0.128	3.0	42.7	24.6
C	0.010	1.1	9.1	5.3

[a] Extract of the TLC band (R_f 0.2–0.3) in Table 5.

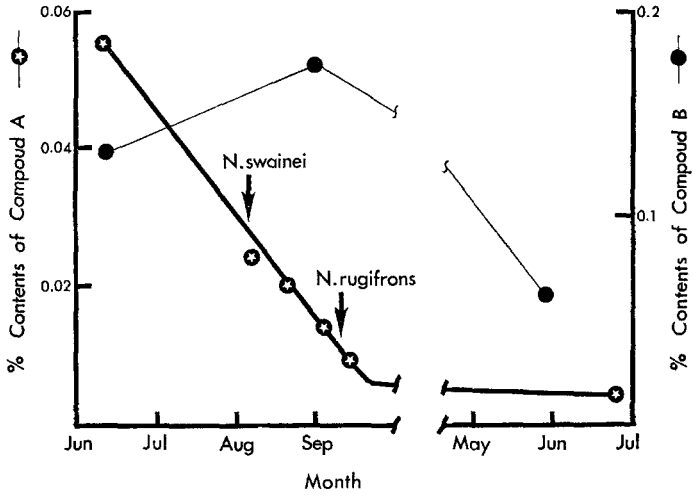

FIG. 6. Seasonal variations of contents of compounds A and B.

of needles were collected periodically at the same location in the University of Wisconsin Arboretum and were extracted with 500 ml of MeOH. Methanol extracts were condensed and chromatographed on TLC and developed with the solvent system of benzene–acetone (2:1). The portion containing compound A was scraped off and treated with diazomethane to form the methyl ester, and then chromatographed again on TLC with the same solvent system. The band corresponding to the methyl ester of A was clearly separated from other materials on TLC. For compound B, on the other hand, 50 g of needles were collected each time and extracted with 300 ml of MeOH. Methanol extracts were concentrated and extracted with ether (125 ml), then dried over anhydrous sodium sulfate. The condensed ether extracts were treated with diazomethane and chromatographed on TLC with the solvent system of *n*-hexane–ethyl acetate (4:1) to obtain a pure methyl ester of compound B.

The contents of compound A in one growing season current year foliage decrease to one seventh by the middle of September and to less than one tenth in a year, while those of compound B slightly increased in September and then decreased to one half over a one-year period (Figure 6).

DISCUSSION

N. rugifrons and *N. swainei* begin feeding on juvenile jack-pine foliage at a stage of foliage development that is different for each species (All and

Benjamin, 1975b); *N. swainei* begins acceptance in early August, and *N. rugifrons* in mid-September. We suggest that this behavior reflects the change of contents of certain chemical components (antifeedants) present in current year foliage. The difference in sensitivity of the two sawfly species for current-year foliage (or its methanol extract) are indicated in Table 1. Actually, the concentration of the antifeedant, compound A, in current-year foliage decreases remarkably during the growing season (Figure 6). Another antifeedant, compound B, on the other hand, does not show such a notable seasonal change of concentration as compound A. This indicates that compound A mainly determines the acceptability of new foliage phenologically for both species.

In comparing the biological activity of compound A with *N. rugifrons* and *N. swainei*, we observed that the former species is more sensitive to compound A in choice tests; the minimum effective concentration was in the order of 0.2–0.5 mg/ml against the former species, and 1.0 mg/ml against the latter (Table 6). In relating the laboratory data on the minimum effective dose to the contents of the antifeedant in the naturally occurring foliage the following calculation is necessary. Since in choice tests 0.2 ml of solution was used to coat 10 pairs of pine needles weighing 800 mg, the dose of the minimum effective concentration (1 mg/ml) against *N. swainei* corresponds to 0.025% of the needle weight. This value coincides well with the natural concentration of compound A (the beginning of August), when *N. swainie* begins to accept the current-year foliage (Figure 6). The corresponding minimum effective dose stimulating *N. rugifrons* was 0.2–0.5 mg/ml, which corresponds to 0.005–0.0125%, indicating that current-season foliage will reach that content of compound A at sometime in September when the second-generation larvae of *N. rugifrons* begin naturally to accept the current-year foliage. It is likely that the decrease in the amount of antifeeding compound A in current-year foliage alone permits the larvae to accept it late in the growing season.

Compound A, 13-keto-8(14)-podocarpen-18-oic acid (Ia), has not been reported previously as a naturally occurring substance. The compound is characterized by the presence of two functional groups, a carboxylic acid and an α, β-unsaturated ketone, in the podocarpen ring structure. Although compound A had strong biological activity at 0.2–0.5 mg/ml, its methyl ester (Ib) did not show activity, even at the high concentration of 5 mg/ml. It is likely that the carboxylic acid moiety is one of the most effective functional groups inhibiting larval feeding (Figure 5). The second active compound B (dehydroabietic acid, III), showed activity at the concentration of 3 mg/ml, while two closely related resin acids, neoabietic acid (II) and abietic acid, had no activity at concentrations of less than 8 mg/ml despite the presence of

the carboxylic acid moiety and the overall similarity of the ring structure to compound B.

It has been well documented that plants produce allelochemics, often referred to as secondary plant chemicals, and that these chemicals play key roles in insect–plant relationships (Beck and Reese, 1975). These substances may originally have acted as defensive substances for the plants, but some insects have evolved to utilize such substances as kairomones to aid their host selection. According to Feeny (1976) and Rhoades and Cates (1976), ephemeral plants, particularly the ones difficult to find (i.e., "unapparent" plants), tend to produce specific toxic substances, and perennial and easy-to-find plants (i.e., "apparent" plants) tend to produce large quantities of deterrents that are not palatable to many biological systems. Certainly, pines belong to the latter category. They are known to produce large quantities of resin acids that deter feeding by most animals. Diprionid sawflies are one of the few defoliator groups that could crash through this barrier. Evolution of these specialized plant feeders (i.e., "specialists") on "apparent" plants requires a well-timed and well-adapted mode of life on the part of the insects, since such specialists must depend upon the availability of limited plant materials both in time and space. The tendency is expected to be more pronounced for monophagous species. Our observation, that there is a single chemical that controls the feeding selection of these specialized insects, can be understood within this framework of plant–insect relationship. It has been said often that the existence of a single chemical factor responsible for defense in a plant against insect pests is not likely. However, in this case, the chemical is meant for specialists as explained above and, moreover, involves only minor changes in chemical structure from an ordinary resin acid that already has been produced in a large quantity against generalist pests.

Assuming that production of these defensive chemicals requires metabolic energy, it can be understood why the plants evolve such a minimum defensive change against specialists. At the same time such consideration for plants' economy budgeting for metabolic energy gives a clue to how they evolved to limit the production of compound A to early stages of juvenile foliage, which is relatively more vulnerable and indispensable for the plant than mature foliage.

The resulting plant–host relationship in the case of these *Neodiprion* sawflies (there are at least nine species that show such a preferential feeding habit) is a mutually beneficial one. It offers ample opportunity for the host tree to recover and resume further growth. In turn, by limiting their feeding, the sawflies are able to assure the availability of feeding materials for the next season for their off-spring.

It has often been suggested that phytophagous insect pests of the forest must develop a long-term relationship with their host plants to ensure

consistent plant and insect survival (Mattson and Addy, 1975). Our observation on two species of *Neodiprion* sawflies appears to be consistent with such a general viewpoint.

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FOVEAL GLANDS, SOURCE OF SEX PHEROMONE PRODUCTION IN THE IXODID TICK *Dermacentor andersoni* STILES[1]

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Abstract—The foveal glands of the Rocky Mountain wood tick, *Dermacentor andersoni* Stiles, are the sex pheromone glands from which the sex pheromone is released via the foveae dorsales. The sex pheromone, 2,6-dichlorophenol, was recovered from extracts of these glands by GLC. Other evidence of the role of these glands in sex pheromone production is described. A ^{36}Cl-labelled volatile compound (or compounds) was (were) collected from partially engorged female *D. andersoni* fed in ^{36}Cl-labelled hosts, but no labelled compounds were collected when the foveae dorsales were blocked. X-ray analysis revealed unusual concentrations of chlorine in the foveal glands compared to other tissues. Autoradiography also revealed significant accumulations of radiochlorine in the vicinity of these glands.

Presumably, the foveal glands of the American dog tick, *Dermacentor variabilis* (Say), are the sex pheromone glands of that species also, since a ^{36}Cl-labelled volatile was collected from female ticks fed on a ^{36}Cl-labelled host. However, attempts to recover 2,6-dichlorophenol from gland extracts or volatile emissions from *D. variabilis* were unsuccessful.

Key Words—Foveal glands, sex pheromone gland, 2,6-dichlorophenol, *Dermacenter andersoni*, *Dermacentor variabilis*, tick, Ixodidae.

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INTRODUCTION

The foveae dorsales are paired structures containing clusters of minute cuticular pores; the associated foveal glands have been implicated in sex pheromone activity in ixodid ticks (Sonenshine et al., 1974; Layton and Sonenshine, 1975). However, conclusive evidence of the role of these tick body structures was lacking. Other studies of sex pheromones in ticks failed to identify the origin of the active compounds, or ignored this question (Berger, 1972, 1974; Chow et al., 1975; Gladney et al., 1974; Sonenshine et al., 1976; Wood et al., 1976).

This paper reports new evidence for the role of the foveal glands as the site of sex pheromone activity in the ixodid tick *Dermacentor andersoni* Stiles. The resemblance in foveal gland structure between *D. andersoni* and *D. variabilis* suggests a similar function for the foveal glands in the latter species.

METHODS AND MATERIALS

The Rocky Mountain wood tick, *D. andersoni*, was colonized with specimens obtained near Hamilton, Montana, and furnished by personnel of the U.S. Public Health Service, Rocky Mountain Laboratory, Hamilton, Montana. The American dog tick, *D. variabilis*, used in these studies 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 were fed on laboratory rabbits (*Oryctalagus cuniculi*) or on dogs. Ticks and developing eggs were held in an Aminco-Aire Climate Lab (American Instrument Co., Silver Spring, Maryland) at 27 ± 1°C. and $95 \pm 2\%$ relative humidity during the nonparasitic periods of their development.

Foveal Glands. These were removed by dissection of partially engorged female ticks of both species (fed 5 or 6 days) and placed in cold saline or fixative (2% glutaraldehyde). A sample of 42 foveal glands and attached cuticle fragments was collected in cold saline and held for GLC analysis. Other samples were dehydrated gradually in ethyl alcohol–water solutions, pure ethyl alcohol, Freon TF and Freon-13 (Dupont Co., Wilmington, Delaware), and dehydrated further in a critical-point drying apparatus, Bomar model SPC-900, prior to examination with a Joel Co. model JSM U-3 scanning electron microscope (SEM). Specimens were coated with 100–200 Å of gold with a Technics, Inc. (Alexandria, Virginia) sputter-type vacuum evaporator prior to SEM examination. An analysis of the elemental contents of one lobe of the gland of *D. andersoni* in an area of 0.1 μm^2 was made for 400 sec with an EDAX Energy Dispersive X-ray analyzer model 505 and Teletype Interface model 604. The analysis was repeated with a different lobe of the gland and with unrelated muscle and tracheal segments. The analyzer

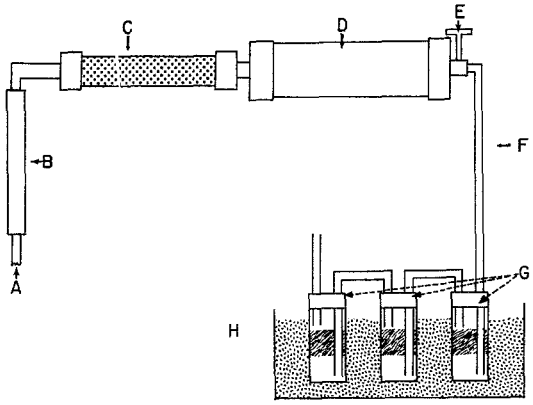


FIG. 1. Diagrammatic sketch of the emission-collecting apparatus for trapping the sex pheromone emitted by female ticks. A = compressed air flow, B = air flow meter, C = stainless-steel chamber containing activated charcoal filter, D = stainless-steel chamber for confining partially engorged female ticks, E = valve to allow escape of gases from the tick chamber, F = stainless-steel tube to collecting vials, G = gas trap consisting of glass vials, connected in series, containing pentane or hexane in the upper layer (wavy lines), 2% NAOH in the lower layer (clear area), H = ice bath to cool collecting vials. Stainless-steel tubing and swage-lock fittings were used between parts B, C, and D; Teflon tape was used to seal the fittings.

presents counts of the photon emissions from the electron shells and identifies the energy peaks of the different elements present. Known elemental energy peaks were compared with those peaks detected by the analyzer to facilitate identification. A computer program relating photon counts and energy peaks of the dominant elements present was used to compute a ratio of their relative abundance. The comparison was limited to gold (used to coat the specimens) and chlorine.

Collections of Volatiles from Partially Engorged Female Ticks. Partially engorged *D. andersoni* female ticks were detached forcibly and placed in the stainless-steel tick chamber of the collecting apparatus (Figure 1). Charcoal-filtered air was passed over the ticks for 5-min periods at the rate of 50–100 ml/min and into a series of collecting vials containing triple-distilled pentane or hexane. The vials were cooled with ice to minimize evaporation. Bubbling of the solvent provided visual confirmation of air flow. Collections of the effluent air were done intermittently at 2- or 3-hr intervals to minimize

evaporation of solvent from the collecting vials. The terminal openings of the collecting vials were sealed with Teflon tape between collections. In some cases, a layer of 2% NaOH was deposited under the hexane or pentane to enhance collection of phenolic compounds. These mixtures were treated with CO_2 gas to regenerate phenols. After termination of the collections, the solvent was analyzed by GLC (electron-capture detector) for the presence of 2,6-dichlorophenol. As a control, filter paper that had been treated with a solution of purified 2,6-dichlorophenol in hexane was substituted for the female ticks, and the collection procedures were repeated. The control was replicated with 0.658, 0.94 (2×), 8.8, and 35.2 μg of 2,6-dichlorophenol. After these experiments with normal hosts, female ticks were allowed to feed on a rabbit inoculated with 100 μg of $Na^{36}Cl$ (Amersham-Searle, Arlington Heights, Illinois; 3 mCi/g Cl) administered in daily increments of 20 μCi/day. Fifty females fed on a radiolabeled rabbit were confined in the collection apparatus, and collections were made as described above. After termination of the collection procedure, samples of the capture solvent were dissolved in PCS cocktails (Amersham-Searle) and radioassays for the presence of ^{36}Cl were done with a Beckman LS-250 scintillation spectrometer (Beckman Instruments, Fullerton, California). Samples were recounted at least 3 times during a 2-week period to minimize errors due to chemiluminescence or photoluminescence. To determine whether presumed sex pheromone emissions were associated with the foveae dorsales, the experiment was repeated with female ticks in which these body sites were sealed with lacquer. Finally, other ticks fed on a radiolabeled rabbit were held for autoradiographic studies and radioassay of GLC fractions from extracts obtained by the sonic freeze method (Sonenshine et al., 1976).

Gas–Liquid Chromatography. Aliquots of tick materials were injected on a Varian model 2740 gas chromatograph equipped with an electron-capture detector (tritium–titanium foil). Nitrogen was used as the carrier gas at a flow rate of 54 ml/min through a 5.5 m × 6.0 mm OD glass column (4% Carbowax on Chromosorb G) at 180°C. The detector temperature was 140°C, and the injector port was 170°C. The peak representing 2,6-dichlorophenol appeared at 40–42 min after injection. Coinjection of known amounts of pure 2,6-dichlorophenol was done to confirm the identification and to quantitate the amount of sex pheromone/gland pair or the amount in the emissions from living female ticks.

Autoradiography. Partly engorged female ticks fed on a ^{36}Cl-labeled rabbit were fixed in 10% neutral formalin. Paraffin sections 8 mm thick, or frozen sections 30 mm thick, were prepared by routine methods. In several preparations, the sections were stained with Mayer's hematoxylin and eosin Y before exposure to the nuclear emulsion. In the remaining preparations, unstained sections of the ticks fed on the radiolabeled rabbit and on an unlabeled control

were dipped in liquefied emulsion, NTB (Kodak Corp., Rochester, New York) in a dark room. The specimens were dried, sealed, and exposed to incorporated radioactivity for 94 hr at 4°C. The coated slides were developed in 1:1 Dektol solution (Kodak Corp.), fixed, stained with Mayer's hematoxylin and eosin Y, and examined microscopically for evidence of silver grain accumulation.

Bioassay. This was done using the method of Sonenshine et al. (1976).

RESULTS

Identification of the glands in dissections of whole female *D. andersoni* was aided by scanning electron microscopy, which revealed clusters of subspherical lobes connected by ducts to pores of the fovea dorsales (Figure 2). Similar gland structure was observed in dissections of *D. variabilis.*

Foveal glands were excised under saline from 42 *D. andersoni* females, and GLC analysis of a hexane extract of these glands revealed approximately 750 ng of 2,6-dichlorophenol, representing 17.9 ng/gland. The extractions were repeated, but no additional 2,6-dichlorophenol was found.

Sex pheromone (2,6-dichlorophenol) was found in the hexane-trapped volatiles only when 2% NaOH was used; it was collected from 218 partially

TABLE 1. RADIOCHLORINE ACTIVITY IN AIRBORNE EMISSIONS COLLECTED FROM *D. andersoni* FEMALES FED ON A ^{36}Cl-LABELED RABBIT[a]

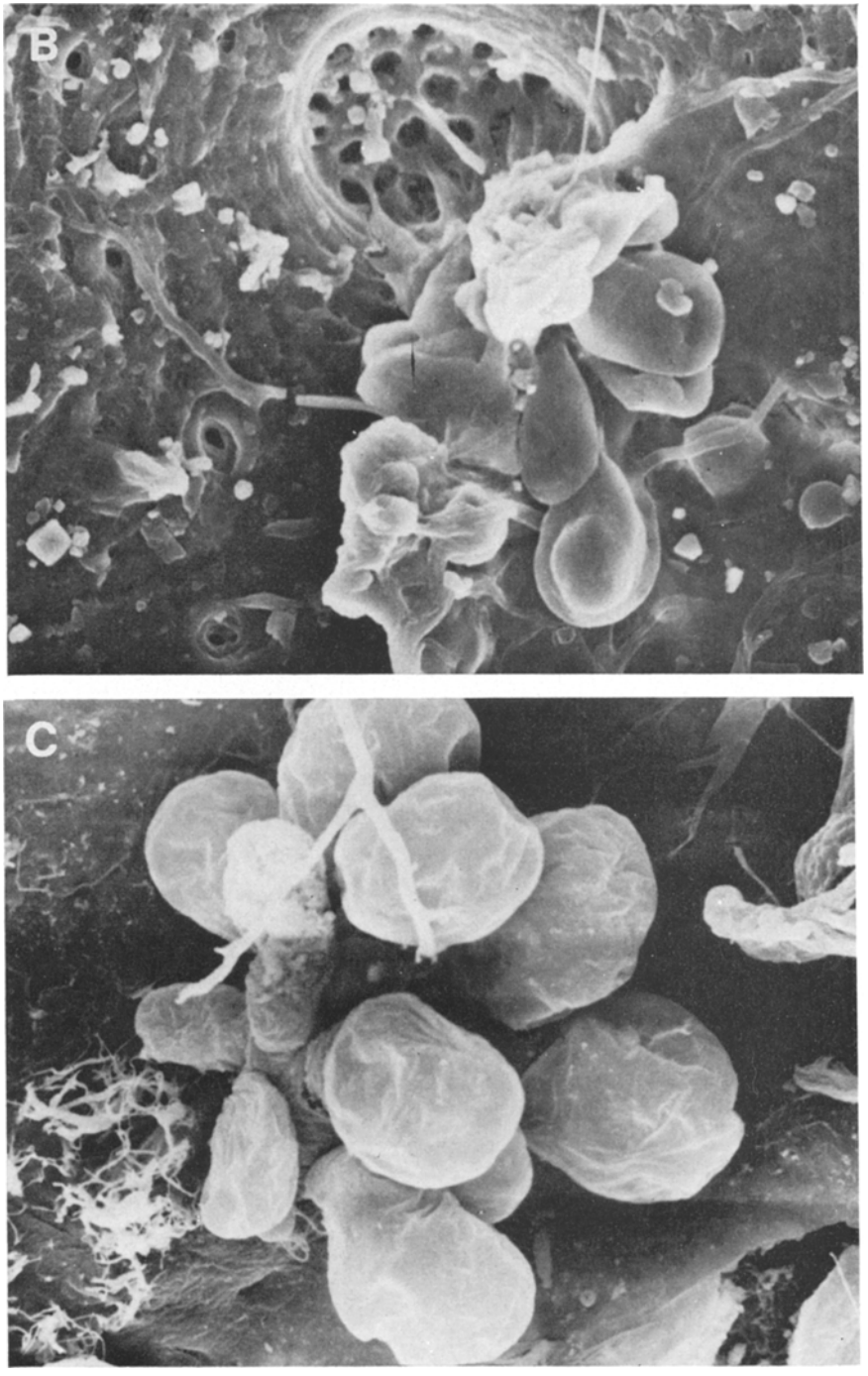
Cocktail	Observed $\bar{X}$ CPM minus background[b] ± percent error	Sample activity (μCi)	Estimated ^{36}Cl in sample (ng)	Percent efficiency (biological)
Solubilized ticks $\bar{X}$	3,277.2 ± 164	1.5×10^{-3} per tick	515/tick	—
Emissions: 50 normal females	202.3 ± 6	9.5×10^{-5} per 50	31.5/50	—
		1.9×10^{-6} per tick	0.63/tick	0.12
25 normal females (control)	223.8 ± 3	1.04×10^{-4} per 25	34.0/25	—
		4.26×10^{-6} per tick	1.4/tick	0.07
50 females (foveae obstructed)	2.5 ± 0.1	— None (?)	— None (?)	—

[a] Collected for 24 hr.
[b] Background = 57.8 CPM ± 0.6 (1%); instrument efficiency = 96.4%

FIG. 2. (above and facing page.) (A) Scanning electron microscopic view of the foveal gland of *Dermacentor andersoni* showing the lobular organization of the gland. (B) SEM view of a partially dislodged foveal gland of *D. andersoni* revealing its associations with the underlying pores of the fovea dorsale. (C) Enlargement of (B) showing membranous tubules extending from the lobes of the gland to the pores of the fovea.

fed *D. andersoni* females (13 ng/tick/14 hr of collection, or 22.2 ng/tick/day). Bioassay of a 100-μl aliquot of this hexane collection produced an extremely vigorous response with almost all males orienting to it (97%), and many attempts to copulate with the preserved female tick adjacent to the extract spot. The results indicate that partially engorged female *D. andersoni* emit 2,6-dichlorophenol vapor. However, only 0.5 μg of 2,6-dichlorophenol was collected from one of the controls (8.8 μg) and none from the other 4 replications. The results of the attempts to recover this compound from the controls suggest that the aeration system is not always reliable and cannot be used without refinement for quantification of the rate of emission.

Scintillation spectrophotometric analysis of the hexane-trapped volatiles showed that partially engorged female *D. variabilis* fed on a ^{36}Cl-labeled rabbit emitted a radioactive volatile compound (or compounds). Presumably,


B
C

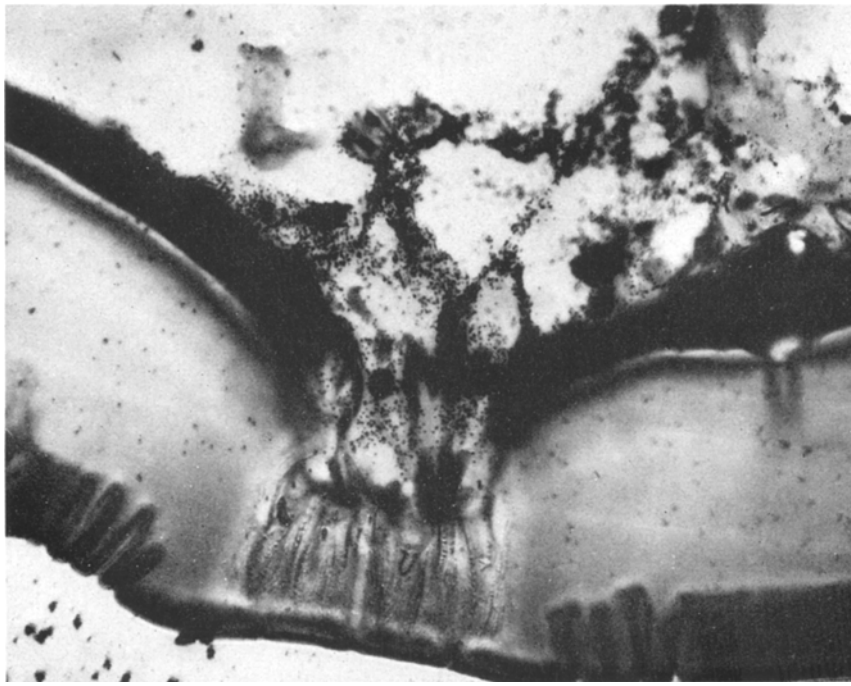


FIG. 3. Autoradiograph of a section of a female *D. andersoni* fed on a ^{36}Cl-labeled rabbit, revealing accumulations of silver grains in the vicinity of the gland, and extending into the pores of the fovea.

the material collected contained radioactive sex pheromone, since, according to Berger (1974), ticks will utilize $Na^{36}Cl$ to synthesize radioactive 2,6-dichlorophenol.

Table 1 summarizes the results of an experiment to measure the rate of emission of the labeled vapor from *D. andersoni* females. In the first replicate of the experiment, an estimated 31.6 ng of ^{36}Cl was collected in a 24-hr period, or 0.63 ng/tick; in the second replicate, 34.0 ng of ^{36}Cl was collected, or 1.4 ng/tick. If all the ^{36}Cl-labeled vapor is represented by 2,6-dichlorophenol, these collections represent 1.43 and 3.17 ng of labeled pheromone/tick/24 hr, respectively, or approximately 10% of the total estimated daily emission rate reported above. When the foveae dorsales of partially engorged *D. andersoni* females were obstructed with lacquer, no radiolabeled emissions were collected.

Elemental analysis of *D. andersoni* foveal gland contents with the X-ray analyzer demonstrated large amounts of chlorine, much more than in other

TABLE 2. ^{36}Cl-ACCUMULATION IN VARIOUS TISSUES OF *D. andersoni* FEMALES FED ON A ^{36}Cl-LABELLED HOST

Tissue	Number Ag grains/0.01 mm^2 ± SE[a]
Foveal gland	587 ± 29.8
Cuticle	98 ± 2.8
Epidermis	140 ± 4.5
Muscle	135 ± 8.9
Salivary glands	105 ± 7.4
Midgut	[b]
Dermal glands	108 ± 5.4
Brain	116 ± 5.1
Ovary	95 ± 5.5

[a] Background = 25 Ag grains/0.01 mm^2
[b] Too numerous to count.

tissues examined (Table 2). The ratio of chlorine to gold (Cl : Au) in the first foveal gland sample examined was 0.3063:1; in the second, 0.4278:1. In contrast, muscle and tracheal samples had much lower values, from 0.0056:1 to 0.0648:1. Chlorine was 4.7–76.4 times more abundant in the samples of foveal glands than in the samples of muscle or trachea analyzed by this method.

Autoradiographs of sections of *D. andersoni* females fed on a ^{36}Cl-labeled rabbit revealed accumulations of silver grains above the foveal glands and vicinity, the midgut, but not above other tissues examined (Figure 3). Table 2 compares the counts of silver grains in the vicinity of the foveal glands with other tissues from 10 individuals. The counts in the foveal glands averaged 587 grains/10 nm^2, or approximately 5 times more than in other tissues except the midgut.

DISCUSSION

Arthropod pheromones may be produced in specialized glands, epithelial linings of different organs, or in the digestive tract. The abdominal sex pheromone gland of the lepidopteran *Rhyacionia frustana* (Comstock) is sac-like and lined with a simple columnar epithelium (Baer et al., 1976). The gland is extended from the abdomen by hydrostatic pressure, facilitating pheromone release. The genital atrium serves as the sex pheromone gland in the cockroach *Bryostria fumigata* (Guerin) (Moore and Barth, 1976). The pheromone-producing tissue in this insect is believed to be a segment of the atrial

epithelial lining, which undergoes columnar expansion during the active period. In six species of the beetle genus *Trogoderma*, the sex pheromone gland consists of the enlarged columnar epithelial lining of the inner surface of the 7th abdominal sternite, associated with minute cuticular pores (Hammack et al., 1973). The highly developed mandibular gland of certain honeybees produces an array of pheromones, including the sex pheromone, while the poison gland produces a trail pheromone in attine ants (Blum, 1970). In contrast, no specific pheromone gland has been reported for the cockroach *Periplaneta americana* L.; rather, the sex pheromones are regarded as by-products of digestive activity and are voided with the wastes (Bodenstein, 1970).

The foveal glands of *Dermacentor* ticks do not resemble any of the pheromone glands described above. The foveal glands consist of lobular clusters of large secretory cells, much like the acini of the salivary glands, rather than saclike glands or cellular sheets. Moreover, these tick glands release their pheromone via tiny pores in the fovea dorsales. Except in the case of the *Trogoderma* beetles noted above, this pheromone emission method is different from that previously reported for insects. The unique chemical feature of the tick pheromone, as noted originally by Berger (1972), is the presence of chlorine.

According to Berger (1976), 2,6-dichlorophenol is the only chlorinated organic molecule "having as its source a land animal." 2,5-Dichlorophenol, from the lubber grasshopper, *Romalea microptera* (Palisot de Beauvois), is believed to be a by-product of the herbicide 2,4-dichlorophenoxyacetic acid, since the insect does not synthesize the chlorinated phenol (Berger, 1976). Morrison and Schonbaum (1976), discussing the chemistry of peroxidase-catalyzed halogenation, note that "chloro- and myeloperoxidase catalyze oxidation of all halide ions except F^-." Myeloperoxidase-catalyzed oxidation of chloride may produce HOCL; HOCL can, according to these authors, "chlorinate phenolic compounds . . ."

Much more 2,6-dichlorophenol was collected when extractions were made directly from excised foveal glands (17.9 ng/gland) and from the hexane-trapped emissions of the living partially fed ticks (22.2 ng/tick/day) than the amount reported by Sonenshine et al. (1976) based on the "sonicate–freeze–thaw" technique (only 2 ng/tick). It is not known whether this difference merely reflects variation expected with different techniques or a real biological difference.

In summary, the paired foveal gland is the sex pheromone gland of *Dermacentor andersoni* and, presumably, *Dermacentor variabilis* as well.

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STARCH: A POTENT FEEDING STIMULANT FOR THE TERRESTRIAL SLUG *Ariolimax californicus*

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Abstract—Laboratory bioassays demonstrate that soluble and insoluble fractions of starch are potent feeding stimulants for the terrestrial slug *Ariolimax californicus*. The stimulatory efficacy of starch is sufficiently great to account for the palatability of potato tubers—a favorite food of terrestrial slugs.

Key Words—*Ariolimax californicus*, feeding stimulant, starch, gastropod mollusk, slug.

INTRODUCTION

Feeding stimulants are chemical compounds that initiate and sustain ingestional behavior (Dethier et al., 1960). Chemical identification of feeding stimulants for invertebrate species has been largely restricted to members of the phylum Arthropoda, especially those belonging to the class Insecta (Minnich, 1929; Dethier, 1970; Bernays and Chapman, 1970). Although several amino acids and protenaceous compounds have been reported to function as food attractants in gastropod mollusks (Jahan-Parwar, 1972; Gurin and Carr, 1971; Carr et al., 1974; S. Kater, personal communication), no chemical compound has been shown to be an effective feeding stimulant in this large group of animals. The identification of feeding stimulants for mollusks, particularly pulmonate mollusks, is especially important in view of its recent use in cellular neurophysiological studies of feeding behavior (e.g., Senseman, 1977), neuroethological investigations of food aversion learning (Gelperin, 1975; Gelperin and Forsythe, 1976), and experimental ecological investigations of herbivore–host coevolution (Cates, 1975; Cates and Orians, 1975; Grime et al., 1970). Evidence presented below demonstrates

starch to be a potent feeding stimulant for the terrestrial slug *Ariolimax californicus*.

METHODS AND MATERIALS

Slugs, *Ariolimax californicus*, 15–30 g in weight, were collected from stream banks in the vicinity of Stanford University, Stanford, California. Animals were freshly collected 48 hr prior to an experiment, with an individual animal being tested in only one of the three experiments described below.

In the laboratory, slugs were individually housed in large plastic petri dishes (15 cm diam. × 4 cm). A small quantity of standing water was maintained on the bottom of each cage to ensure a high relative humidity. Experiments were performed at ambient temperature (20±2°C) with the animals being exposed to a 12-hr light–12-hr dark cycle.

The following compounds were assayed for their ability to initiate and sustain feeding behavior: insoluble potato starch (ICN Pharmaceuticals), reagent-grade soluble starch (ICN Pharmaceuticals), maltotriose (Sigma), maltose (ICN Pharmaceuticals), and *d*-glucose (Fisher). Test compounds were suspended in nonnutritive agar (Gibco) and presented to slugs in the form of small pellets (2 cm diam. × 2 cm). Also tested were whole potatoes (*Solanum tuberosum*) which were cut into pellets similar in size and shape as those described above.

The same protocol was followed in each feeding experiment. A preweighed sample pellet was placed in the center of the slug's cage and the slug positioned on its side so that upon righting, its oral region would come into contact with the pellet. The duration of the feeding bout was visually monitored. A meal was considered terminated whenever an animal ceased to feed for >2 min or moved >2 cm from the pellet. Immediately after meal termination the remaining material was weighed to determine meal size. The meal size divided by meal duration was defined as the mean consumption rate for the meal.

Animals were tested on a different sample diet every 48 hr until each slug had been exposed to each of the various diets employed in a given experiment. Care was taken to randomize the order in which individual animals received the various diets to prevent possible systematic biases. Slugs were food-deprived 48 hr prior to the first experimental trial and received no food, except for the test diets, during the experimental period.

Further details concerning culture conditions, diet preparation, and testing procedures will be published elsewhere (Senseman, in preparation).

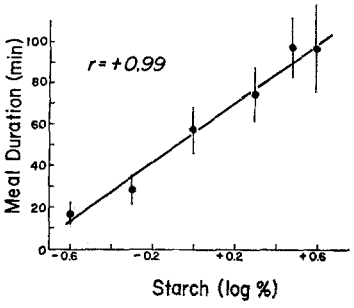

FIG. 1. Relationship between meal duration and soluble starch concentration at low consumption rates. Meal durations represent means (±SEM) determined for a population of slugs ($N = 11$). Starch was incorporated into 15% agar pellets that limited consumption rate to a mean value of 27±1 mg/min (±SEM, $N = 66$).

RESULTS

Relationship between Meal Duration and Starch Concentration

A direct measure of a compound's ability to function as a feeding stimulant is the duration of a feeding bout that can be elicited by a given concentration of that compound. Since in many animals (including *Ariolimax*), meal duration is governed in part by inhibitory postingestional feedback, such inhibitory effects must be taken into account if meal duration is to be a reasonable measure of palatability. In *Ariolimax* the significance of postingestional factors can be substantially reduced by feeding slugs relatively hard agar pellets that restrict meal size by reducing the rate of food intake (Senseman, 1976; Senseman, in preparation).

Figure 1 shows the relationship between meal duration and starch concentration for a random sample of 11 slugs. Mean consumption rate was reduced to 12±1 mg/min (±SEM, $N = 66$) by incorporating the starch into 15% agar pellets. The duration of a feeding bout was found to be highly correlated ($r = +0.99$, $N = 6$) with the logarithm of the starch concentration between 0.25% and 4.0% (w/w). Higher concentrations of starch significantly degraded the hardness of the agar pellets and therefore could not be properly evaluated.

Stimulant Efficacy of Potato Starch Compared to Whole Potato

The previous data strongly suggest starch to be a feeding stimulant for *Ariolimax*. However, these results do not provide information as to the relative potency of starch as feeding stimulant compared to natural food items. One very palatable food item for terrestrial slugs is potato (Fromming, 1952), which contains an appreciable starch content (~14.5%, Chatfield and Adams, 1931). An attempt was made in this experiment to determine the relative effectiveness of starch to sustain feeding behavior compared to fresh potato.

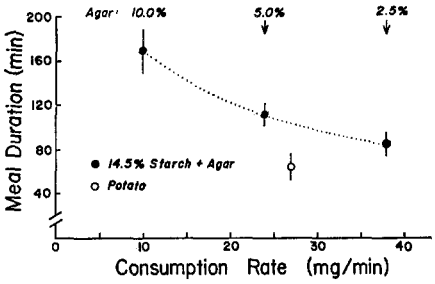

FIG. 2. Relative potency of insoluble potato starch (●) to sustain feeding compared to whole potato tubers (○). Mean meal durations (±SEM) have been plotted as a function of the mean rate at which each diet was consumed. Consumption rates for the potato starch diets were manipulated by incorporation into 2.5%, 5%, or 10% agar pellets. Dotted line shows the decline in meal duration for the starch/agar diets resulting from increased postingestional feedback at higher consumption rates. Mean values for meal durations were determined from a population of slugs ($N = 14$).

As before, it was necessary to take into account the inhibitory effects of postingestional feedback on meal duration. *Ariolimax* can consume potato tubers at a relatively fast rate (~27 mg/min) so that appreciable levels of feedback inhibition are likely to be generated during the meal. Thus, meal durations obtained from slugs feeding on whole potatoes and on potato starch incorporated into agar pellets can be compared only if animals encounter quantitatively similar levels of inhibitory feedback during meals on each of the two foods. Therefore, an attempt was made to indirectly manipulate the contribution of postingestional factors in the termination process for the starch/agar pellets by directly varying the rate at which the pellets were consumed. This was accomplished by incorporating the potato starch into pellets containing 2.5%, 5%, and 10% agar (w/w) which led to a series of consumption rates that "bracketed" the consumption rate observed for potato tubers.

Figure 2 shows the mean meal durations for a random sample of 14 slugs feeding on fresh potato and insoluble potato starch/agar pellets. Meal durations have been plotted as a function of the rate at which each diet was consumed. Even though the potato starch/agar pellets contained similar concentrations of the feeding stimulant (i.e., 14.5% potato starch), mean meal duration declined with increasing rates of consumption. This reduction in meal duration reflects the greater importance of postingestional feedback inhibition in the termination process at higher consumption rates.

The most important aspect of the data shown in Figure 2 is that at comparable rates of consumption (and therefore at presumably similar levels of

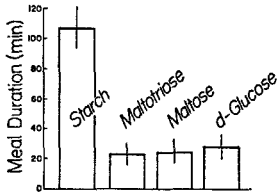

FIG. 3. Relative potency of equal concentrations (2% w/w) of soluble starch and its mono-, di-, and trisaccharide constituents to sustain feeding. Mean values (± SEM) for meal durations determined for a population of slugs ($N = 10$). Test compounds were incorporated into 15% agar pellets that restricted consumption rate to mean values (±SD) of 7.7±2.3 mg/min for starch, 11.7±8.6 mg/min maltotriose, 9.6±2.8 mg/min for maltose, and 10.6±7.3 mg/min for *d*-glucose ($N = 10$ for each diet). Paired-sample *t* test analysis using an animal's response on starch compared to its responses on the sugars showed meal durations on starch to be significantly greater than on the sugars ($P<0.005$).

postingestional feedback inhibition), 14.5% potato starch appears to be slightly *more* stimulating than whole potato. Two factors may contribute to the greater stimulant efficacy of potato starch relative to whole potato. First, since a direct measurement of the starch content of the potato tubers used was not made, it is possible that their starch content fell somewhat below the average value of 14.5%. However, since meal duration is linearly related to the *log* concentration of the starch (Figure 1), it seems somewhat doubtful that the difference in stimulant efficacy can be completely explained by a difference in the starch content of the whole potato and the potato starch/agar pellets. A more likely reason for the difference in palatability may be due to the presence of secondary plant substances in the whole potato such as chlorogenic acid that might function as antifeedants for terrestrial slugs (Stephenson, 1970).

Possible Involvement of Salivary Enzymes in the "Starch Taste"

Since pure starch is generally considered to be a "tasteless" compound, I considered the possibility that slugs did not taste starch per se, but rather utilized salivary enzymes (e.g., amylase) to hydrolyze starch into the low-molecular-weight sugars: maltose, maltotriose, and possibly glucose. To test this hypothesis, the ability of these mono-, di, and trisaccharides to stimulate feeding was compared to an equal concentration (2% w/w) of a purified soluble starch fraction. Test substances were incorporated into hard (15%) agar pellets. The results of this experiment are summarized in Figure 3. The low-molecular-weight saccharides were substantially less effective in stimulating feeding than the soluble starch fraction. This suggests that starch need

not be enzymatically degraded prior to its chemoreception. However, the possibility remains that salivary enzymes produce rather specific concentrations of oligosaccharides that are intensely synergistic with respect to their capacity to sustain feeding. Such intense and unexpected synergisms have been observed in rats offered an aqueous solution containing 0.125% saccharin and 3.0% glucose (Valenstein et al., 1967).

DISCUSSION

The ability of high-molecular-weight polysaccharides to elicit appetitive components of the feeding response has been previously reported for two species of prosobranch gastropods. Soluble starch and glycogen were observed by Henschel (1932) to evoke proboscis extension in the marine snail *Nassa reticulata*; Carr (1967) found that glycogen was an effective stimulus for the proboscis search response in another prosobranch gastropod, *Nassarius obsoletus*. It has not been made clear, however, whether these compounds initiate and sustain the ingestive components of the feeding response. The present work appears to be the first demonstration that starch can also function as a feeding stimulant.

Food selection represents an approach–avoidance situation for a generalized herbivore such as *Ariolimax*. The slug must avoid potentially toxic materials while selecting foods that will provide adequate metabolic energy and essential nutrients to ensure reproductive success. The recognition of potentially toxic plant materials by generalized herbivores has received considerable attention by ecologists interested in herbivore–host coevolution (e.g., Jones, 1962, 1966; Eisner, 1964; Feeney, 1970; Schoonhoven, 1972; Cates, 1975; Cates and Orians, 1975). The results of these and similar investigations support the hypothesis that certain plant species utilize secondary plant substances as chemical deterrents against herbivore predation.

While secondary plant substances may play a major role in mediating the rejection of a potentially toxic food plant, it does not follow that the slug, or any herbivore for that matter, simply selects plant materials which contain negligible levels of chemical deterrents. The metabolic resources available to a slug within its foraging range are not uniformly distributed between various plant species nor between the tissues within a given plant. Starch, for example, is the principal form of stored metabolic energy in vascular plants, yet its concentration varies considerably from as little as a few percent in some plant tissues to over 70% in certain cereal grains (Greenwood, 1970). Significant quantitative differences in crude protein, fats, and other nutrients can also be found between various seeds and fruits occurring within a given region (Short and Epps, 1976). Considering this variability in nutrient density among

potential food sources, it would be advantageous for the slug to be able to select food items on the basis of their metabolic potential. However, if selection is to be based on nutrition, the slug must be able to assess the levels of important nutrients within a food at the onset of a feeding bout. Such an assessment could be accomplished by a relatively "simple" organism as the slug if the animal was capable of perceiving such nutrients via its chemical senses: the nutrient concentration of a food would be encoded in the intensity of the evoked chemosensory response.

The present work demonstrates that the slug is capable of perceiving at least one important nutrient, i.e., starch, presumably through its contact chemoreceptors. The perception of starch reliably initiates and sustains the feeding response in animals deprived of food for 24 hr. Furthermore, the slug displays the ability to discriminate between foods on the basis of their starch concentrations, devoting more time and energy to the consumption of foods containing the greatest density of the polysaccharide (Figure 1).

Studies similar to those presented above have shown that starch is not the only feeding stimulant in *Ariolimax*. Caesin (milk protein) is at least as effective as starch on a per weight basis (unpublished observations). Although the nutritional requirements of slugs are poorly understood (Runham and Hunter, 1970), it seems reasonable to suspect that slugs require some protein intake for normal growth and development. Thus caesin might represent another example of an important nutrient functioning as a feeding stimulant in *Ariolimax*.

Finally it should be emphasized that the presence of a feeding stimulant within a plant tissue need not ensure its palatability. In the absence of contrary data, it seems reasonable to suspect that a slug's decision to feed and perhaps the duration of the ensuing feeding bout would be determined by the concentration of feeding stimulant(s) *relative* to the concentration of any feeding deterrent(s) within the food item. Behavioral experiments designed to study the interaction of stimulants and deterrents on the feeding response of terrestrial slugs are currently in progress.

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SEX PHEROMONE OF THE FALSE CODLING MOTH *Cryptophlebia leucotreta* (LEPIDOPTERA: TORTRICIDAE) Evidence for a Two-Component System

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Abstract—The sex pheromone of *Cryptophlebia leucotreta* females is a mixture of (E)-8- and (Z)-8-dodecenyl acetate (dda). The compounds have been isolated from extracts of *C. leucotreta* females and were identified by means of chromatography on a silver-impregnated column, and by combined GC-MS analysis and ozonolysis. Optimal catches of males were obtained with mixtures of (E)-8- and (Z)-8-dda in the range of 70:30 to 30:70.

Key Words—Sex pheromone, false codling moth, Tortricidae, *Cryptophlebia* (*Argyroploce*), (Z)-8-dodecenyl acetate, (E)-8-dodecenyl acetate.

INTRODUCTION

In reports by Read et al. (1968, 1974), it is suggested that the sex pheromone of the false codling moth, *Cryptophlebia leucotreta*, is (E)-7-dodecenyl acetate [(E)-7-dda]. In field experiments, however, Rauch (Procida, Marseille, France) found this compound to be inactive. A reinvestigation of the problem (Persoons et al., 1976) yielded evidence that the true pheromone is a positional isomer of (E)-7-dda, namely (E)-8-dda. When the latter compound was tested in field experiments, it was found to be strongly synergized by (Z)-8-dda.

Conclusive evidence that the two compounds make up the sex pheromone of the insect was lacking at the time. Neither compound could be isolated from an extract in aqueous ethanol of virgin females, which had been standing for a long time before it could be analyzed. However, upon acetylation the

extract regained its biological activity, and the E isomer could be isolated from it. Fresh extracts of virgin females of *C. leucotreta* have now been shown to contain both isomers, E-8-dda and Z-8-dda.

METHODS AND MATERIALS

Extraction of the Pheromone

A sample of 2- to 3-day-old laboratory-reared virgin females of *C. leucotreta* immersed in methylene chloride was supplied by Procida Laboratories, Saint Marcel, Marseille, France. The insects were homogenized three times in the methylene chloride suspension, and the solid residue was extracted with freshly distilled methylene chloride for 8 hr in a Soxhlet extractor. The extracts were combined and dried over anhydrous $MgSO_4$, and the residue remaining after removal of the solvent at reduced pressure was taken up in 0.5 ml of acetone.

Isolation

The acetone extract was subjected to preliminary purification by gel permeation chromatography on a Sephadex LH 20 column (150×0.7 cm, flow rate 20 ml acetone/hr), 5-ml fractions being collected. The geometric isomers were separated on a silver-loaded Nucleosil 10-SA column [Custom-packed with silver-loaded Nucleosil 10 SA (Mackerey, Nagel & Co., Düren, G.F.R.) by Chrompack Nederland, Middelburg, The Netherlands.] (25×0.46 cm ID, eluent hexane/ethyl acetate 9:1, flow rate 0.5 ml/min). Those fractions that had elution times corresponding to those of synthetic (Z)-8-and (E)-8-dda were collected for further purification by gas chromatography with PDEAS as the stationary phase (3% phenyldiethanolamine succinate on Chromosorb W(AW), 650×0.23 cm, 175°C, 11.5 ml N_2/min), followed by chromatography on a column of OV 101 (5% on Chromosorb G, 200×0.4 cm, 194°C, 40 ml N_2/min). The products of ozonolysis were separated on an OV 101 column at 175°C.

The retention times of the isolated pheromones were compared with those of synthetic (Z)-8-dda and (E)-8-dda on the PDEAS column described above, and also with the retention times on a SP 2340 column (15% cyanosilicone on Chromosorb P AW -DMCS, 600×0.23 cm, 210°C, 15 ml N_2/min).

Mass Spectrometry

Mass spectra of the compounds were run with a Mat 112 GC-MS combination fitted with a 35-m SE SCOT column maintained at 180°C.

TABLE 1. COMPARISON OF RETENTION TIMES OF SYNTHETIC (E)-8-dda AND (Z)-8-dda ON COLUMNS OF SP 2340[a] AND PDEAS[b] WITH THOSE OF COMPOUNDS ISOLATED FROM A SERIES OF PURIFIED[c] FRACTIONS OF AN EXTRACT OF VIRGIN FEMALES

	Retention times (min)	
Fraction	SP 2340	PDEAS
2	—	—
3	12.4	8.4
4	12.4+13.7	8.4+8.8
5	13.7	8.8
6	—	—
Synthetic compounds		
(E)-8-dda	12.4	8.4
(Z)-8-dda	13.7	8.8

[a] 15% cyanosilicone on Chromosorb PAW-DMCS, 6 m×2.3 mm, 210°C, N = 15 ml/min.
[b] 3% phenyldiethanolamine succinate on Chromosorb W(AW), 6.5m×2.3 mm, 175°C, N = 11.5 ml/min.
[c] By chromatography on a silver-impregnated column.

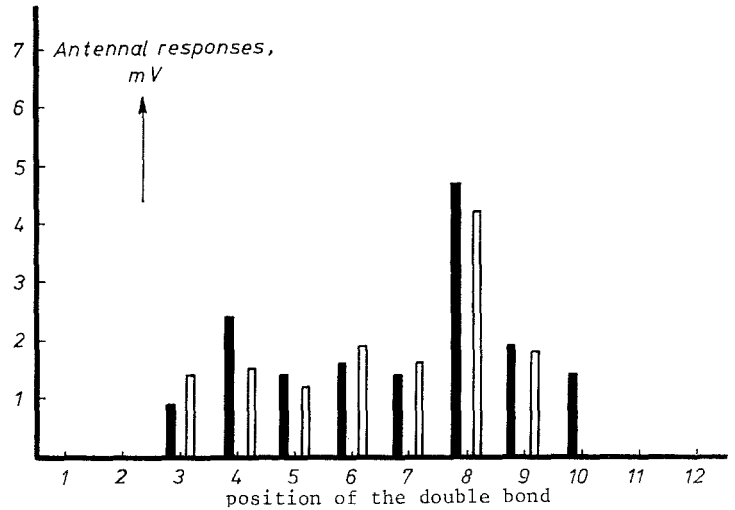

FIG. 1. Antennal responses of *C. leucotreta* male moths to various monounsaturated *cis*- and *trans*-dodecenyl acetates. Black bars: cis isomers. White bars: trans isomers, 5 duplicates, 10-μg samples (Persoons et al., 1976).

Ozonolysis

This was carried out according to the method of Beroza and Bierl (1967), with carbon disulfide as the solvent.

RESULTS

The fractions (nos. 23–28) obtained by gel permeation chromatography of the extract and having elution times similar to those of synthetic (Z)-8-dda and (E)-8-dda were each concentrated to a residue of about 100 μl and subjected to chromatography on the silver-impregnated column. Those fractions which had the same elution times as the synthetic compounds (11.7 and 9.5 min for (Z)- and (E)-8-dda, respectively) were again collected and subjected to gas chromatography on SP 2340 and PDEAS. The results are summarized in Table 1.

Compounds with retention times identical to those of (E)-8-dda and (Z)-8-dda were isolated from fractions 3 and 5, respectively, fraction 4 containing a mixture of the isomers. Both compounds occur in the extract in about equal amounts. The mass spectra of the compounds isolated from fractions 3, 4, and 5 were identical with those of synthetic (Z)-8-dda and (E)-8-dda. Since the mass spectra of compounds of this type are very similar, the only conclusion that can be drawn from them is that the isolated compounds are monounsaturated dodecenyl acetates (m/e = 166 = M-CH_3COOH).

From mass spectra of the type in question, the positions of double bonds cannot be deduced. To this end the compounds isolated from fractions 3, 4, and 5, and purified over columns of PDEAS and OV 101, were subjected to ozonolysis. The reaction products were separated by chromatography on an OV 101 column and had retention times identical to that of acetoxy octadecanal (7.1 min), which can also be obtained by ozonolysis of synthetic (Z)-8-dda or (E)-8-dda. The ozonolysis products of (E)-7-dda and (E)-9-dda, on the other hand, have retention times on an OV 101 column of 4.2 and 10.2 min, respectively. As (E)-7-dda is not separated from (E)-8-dda under the chromatographic conditions employed, the results of the ozonolysis demonstrate the absence of (E)-7-dda in the extracts.

The results of the electroantennographic experiments with model compounds, including (E)-7-dda, confirm the conclusion that the latter compound is not the sex pheromone of *C. leucotreta* (Figure 1). These data, together with the earlier finding (Persoons et al., 1976), that butanal is a second product of ozonolysis, complete the evidence that the compounds isolated from the insect are (Z)-8-dda and (E)-8-dda. The fact that they are

TABLE 2. FIELD-TRAPPING RESULTS OF C. *leucotreta* MALES; TRAPS BAITED WITH PHEROMONE WERE EXPOSED FOR 104 DAYS, AND THOSE WITH VIRGIN FEMALES, 94 DAYS; THREE DUPLICATES; PURITIES OF COMPOUNDS ⩾ 99%; 20 mg/TRAP (PERSOONS ET AL., 1976)

Ratio *cis*-8/*trans*-8	Total numbers caught
100/0	115
70/30	2893
50/50	3009
30/70	3042
0/100	41
Traps baited with two virgin females each	2341

active as sex pheromones is demonstrated by the field data given in Table 2.

DISCUSSION

The experimental data presented above show unambiguously that the double bond in the sex pheromone of *C. leucotreta* is not at the 7 position, as reported by Read et al. (1968, 1974), but at the 8 position. In addition, the data show that the insect contains the (E) as well as the (Z) isomer and that these two compounds are present in fresh extracts of virgin females. The conclusion that it is not (E)-7-dda, but a mixture of (E)-8-dda and (Z)-8-dda which constitutes the sex pheromone of *C. leucotreta* is also supported by the negative results of field experiments with (E)-7-dda and by the positive results of similar experiments with (E)-8-dda, (Z)-8-dda, and particularly, mixtures of the two.

As far as we are aware, the false codling moth is the only insect whose sex pheromone contains (E)-8-dda, whereas (Z)-8-dda has been found in the pheromones of several other insects. For example, the oriental fruit moth, *Grapholitha molesta*, has (Z)-8-dda as the main component of its pheromone (Roelofs et al., 1969). The activity of the (Z) isomer in this insect is strongly synergized by the (E) isomer, although the presence of the latter in the insect has not been established (Beroza et al., 1973a,b, Roelofs and Cardé, 1974).

A number of other tortricid moths are also attracted by mixtures of

(Z)-8-dda and (E)-8-dda, or by the individual compounds, but the presence of these compounds in the insects has not been established (Inscoe and Beroza, 1976; Ritter and Persoons, 1976). Although mixtures of geometrical isomers have been found in several members of the Tortricinae subfamily (Inscoe and Beroza, 1976; Ritter and Persoons, 1976), the false codling moth is the only member of the Olethreutinae subfamily which has been proved to produce a binary sex pheromone composed of about equal amounts of geometrical isomers.

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EFFECTS ON BEHAVIOR OF *Apanteles melanoscelus*[1] FEMALES CAUSED BY MODIFICATIONS IN EXTRACTION, STORAGE, AND PRESENTATION OF GYPSY MOTH[2] SILK KAIROMONE

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Abstract—Choice experiments were performed to investigate details of female *Apanteles melanoscelus* (Ratzeburg) behavior when exposed to gypsy moth silk kairomone [*Lymantria dispar* (L.)] and to host larvae when kairomone is present. Female parasites only responded to the kairomone when it had been placed on thin strands such as cotton fibers. Both gypsy moth silk and silk glands contain the same or similar kairomones. Silk gland extracts were more active than head, alimentary canal, or hemolymph extracts of host larvae. Female responses decreased when low concentrations of silk gland extract or small numbers of treated fibers were presented to them. Silk gland extract was stable when frozen for 2 weeks, heated to 100°C for 0.5 hr, freeze dried, or treated with 95% ethanol. The active component was nondialyzable. Silk deposited on the substrate increased host contacts and oviposition attempts, more so if wider areas were covered with silk, and even if the areas having silk were separated from the host. A theory of host selection in *A. melanoscelus* is proposed.

Key Words—Gypsy moth, *Lymantria dispar*, *Apanteles melanoscelus*, insect silk, kairomone, parasite behavior, examination behavior.

INTRODUCTION

Apanteles melanoscelus (Ratzeburg) females examine with their antennae

[1] Hymenoptera, Braconidae.
[2] *Lymantria dispar* L. (Lepidoptera: Lymantriidae).

surfaces containing gypsy moth silk [*Lymantria dispar* (L.)]. Evidently this response is due to one or more water-soluble chemicals (kairomone) specific to gypsy moth silk and silk glands. Silk gland water extracts are only active if presented to the parasite in association with deactivated (i.e., water-washed) silk. Why deactivated silk is necessary has not been determined, but characteristics of silk besides the water soluble kairomone are important. Also, it has not been proved that the silk itself contains the kairomone or that the silk gland is the only source of it. Pieces of oak leaves, which have received silk naturally as caterpillars feed are more effective in eliciting examination behavior than are filter-paper squares artificially wrapped with silk. Whether or not this is due to a greater amount of silk on the leaves or to properties of the leaves themselves has not been determined. Substrate silk influences the response of parasites to hosts but the effects may be rather small (Weseloh, 1976c).

The experiments reported herein were designed to eliminate these ambiguities and follow up promising leads. Other experiments were performed to gain more information about the characteristics of the kairomone.

METHODS AND MATERIALS

A colony of *A. melanoscelus* was maintained on gypsy moth larvae reared on artificial diet. For some tests 1st or 2nd gypsy moth instars were induced to spin down on a silk thread which was wrapped 10–20 times around 1-cm squares of filter paper or Parafilm. Silk gland water extracts were prepared by dissecting silk glands from 5th or 6th gypsy moth instars, grinding them in a tissue grinder with water, centrifuging, and saving the supernatant. Parasite responses were recorded by placing 2-day-old female parasite adults in a 20×200-mm glass petri dish which contained the test materials and recording the number of times they examined each in 15 min or until 10 contacts occurred. These techniques were detailed by Weseloh (1976c); modifications of them are described where appropriate.

One method for presenting silk gland extracts to parasites was used in most experiments. A small quantity of absorbent cotton strands was soaked in the extract and spread over a glass surface to dry (this required only 0.01 ml extract, but usually larger volumes were used). Approximately 20–50 treated strands were placed on 1-cm squares of Parafilm and pressed down with forceps so they remained in place. The Parafilm squares adhered to the petri dish bottoms used as test chambers when pressed down on the corners.

Physical Context of Kairomone Presentation

Eight experiments were performed to determine if physical characteris-

TABLE 1. RESPONSE OF *A. melanoscelus* FEMALES TO SILK AND SILK GLAND EXTRACT PRESENTED IN VARIOUS PHYSICAL SITUATIONS

Experiment number	Description of test materials	Number of replicates	Silk gland extract concentrations (glands/ml)	Average % contacts leading to examinations
1	Silk on parafilm squares	8	—	78.1a[a]
	Silk on filter paper squares		—	62.1a
2	Silk on oak leaf squares	14	—	50.1a
	Silk on filter paper squares		—	43.8a
3	Extract on cotton fibers only	6	140	63.6a
	Extract on parafilm only, + cotton fibers			17.2b
	Extract on parafilm only			17.3b
	Water on cotton fibers only			12.2b
4	Extract on cotton fibers only	6	140	95.2a
	Extract on cotton fibers and parafilm			23.3b
5	Extract on glass fibers only	8	50	40.3a
	Extract on glass fibers and parafilm			29.4ab
	Glass fibers on parafilm only			14.6b
6	Extract on commercial silk strands only	8	50	37.1a
	Extract on commercial silk strands and parafilm			16.4b
	Commercial silk strands on parafilm only			12.9b
7	Gypsy moth silk	8	—	86.6a
	Extract on cotton fibers		140	58.1b
8	Extract on tangled cotton fibers	6	72	58.3a
	Extract on straight cotton fibers			36.2a

[a] Numbers in same experiment followed by different letters significantly different at $\alpha = 0.05$ in this and subsequent tables.

tics of silk and substrate are important in the behavioral response of the parasite.

Methods. The same quantity of 1st instar gypsy moth silk was wrapped around 1-cm Parafilm squares and filter paper squares (experiment 1), or around 1-cm greenhouse-grown oak-leaf squares and filter-paper squares (experiment 2). In experiment 3, silk gland extract-treated cotton fibers were placed on Parafilm squares. Drops (10–15 μl) of extract were placed on other

Parafilm squares and let dry, and nontreated cotton fibers were placed on half of these. Cotton fibers were also soaked in water and dried before being placed on Parafilm squares. In experiment 4, extract was placed on cotton fibers only or as a 10–15-μl drop on Parafilm squares after untreated fibers had been placed on them, so that both fibers and square were covered with extract. These were tested after extract dried. Experiments 5 and 6 were similar to 4 except that glass-wool fibers (experiment 5) or strands of commercial silk (experiment 6) rather than cotton fibers were used. Fibers mounted on Parafilm but without extract were tested as well in these two experiments. Experiment 7 was a comparison between gypsy moth silk wrapped around Parafilm squares and extract-treated cotton fibers mounted on Parafilm squares. A comparison between extract-treated cotton fibers mounted as usual and similar fibers carefully straightened out before being mounted (10 fibers/square) was made in experiment 8.

In each experiment one example of each material was placed in the test chamber (a 200-mm-diam. glass petri dish) and the average percent of contacts with each test material which resulted in antennal examination by each female was recorded. A test with one female constituted one replicate.

Results. Experiments 1 and 2 showed that response of the parasite to silk was not dependent on the nature of the substrate (Table 1). Thus it appears that any convenient substrate can be used in tests. Properties of oak leaves did not influence the parasite, and the response was strictly to the silk deposited thereon. Experiments 3 through 6 demonstrate that the physical manner in which kairomone is presented to parasites is extremely important. Except for experiment 5, parasite responses were greatest when extract was present only on fibers. Extract applied to substrate only or to substrate plus fibers was never more effective than when extract was completely absent. (The marginal response in experiment 5 may have been caused by spotty adherence of extract to the glass fibers or to some other physical property of the combination.)

Because of these results and the diverse nature of the fibers used, it is clear that the water-soluble kairomone present in gypsy moth silk glands need not be combined with other chemicals to be active. It need only be presented to the parasite on thin strands. However, experiment 7 shows that silk itself is more effective than the extract even when the latter is presented on cotton strands. This may be because treated cotton fibers do not have precisely the same physical configuration as gypsy moth silk. As one way of investigating this, straightened fibers were tested against tangled fibers. The former were presumably closer in physical properties to silk deposited on a substrate than were the latter. No statistically significant differences occurred between these, indicating that this aspect of the physical nature of the silk was not important (experiment 8).

TABLE 2. RESPONSE OF *A. melanoscelus* TO COTTON FIBERS SOAKED IN WATER EXTRACTS FROM VARIOUS PARTS OF THE GYPSY MOTH

Experiment number	Source of extract	Number of replicates	Concentration of extract	Average % contacts leading to examinations
9	2nd instar silk	7	12,600 meters/ml	39.0a
	Water only			19.5b
10	Silk gland	8	60 glands/ml	46.8a
	Alimentary canal		30 guts/ml	0b
	Head		30 heads/ml	2.2b
	Hemolymph		60 drops/ml	26.8c

Source of Kairomone

Experiments were performed to determine (1) if gypsy moth silk itself contained a water-soluble kairomone such as is present in silk glands, and (2) if silk glands are the only source of the kairomone.

Methods. One hundred and twenty-six meters of 2nd instar gypsy moth silk was collected by winding it on the enlarged 1-cm-diam. ground-glass portion of a tissue grinder rod. The end containing the silk was immersed in 0.2 ml distilled water (without grinding) for 2 min and the resulting extract freeze-dried using a Virtis freeze-drying apparatus (Gardiner, New York). The residue was redissolved in 0.01 ml distilled water and deposited on cotton strands for testing. Cotton strands soaked in water served as the check (experiment 9).

In experiment 10, 2 drops of hemolymph were bled from 9 large 5th and 6th instar gypsy moth larvae by cutting off one proleg and the pooled hemolymph diluted with 0.3 ml distilled water. Water extracts were made from macerated and centrifuged silk glands, alimentary canals, and heads of these same 9 larvae. Supernatants from all sources were dried on cotton fibers and mounted on 1-cm Parafilm squares.

Results. Experiment 9 demonstrates that a water-soluble kairomone is on the silk itself (Table 2). The silk gland kairomone is probably the same chemical or a precursor of it. Experiment 10 shows clearly that the silk gland is the primary source of the kairomone. Secretions from other glands in the head or digestive system are definitely ruled out. It is interesting that hemolymph has activity. Perhaps the kairomone is only concentrated in the silk glands, or a partially active form is present in the hemolymph, or the parasite responds to more than one chemical.

TABLE 3. RESPONSE OF *A. melanoscelus* FEMALES EXPOSED TO DIFFERENT CONCENTRATIONS OF SILK GLAND EXTRACT AND DIFFERENT NUMBERS OF COTTON FIBERS SOAKED IN SILK GLAND EXTRACT

Experiment number	Description	Number of replicates	Average % contacts leading to examinations					
			500	50	5	0.5 glands/ml		
11	Cotton fibers soaked in different concentrations of extract	6	51.7ab	66.7a	38.3b	18.3c		
			20 fibers	Blank	10 fibers	Blank	1 fiber	Blank
12	Cotton fibers soaked in extract, different numbers of fibers tested	6	60.2a[a]	6.7b	55.3a	3.0b	18.3a	4.8a

[a] Comparisons of statistical significance are made only between fibers and corresponding blank.

Quantitative Effects

To increase efficiency of bioassays, tests using different quantities of kairomone were performed.

Methods. In experiment 11 a silk gland extract was serially diluted 4 times (0.5–500 glands/ml), and cotton fibers were treated with each dilution. Females were not presented a choice but only exposed to fibers soaked in one dilution. Different females were used for different dilutions. The exposure of 4 females to four different kairomone concentrations constituted one replicate. One-way analysis of variance was used to assess differences in responses to different concentrations.

In experiment 12, cotton strands were treated with an extract (53 glands/ml) and either 1, 10, or 20 strands were placed on Parafilm squares. Each female was exposed to a square containing a given number of strands and to a blank containing no extract-treated strands. One replicate constituted tests of 3 females, each exposed to different numbers of strands. Results were analyzed by comparing responses of each female to fibers and blank with a paired *t* test.

Results. There was clearly a differential response to different extract concentrations (Table 3, experiment 11). Decreased activity occurred at 5 glands/ml, but this was still greater than the lowest concentration tested.

Responses to number of extract-treated strands were also quantitative (experiment 12). As previously shown using strands of gypsy moth silk (Weseloh, 1976c), 1 strand was not detectable but 10 and more strands were.

Stability and Characteristics of Kairomone

Experiments were performed to determine the stability of the silk kairomone under a variety of conditions and some of its characteristics.

Methods. A number of experiments (13–18) involved holding dissected silk glands or silk gland water extracts under a variety of conditions and testing the parasite's responses to extract-treated cotton fibers. Extract or dissected silk glands were stored in a freezer (−18°C), a refrigerator (4°C), or at room temperature (25°C) for varying numbers of days before being used to treat cotton strands. Extract was heated to approx. 100°C by placing test tubes containing extract in a boiling-water bath. It was freeze-dried in a LabConCo Corporation freeze-dry apparatus and redissolved in water for testing.

Dialysis (experiment 19) was carried out by placing 0.4 ml of extract in dialysis tubing (Union Carbide, 48 Å pore diam.), immersing in 3 ml distilled water, and stirring continuously for 1.5 hr. Both the inside and outside solutions were placed in a boiling-water bath and the outside solution vacuum evaporated to 0.4 ml. Both solutions were tested.

TABLE 4. RESPONSE OF *A. melanoscelus* TO COTTON FIBERS SOAKED IN SILK GLAND EXTRACT TREATED IN VARIOUS WAYS

Experiment number	Extract treatment	Number of replicates	Silk gland extract concentration (glands/ml)	Average % contacts leading to examinations
13	Extract at −18°C 1 day	8	50	20.3a
	Extract at 4°C 1 day			28.4a
	Extract at 25°C 1 day			24.4a
14	Extract at −18°C 17 days	8	53	38.8a
	Water			4.6b
15	Silk glands at −18°C 37 days, extracted day of test	5	50	68.4a
	Water			27.7b
16	Extract freeze-dried then 25°C 5 days	8	50	37.8a
	Extract fresh			42.0a
	Water			11.0b
17	Extract at 100°C ½ hr	8	50	46.7a
	Extract at 0°C ½ hr			46.8a
	Water			4.0b
18	Extract at 100°C ½ hr, then −18°C 1 day	8	50	41.3a
	Extract at −18°C 1 day			31.5a
	Extract fresh			37.3a
19	Extract dialyzed, inside solution	8	75	47.8a
	Extract dialyzed, outside solution			4.8b
	Water			6.1b
20a	Extract with 95% ethanol supernatant	8	50	18.2a
	95% ethanol only			6.4a
b	Extract with 95% ethanol precipitate extracted with water	8	50	24.6a
	Water only			0b
21a	Freeze-dried extract in 95% ethanol	7	50	29.0a
	95% ethanol only			14.3a
b	Freeze-dried extract in 95% ethanol	8	122	45.9a
	95% ethanol only			12.5b

In two experiments 95% ethanol was used as the extracting solvent to determine its affect on the kairomone. The supernatant was tested directly (experiment 20a). The precipitate was resuspended in water, centrifuged, and the resulting supernatant tested (experiment 20b). In experiment 21a and b, a freeze-dried water extract was suspended in 95% ethanol and tested.

Results. Extract and silk glands were stable when frozen for more than 2 weeks (Table 4, experiments 14 and 15), and extract was as stable at room temperature as it was when frozen (experiment 13). Freeze-drying did not alter the activity of the kairomone (experiment 16), nor did heat treatment (experiment 17), nor heat treatment followed by freezing (experiment 18). The kairomone remained inside the dialysis bag, indicating that it is a large molecule or attached to a large molecule (experiment 19). Experiment 20a shows that ethanol is either a poor solvent for the kairmone or deactivates it. Because the water extract of the ethanol precipitate was active (experiment 20b), any deactivation was reversible. Experiment 21b confirms that ethanol does not drastically affect the kairomone and the same trend is evident in experiment 21. (If data for one anomalous female in experiment 21a are deleted these results are significant as well.) These experiments strongly suggest that ethanol extracts are not active only because ethanol is a poor solvent for the kairomone.

Effects of Silk on Host-Related Behavior

Previously it was shown that silk deposited on a substrate increased the number of contacts with and ovipositions on gypsy moth larvae (Weseloh, 1976c). However, the increase over the situation where silk was not present was quite small. To obtain more insight into the mechanisms involved and to determine how important this phenomenon is in the host-selection process of the parasite, additional experiments were run.

Methods. These experiments differed from those already reported in that a large, straight-sided 23.7-cm-diam. cylindrical glass battery jar 8.1 cm high was used as the test chamber rather than a petri dish because the test substrates were quite large. First instar gypsy moth silk was wrapped around squares of filter paper at a rate of 10 strands/cm of square side length. Paper squares of different sizes with and without silk were glued together in different combinations with polyvinyl glue to form surfaces parts or all of which were silk covered. In all experiments but number 22 the smaller square was always glued to the center of the larger square. In experiment 22 only squares 3 cm on a side were tested with or without silk (see Table 5). One 1st instar gypsy moth larva, previously killed by freezing, was glued to the center of each combination. [*A. melanoscelus* responds to such larvae as readily as to living ones (Weseloh, 1974)]. Two specimens of one combination were placed opposite each other in the chamber bottom and held down with drops of honey. Two specimens of another combination were placed at right angles to the first one. The chamber was inverted over a large mirror so the parasites could be easily observed and an *A. melanoscelus* female released. Her activity was observed for 0.5 hr or until she contacted each kind of test material 10

TABLE 5. RESPONSE OF *A. melanoscelus* FEMALES TO GYPSY MOTH LARVAE AS INFLUENCED BY SILK ON SUBSTRATE

Experiment number	Description of filter paper squares, (Small square glued to larger square)				Number of replicates	Average % substrate contacts resulting in:		
	Size of smaller square (cm)	Silk wrapped	Size of larger square (cm)	Silk wrapped		Examination of substrate	Host contacts	Attempted ovipositions
22	—	—	3	Yes	9	68.0a	44.9a	31.8a
	—	—	3	No		28.6b	23.7b	12.2b
23	1	Yes	3	Yes	7	90.7a	60.0a	44.1a
	1	Yes	3	No		70.0b	38.6b	27.1a
24	1	Yes	3	Yes	8	54.8a	36.8a	25.5a
	1	No	3	Yes		52.4a	41.5a	37.6a
25	3	Yes	5	Yes	8	74.8a	41.5a	30.2a
	3	No	5	Yes		79.2a	44.6a	38.6a

times, whichever came first. Data recorded were: number of contacts with test substrates, number of antennal examinations on the substrate, number of host contacts, and number of attempted ovipositions in hosts.

Results. The average percent of substrate contacts which were followed by substrate examination, host contacts, and attempted oviposition are given for each experimental situation in Table 5. Experiment 22, using 3-cm squares, confirms the results of Weseloh (1976c) where 1-cm squares were used that a substrate with silk results in greater numbers of host contacts and attempted ovipositions than a substrate without silk. Silk present at the same density over a wider area was more effective in doing this than silk present over a smaller area (experiment 23). Experiments 24 and 25 suggest that the silk need not immediately surround the host to be effective. Even when no silk was closer than 1.5 cm to a larva, the response was the same as when silk covered the entire area (experiment 25).

CONCLUSIONS AND DISCUSSION

The physical context in which the silk gland kairomone is presented to *A. melanoscelus* females is very important. However, silk spun directly from caterpillars was more active than the most active kairomone preparation. This is probably due to additional physical factors associated with silk, because the silk does definitely have a water-soluble kairomone, and the response of female parasites to this kairomone is the same as their response to the water-soluble kairomone from silk glands. However, only chemical characterization will definitively show whether or not the kairomone of the silk gland and silk itself are the same.

As the silk glands are an abundant source of kairomone, and because the kairomone is stable under a variety of conditions, characterization of it should be straightforward. However, the dialysis experiment suggests that the active component has a high molecular weight, and this could increase the difficulty of identifying it. But the kairomone is also heat-stable and not deactivated by ethanol. Thus if it is proteinaceous, for instance, it does not depend on tertiary structure for activity. Perhaps the kairomone is a relatively small functional group attached to a larger molecule. This would increase the likelihood of identifying it considerably.

Characterization of the kairomone is an important priority because of the effect the presence of silk has on the host-related behavior of the parasite. Silk present on the substrate increases the parasite's ability to find and thus oviposit in a host. The parasite vigorously examines areas nearby, leading to parasitism of hosts in adjacent areas as well.

A general theory of host selection in *A. melanoscelus* can now be given,

based on this and previous studies. *A. melanoscelus* females probably respond to the color of tree leaves (Weseloh, 1972a). This response restricts their activities to the foliated parts of trees in a woodland (Weseloh, 1972b). Females probably fly from leaf to leaf, examining each briefly. Once they contact a leaf with fresh gypsy moth silk on it (i.e., silk which has not had the kairomone washed off by rain or dew) they begin intensely examining the leaf and the immediate vicinity. Because gypsy moth larvae tend to spin silk on leaves they are feeding on (Leonard, 1967) and because young gypsy moth larvae which *A. melanoscelus* females preferentially attack (Weseloh, 1976b) tend to remain in or near such leaves even when resting (Leonard, 1970), chances are good that the parasite will encounter a host during this period of intensive examination. Once the host is contacted, a lipid-soluble kairomone in the host's integument (Leonard et al., 1975) and host hairyness (Weseloh, 1974) enable the parasite to recognize the host and an egg is laid. The parasite is also able to distinguish between already parasitized and nonparasitized hosts and tends to avoid the former (Weseloh, 1976a).

The portion of this theory concerning the initial response to foliage is speculative at present, but the response to silk is firmly established and fits the presumed initial response. With this selection mechanism, *A. melanoscelus* has the potential to parasitize hosts even at low host population densities (Hoy, 1975; Weseloh and Anderson, 1975).

The uses of the kairomone, should it prove easily synthesizable, are obvious. *A. melanoscelus* (either intentionally released or natural populations) could be induced to intensively search regions where the kairomone has been artificially applied, perhaps impregnated in polymer fibers similar to microencapsulated insecticides, and so increase parasitism of gypsy moths in such areas. This has been done with other parasites (Lewis et al., 1975). Experiments to investigate the procedure's feasibility with *A. melanoscelus* await characterization, synthesis, and formulation of the kairomone.

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