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A NONDESTRUCTIVE TRAP FOR *Dendroctonus frontalis* ZIMMERMAN (COLEOPTERA: SCOLYTIDAE)

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Abstract—The bucket trap is a lightweight device for capturing southern pine beetles in flight and retaining them either alive or dead for later examination. It is not messy like the sticky trap and not cumbersome like conventional live traps. Placing the bucket against a vertical silhouette increases the number of beetles caught. Few nontarget insects are captured except for the clerid *Thanasimus dubius*. When the trap was baited with Frontalure, about 99% of the beetles trapped were male. When infested pine bolts were used as bait, roughly equal numbers of males and females were captured.

Key Words—southern pine beetle, *Dendroctonus frontalis*, bucket trap, Frontalure, *Thanasimus dubius*.

INTRODUCTION

Southern pine beetles (*Dendroctonus frontalis* Zimmerman) in flight are normally captured with live traps, such as rotating nets and sleeve funnels, or with sticky traps (Gara, 1967; Gara et al. 1965). Live traps, however, are large, bulky, and require power sources. Sticky traps, though lightweight, portable, and suitable for synthetic pheromone baits, catch many nontarget organisms as well as debris; moreover, beetles become so mired in the sticky material that they are of little use for chemical investigations and other studies (Moser, 1976).

This paper describes an improved bucket trap that is as lightweight, portable, and easy to bait as the sticky trap but catches few if any nontarget insects. It can retain trapped beetles either alive or dead, and it has none of the messy features of sticky traps.

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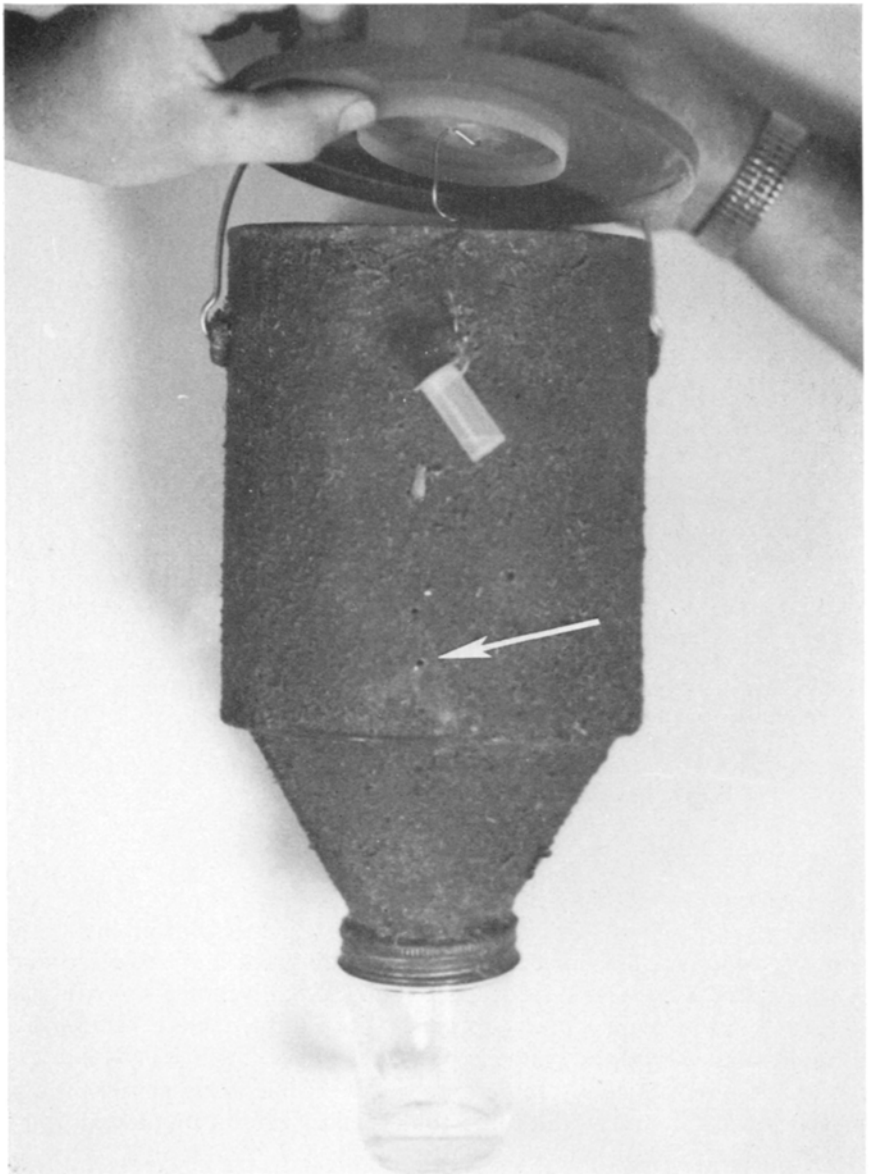

FIG. 1. The bucket trap with Frontalure-containing vial exposed. Beetles enter through the holes (arrow) and drop into jar which contains water.

METHODS AND MATERIALS

Trap Construction

The trap consists of a 1-gal (3.79-liter) paint bucket with top and bottom removed (Fig. 1). A 16-cm (top diameter) metal funnel was soldered to the lower end; a mason jar rim was soldered to the narrow end of the funnel, and a 0.5 pt (0.24-liter) glass jar was connected to the rim. The top of the can was covered by a plastic lid; a 2.5-ml polyethylene snap-cap vial containing 20 drops (0.3 ml) of the attractant Frontalure,[3] a mixture of 1 part frontalin to 2 parts α-pinene (Vité, 1970) was suspended by a stiff wire from the center of the lid. Seventy-two entry holes (2-mm diam) were punched in the can in eight equidistant vertical rows. To minimize rain entering the holes, the upper rim of each was bent out with a punch to form hoodlike shields. One advantage of the trap is that it provides for a walking response in addition to the flying response. To provide a walking surface for the beetles, the can was first painted with water-soluble white glue and then rolled in sawdust; after drying, the can was sprayed with a light coat of red enamel, a color that appears to be highly attractive to flying adults (Nash, 1970). Insects entering the holes fell down the funnel into the glass jar. To collect living specimens, it was necessary to place tissue paper in the jar because the beetles would otherwise concentrate at the bottom and chew off each other's legs and antennae. If dead beetles were desired, water was placed in the jar instead of paper.

Field Placement of Traps

To test the bucket traps, an infestation was artificially started in fall 1973 by baiting several trees with Frontalure in a *Pinus taeda* L. stand in Rapides Parish, Louisiana. The spot was maintained and directed by continuous baiting of new trees just ahead of the line of newly infested trees, which generally advances in only one direction away from the initial attractant source (Gara and Coster, 1968). This line is commonly referred to as a "front" because of its analogy to weather systems. After 10 mo., the spot encompassed about 1 ha and consisted of approximately 250 trees, about one-third of which contained live beetles. Traps were set out November 4, 1974, and tested through November 1, 1975, being inspected twice weekly. Our traps were about 20 m ahead of the front among uninfested trees along a line facing the spot's south end. Traps were placed about 9 m apart and were moved forward as the front advanced. The number of traps varied from 5 to 12 because only those with sustained large catches were retained.

[3] Mention of trade names is solely to identify materials used and does not imply endorsement by the U.S. Department of Agriculture.

FIG. 2. Field placement of bucket trap.

Traps were initially hung with strings from tree branches, but very few beetles were captured until a vertical silhouette was added. Subsequently, buckets were suspended about 4 m high from pulleys against $1 \times 30 \times 400$-cm plywood boards (Fig. 2). These "artificial trees" had the added advantage of being highly mobile.

When a high percentage of the captured beetles turned out to be males, it was necessary to determine if trap design or bait was responsible. Therefore, two bolts of *Pinus taeda* naturally infested with both sexes of southern pine beetles were screened and placed inside each of two bucket traps. In addition, three bucket traps baited with Frontalure and coated with Stickem Special® were set out.

Because large numbers of flying adults have been caught on sticky traps in or near infestations (Reeve, 1975), three pairs of bucket traps were tested to see whether the beetles would enter unbaited bucket traps. Each pair consisted of one trap baited with Frontalure and a twin without Frontalure in the plastic vial. The three pairs were placed in a line along the front of a spot from 6 to 15 m ahead of the infested trees. Individual traps within pairs were separated by about 5 m, and pairs were separated by about 10 m. A total of eight collections were made twice weekly from April 26 through May 20, 1977. After the beetles from each pair of traps were collected, a coin was flipped. If the toss was "heads," positions of the baited and unbaited buckets were switched so that the relative positions of buckets within pairs remained at random.

RESULTS AND DISCUSSION

During a trapping period of 12 mo., a total of 9637 southern pine beetles were caught. Collections improved after the buckets were suspended against plywood boards, apparently because many scolytids (including *D. frontalis*) orient in flight to vertical objects (Henson, 1962; Gara et al., 1965; Rudinsky, 1966; Shepard, 1966; Billings et al., 1976). Traps consistently collecting the most beetles were located in open areas, which provided the beetle with aerial pathways, whereas traps in obstructed areas were inefficient.

Of 1512 beetles examined for sex determination, the majority (98.7%) were males, as determined by clearing the beetles in lactophenol and examining the genitalia. Our data support Hughes' (1976) observation that fewer females than males are attracted to point sources of frontalin. Perhaps the bucket trap simulates a newly initiated female gallery, which, like the trap, attracts only males.

Tests with infested bolts indicated that the bait and not the trap's design was responsible for the high percentage of males captured. One of the buckets containing infested bolts trapped 14 males and 13 females; the other trapped 12 males and 8 females. The traps baited with Frontalure and coated with Stickem Special® captured 242 males, of which 104 were found inside the collecting jar and 138 were stuck to the outside of the can. No females were collected.

Tests with baited vs. unbaited traps likewise showed that the trap's design did not attract beetles. A total of 155 males and 2 females were trapped in the baited buckets during the 4-wk period; no beetles were found in the unbaited buckets. Totals for each collecting date varied from 2 to 41 beetles.

This trap design may be of limited usefulness for attracting other bark beetle species. Small-scale tests with *Dendroctonus brevicomis* (Lec.) in Madera County, California, and with *Scolytus multistriatus* (Marsham) at Delaware, Ohio, showed that bucket traps baited with pheromones that attracted hundreds of beetles to nearby conventional traps caught only one or two beetles each.

In addition to the southern pine beetle, specimens of the following insect species were captured: *Corticeus glaber* LeConte, *Ganascus ventricosus* (LeConte), *Hylastes porculus* Erichson, *Ips avulsus* (Eichhoff), *I. calligraphus* (Germar), *I. grandicollis* (Eichhoff), *Lyctocoris elongatus* (Reuter), *Osmita colon* (L.), *Platydema flavipes* (F.), and *Thanasimus dubius* (F.). Only one or two specimens of each were found, with the exception of *T. dubius*, of which 258 were collected. This species as well as other clerids are known for their attraction to bark beetle pheromones (Wood et al., 1968; Vité and Williamson, 1970; Rudinsky et al., 1971; Whittaker and Feeny, 1971; Pitman, 1973). All except *G. ventricosus*, *H. porculus*, and *O. colon* were previously listed as southern pine beetle associates (Overgaard, 1968; Moser et al., 1971). The euglenid *G. ventricosus* is associated with scolytid-infested trees, where they oviposit in decaying wood (F. G. Werner, University of Arizona, personal correspondence). Adults of the scolytid *H. porculus* are attracted to freshly cut lumber, and breed in stumps and roots of dying pines (Baker, 1972). Furniss and Schmitz (1971) found four related species of *Hylastes* attracted to frontalin and other tree volatiles. W. A. Connel, of the University of Delaware (personal communication), regards the capture of nitidulid *O. colon* as accidental, although this pest of stored products is often attracted to volatiles emanating from decomposing vegetation or fermentation.

Addenda to Proof. Dr. G.D. Amman reports that in August, 1977, at Ogden, Utah, bucket traps baited with linalool, the *Ips pini* (Say) attractant, failed to attract any beetles. Bucket traps containing screened bolts naturally infested with *Ips pini* attracted flying adults of both *Ips pini* and *Pithophthorus* sp.

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ALLELOPATHY AS A FACTOR IN THE SUCCESS OF *Helianthus mollis* Lam.

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Abstract—A method to rapidly screen species suspected of producing allelochemics, using results from simple bioassay tests, is presented. By measuring the osmotic potential of *H. mollis* extracts and using mannitol solutions of comparable osmotic potential, the influence of osmotic potential in the bioassay was eliminated. Nested analysis of variance was used to examine the separate influences of (1) extract concentration, (2) source of plants used in extract preparation (edge or center of clones) (3) osmotic potential of the extract, and (4) the differential development of radicles and shoots of species used in the bioassay tests. Bioassay tests for allelopathy showed that extracts made of whole *H. mollis* plants significantly inhibited both radicle and shoot development of radish and wheat, but only the radicle of little bluestem. There was a significant increase in the inhibition of radish shoots and wheat radicles at high concentration of the extract, but the radicle of little bluestem was inhibited more at the lower concentration. Extracts prepared from plants collected from the clone center inhibited radish radicle development significantly more than extracts made of plants growing at the clone edge.

Key Words—*Helianthus mollis*, allelopathy, bioassay, competition, osmotic potential.

INTRODUCTION

During the past 10 years there has been widespread documentation of the importance of allelopathy as an aspect of plant interference (Muller, 1969; Rice, 1974). In most cases, the toxic chemical produced by a species has been shown to inhibit germination or growth of other species, although, in some studies, autotoxicity has been demonstrated (Curtis and Cottam, 1950).

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Helianthus mollis Lam. (ashy sunflower) is a species of scattered occurrence throughout most of the tall-grass prairie region. This species forms dense colonies from creeping underground rhizomes and tends to exclude other species from those portions of the clones where it occurs with the greatest densities (Steyermark, 1963). In this study, a technique to rapidly screen species suspected of allelochemics is presented by examining the allelopathic properties of *H. mollis*.

METHODS AND MATERIALS

Field Methods

In the field, it appeared that inhibition of other species was greatest in the center of *H. mollis* clones. Consequently, the possibility of plants at the center of a clone having greater allelopathic properties than those at the clone edge was examined. To document the observed pattern of interference, a transect of contiguous 10×10 cm square quadrats was sampled across a single *H. mollis* clone on July 11, 1970, in a tall-grass prairie located on a railroad right-of-way near Hallidayboro, in Jackson County, Illinois. The total length of the transect was 10.5 m, providing 105 individual quadrat observations. In each of these quadrats, density counts were made by species.

Laboratory Methods

Plant extracts were made of whole *H. mollis* plants (shoot, root, rhizome) collected from the center and the edge of the clone. The plant tissue was oven-dried at 70°C, ground, and soaked in glass-distilled water at room temperature for 24 h. The ratio by weight of tissue extracted to water added was 1:10. After soaking, the mixture was twice passed through cheesecloth, and the filtrate was permitted to settle for 12 h. The upper portion of the filtrate was decanted and used for the bioassays. Dilutions of the cleared extract equal to 1/2 and 1/4 full strength were prepared by adding distilled water. These extracts are referred to as 1:10, 1:20, and 1:40 extracts, respectively.

Osmotic potentials of plant extracts much greater than -1.0 to -0.5 atm have been shown to result in inhibition of seed germination and seedling development (Anderson and Loucks, 1966; Bell, 1974). Accordingly, the osmotic potentials of the plant extracts were determined using a Fisk osmometer. The 1:10 extracts had osmotic potentials of -2.34 ± 0.01 (edge) and -2.21 ± 0.03 (center), and were not used in the bioassay. Osmotic controls containing mannitol (Cruden, 1974) were used for the diluted extracts.

Three species were used in the bioassay tests: wheat (*Triticum*), radish (*Raphanus*), and little bluestem (*Schizachyrium scoparium* Nees). Twenty-five seeds of each species were placed in petri dishes containing a single layer of filter paper. The filter papers were moistened with 5 ml of the plant extracts, mannitol solutions, or glass distilled water. Each treatment and control was replicated twice. Both wheat and radish seeds were kept in the petri dishes 3 days, and little bluestem for 5 days, before radicle and shoot lengths were measured and germination percentages calculated.

Analyses of the Field Data

Quadrats obtained from the transect were grouped according to the numbers of *H. mollis* stems they contained. Both average numbers of other species and average numbers of stems of other species were calculated for quadrats containing 1, 2, 3, and 4 or more *H. mollis* stems (it was necessary to combine those quadrats with more than three stems into a single category due to their low frequency). The data were then analyzed using linear regression techniques.

Analyses of the Bioassay Data

Germination rates among the treatments were examined using contingency chi-square. However, since no significant differences were found, germination was not considered further.

Influences of the *H. mollis* extract on the radicle and shoot development of radishes, wheat, and little bluestem were examined using analysis of variance techniques. Preliminary analyses of the data were performed to determine whether effects of osmotic potential significantly contributed to radicle and/or shoot inhibition. The two mannitol treatments and the distilled water control within each experiment were compared by means of one-way nested analysis of variance. Effects of the *H. mollis* extracts (edge and center) and the mannitol solutions were then compared at both low and high concentrations within each experiment by means of a two-way nested factorial analysis of variance. Both treatment and concentration effects were considered to be fixed, and the replicate effects were considered as being random. The treatment sum of squares within each analysis was further partitioned into two a priori comparisons: (1) a comparison of the mannitol solution to the *H. mollis* extracts, and (2) a comparison of the plant extract from the edge to that from the center.

The above analyses of variances are greatly facilitated if the sample sizes are equal among treatments. Since the number of seeds that germinated varied among the treatments, it was necessary to exclude some observations

(chosen at random) from certain replicates to obtain equal subclass numbers. Approximately 7% of the available data was excluded in this manner.

RESULTS

Field Studies

Figure 1 shows the average number and density of other species plotted against the average number of *H. mollis* stems per square decimeter. Linear regression analysis of both sets of data yielded significant negative regression coefficients. These results confirmed the general field observations (Steyermark, 1963) that, as the density of *H. mollis* stems within the clone increased, there tended to be a reduction in the density and number of species present. While these results do not confirm allelopathy, they do suggest interference between *H. mollis* and its associates.

The most abundant of the associates of *H. mollis* included Indiangrass [*Sorghastrum nutans* (L.) Nash] and *Carex hirsutella* Mack., with average

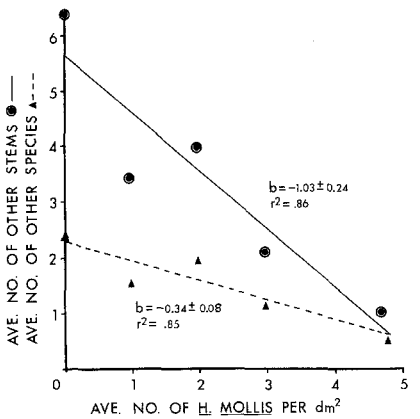

FIG. 1. Relationship between the average number of other species and stems of other species per square decimeter and the density of *Helianthus mollis*. Both regressions are significant ($P < .05$), with coefficients of determination (r^2) of .85 and .86 for species and stems, respectively.

densities of 2.5 and 0.6 stems per square decimeter and frequencies of 81.0% and 20.9%, respectively. The average values for *H. mollis* were 1.3 stems and 69.5%. A total of 21 species was found in the transect. Unfortunately, viable seeds of these species were not available, and they were not used in the bioassay tests.

Bioassay Tests for Allelopathy

The osmotic potentials of the treatments used in this investigation and the mean radicle and shoot lengths per treatment for the radish, wheat, and little bluestem are presented in Table 1. No significant effects of osmotic potential were found on radicle or shoot inhibition in radish, wheat, or little bluestem. These results are in agreement with those of Anderson and Loucks (1966) and Bell (1974), who predicted that little, if any, influence of osmotic potential should be expected within the interval 0 to −1 atm. Significant differences between replicates were found in the radish radicle and wheat shoot experiments. These significant replicate effects may be attributed to differences in the microenvironments among the petri dishes within a treatment.

Significant treatment effects were found in radish for both radicle and shoot (Table 2). The a priori comparisons indicate that plant extracts significantly inhibited both radicle and shoot development in comparison with the

TABLE 1. OSMOTIC POTENTIAL OF PLANT EXTRACTS AND CONTROLS AND THE AVERAGE RADICLE AND SHOOT LENGTH OF THE BIOASSAY TEST SPECIES

Treatment	Osmotic potential ($\bar{X} \pm SE$)	Radicle and shoot length (mm)					
		Radish		Wheat		Little bluestem	
		Radicle	Shoot	Radicle	Shoot	Radicle	Shoot
Control	—	22.3	12.5	41.0	25.0	20.3	13.5
Control mannitol	−0.67±0.02	24.5	13.2	35.5	22.4	15.2	11.7
Extract edge (1:40)	−0.58±0.00	23.8	11.8	33.0	17.9	8.2	9.4
Extract center (1:40)	−0.54±0.01	16.6	10.3	38.2	22.6	10.2	11.0
Control mannitol	−1.13±0.05	22.9	11.7	39.4	24.1	14.9	11.1
Extract edge (1:20)	−1.10±0.02	17.3	9.7	33.0	19.5	13.8	11.7
Extract center (1:20)	−1.03±0.01	11.5	8.2	28.9	19.0	14.8	14.3

TABLE 2. *Helianthus mollis* F STATISTICS[a]

Effect	*Df*	Radish		Wheat		Little Bluestem	
		Radicle	Shoot	Radicle	Shoot	Radicle	Shoot
Treatment	2, 6	7.419*	11.178**	6.371*	5.399*	3.502^{ns}	0.700^{ns}
Mannitol vs. extract	1, 6	8.381*	17.398**	12.622**	8.501*	6.098*	0.015^{ns}
Edge vs. center extract	1, 6	6.458*	4.958^{ns}	0.121^{ns}	2.298^{ns}	0.906^{ns}	1.385^{ns}
Concentration	1, 6	4.451^{ns}	12.013*	2.681^{ns}	0.009^{ns}	6.619*	1.352^{ns}
Treatment × concentration interaction	2, 6	0.480^{ns}	0.129^{ns}	12.732**	2.429^{ns}	2.058^{ns}	0.638^{ns}
Replicates	6, 264	2.661*	0.841^{ns}	0.897^{ns}	1.586^{ns}	$^{a}0.478^{ns}$	$^{a}1.053^{ns}$

[a] Key: * = significant at 5% significance level; ** = significant at 1% significance level; ns = not significant; a = associated degrees of freedom are 6 and 132

mannitol solution. Furthermore, the plant extract taken from the center of the clone resulted in a significantly greater inhibition of the radicle than the extract taken from the clone's edge. Significant concentration effects were also found in the shoot experiment, and significant replicate differences were again observed in the radicle study.

The analyses of the wheat data (Table 2) revealed significant treatment effects for both radicle and shoot. Again, it was found that the plant extracts significantly inhibited radicle and shoot development in comparison with the mannitol control. No significant difference was observed between the plant extracts taken from the center and edge of the clone. A significant interaction between treatment and concentration was found in the radicle experiment. This implies that any differences found between treatments depend on the level of concentration. In this instance, the significant difference between the plant extracts and the mannitol solution in inhibition of radicle growth occurred only at the higher concentration; no significant differences between treatments existed at the lower concentration.

The analyses of the little bluestem data (Table 2) initially revealed no significant treatment effects for either radicle or shoot inhibition. However the a priori comparison of the mannitol solution to the plant extracts for radicle growth revealed a significant difference. No such difference was found between the plant extracts taken from the center and edge of the clone. Significant concentration effects were also indicated in the radicle analysis. Interestingly, a greater inhibition of radicle development occurred at the *lower* concentrations. A similar trend was observed in the shoot analysis (greater inhibition at the lower concentration), although the difference was not significant.

DISCUSSION

In this study, *Helianthus mollis* was shown to have a negative effect on the abundance of associated plant species. The separate roles of competition and allelopathy were not assessed; however, bioassays revealed that extracts of *H. mollis* were inhibitory to several species of plants.

The statistical procedures used in the analyses of the bioassay data permitted the partitioning of the separate influences of a variety of factors on the development of the species examined. In several instances, significant differences were demonstrated between replicates. These differences can be expected and need to be accounted for in the analysis of data. Replicates exhibiting significant differences should not be discarded as has been previously done by some workers (del Moral and Cates, 1971).

Although a negative linear relationship exists between the density of

H. mollis stems and success, as measured by density, of other species, the density of *H. mollis* stems was not related to clone position. This suggests that interference may increase as the proximity to *H. mollis* increases. Wilson and Rice (1968) also reported that inhibition of *Erigeron canadensis* and *Rudbeckia hirta* was associated with proximity to individuals of *Helianthus annuus*. Similarly, Al-Naib and Rice (1971) found that sycamore (*Plantanus occidentalis*) inhibited several understory plants, and that the zone of inhibition was associated with areas under sycamore trees having heavy litter accumulation.

Our work has not delineated the separate influences of competition and allelopathy as aspects of interference in the success of *H. mollis*. However, the methods used to test for allelopathy in this species will permit rapid screening of species suspected of producing allelochemics. The statistical procedures employed allow the assessment of the importance of a variety of factors in the bioassay tests.

Further studies would include more sophisticated bioassay techniques (Rice, 1974) and the use of seeds of plants that are thought to be inhibited by *H. mollis* in the field.

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SOME CHEMICAL CONSTITUENTS OF THE SECRETION FROM THE TEMPORAL GLAND OF THE AFRICAN ELEPHANT (*Loxodonta africana*)[1]

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Abstract—The temporal glands of African elephants were examined macroscopically and histologically, and were found to be modified apocrine sweat glands. The secretion from these glands was analyzed by gas chromatography and mass spectrometry, and some of the major volatile components have been identified as phenol and *m*- and *p*-cresol.

Key Words—African elephant, temporal gland, phenol, *m*- and *p*-cresol, apocrine sweat gland, *Loxodonta africana*.

INTRODUCTION

On both sides of the head of the African elephant (*Loxodonta africana*), midway between the outer canthus of the eye and the external auditory meatus, there is located a relatively large organ identified as the temporal gland. The gland is embedded in the subcutaneous fascia, and its external orifice is a vertical slit located deep in a fissure of the skin in the area of the temporal depression. The name of this gland is derived from its anatomical location (Fig. 1).

The temporal gland, which is present in both sexes, is apparently functional in all age groups, except in the very young calves (Perry, 1953; Laws,

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FIG. 1. A mature African elephant shot during a culling operation, showing copious secretion from the temporal gland on the side of the head appearing as a dark streak.

1970). We have observed temporal gland secretion in calves as young as approximately 1 year of age. Johnson and Buss (1967) reported that the temporal gland increased in weight with the age of the elephant. The largest gland recorded weighed 1590 g in an old bull, and weighed only 230 g in an 11-year-old bull. Laws (1970) stated that the gland grew at a similar rate in both sexes up to an age of 20 years, but then its growth accelerated so that the temporal gland weighed twice as much in the male as in the female. However, Short, Mann, and Hay (1967) failed to find a sex difference in the size and structure of the temporal gland.

One of the earliest morphological descriptions of the temporal gland was made by Eales (1925), who described it as a modified and complicated sweat gland. More recently, Sikes (1971) described the temporal gland as an apocrine cutaneous gland.

Macroscopically, the structure of an excised temporal gland appeared to be a large round mass of encapsulated tissue comprised of numerous

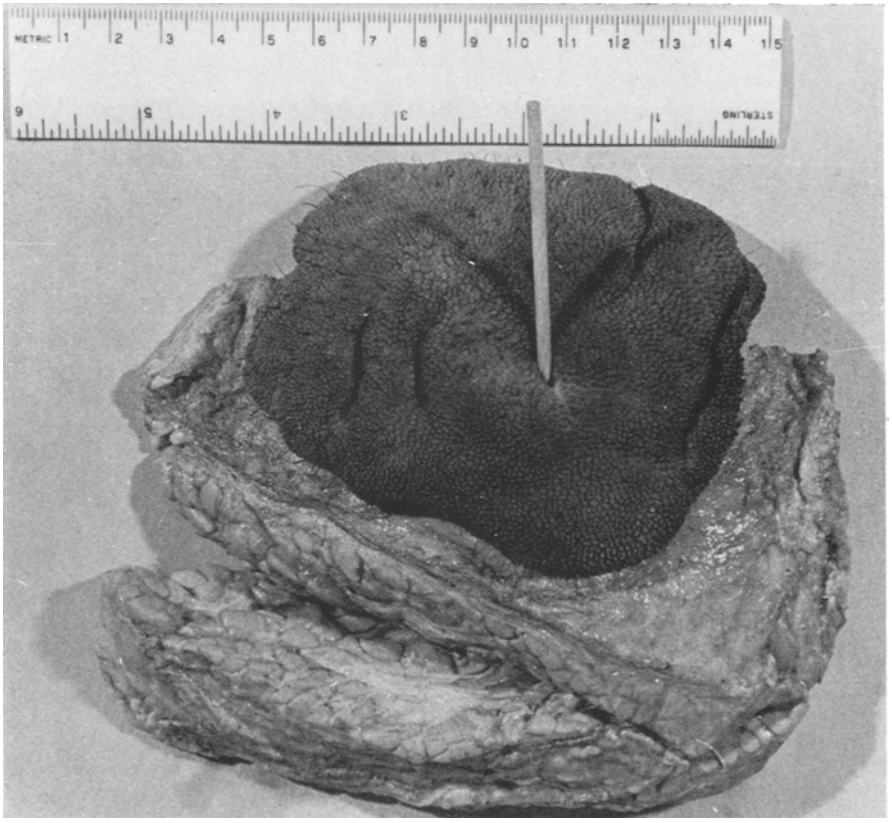

FIG. 2. An excised temporal gland from a young male elephant, showing lobules encapsulated in connective tissue. The wooden dowling is inserted in the opening of the gland on the surface of the skin.

lobules that were held together by connective tissue. A piece of dowel is placed in the opening of the gland deep in the fissure of the skin (Fig. 2).

Histologically, each of the lobules consisted of compound tubular alveoli from which two-layered ducts connected to larger ducts. Several interlobular ducts led to a series of secondary ducts that opened into a main duct out of the temporal gland and into a pore in the fissure of the skin. The alveoli consisted of a row of columnar epithelial cells oriented around the lumen, and myoepithelial cells in the periphery. The columnar epithelial cells were similar to those found in the apocrine glands of other mammals including man (Fig. 3).

Although the presence of the temporal gland has been known for a long

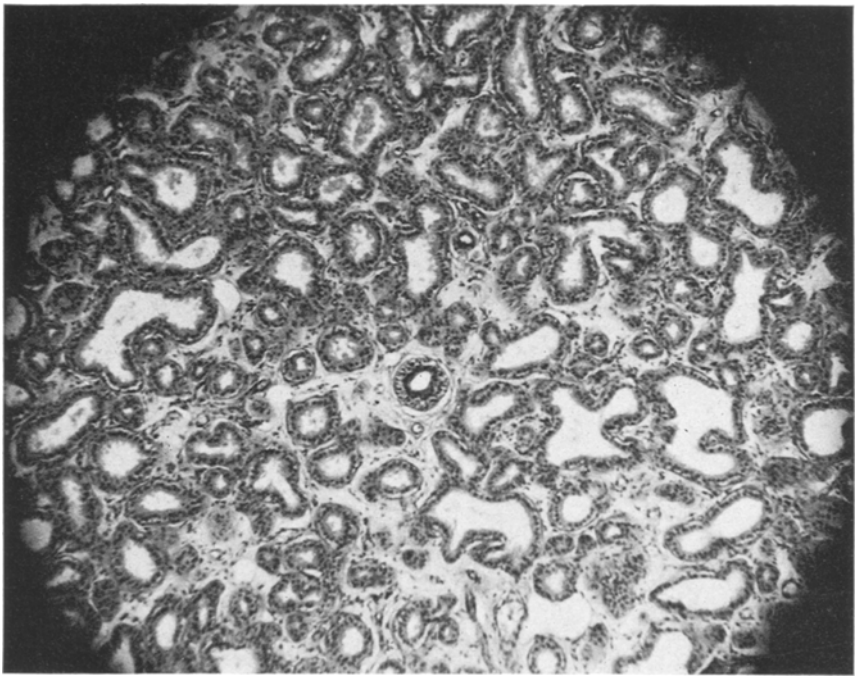

FIG. 3. Histological section through the lobules of the temporal gland, showing columnar epithelial cells of the alveoli oriented toward the lumen of the gland (120 ×, reduced 50% for reproduction).

time, there is considerable disagreement as to its function. Most of the explanations (Pocock, 1916; Bere, 1966; Laws, 1970; Sikes, 1971) are speculative. Reports on the chemical composition of the secretion have not been found.

The purpose of this study was to determine the nature and chemical composition of the secretion from the temporal gland of the African elephant. A preliminary description is provided in this report.

METHODS AND MATERIALS

Subjects

The subjects were two adult male African elephants, each from a separate geographical area: one from Kruger National Park, Republic of South Africa; the other, from Wankie National Park, Rhodesia. The elephants

were captured in open savannah during the winter season (July and August, 1975), when the temperature averaged 30°C.

Procedure

The raw temporal gland secretion was obtained from two dying male adult African elephants that had been shot during a culling program. A stainless steel tip on the end of a meter length of polyethylene tubing was inserted into the external opening of the profusely secreting temporal gland, and the secretion was aspirated into a plastic syringe. Immediately after collection, the solution was poured into glass bottles and placed in an iced container until shipment. The samples were frozen with solid CO_2 and shipped via air-freight to the laboratory in Los Angeles, California. The shipment spent 3 days in transit. Upon receipt in the laboratory, the secretion was divided into five 2-ml samples, each of which was sealed in glass and stored under liquid nitrogen until analysis. The secretion was a viscous grayish liquid with a colloidal appearance. It was primarily aqueous (approximately 92% H_2O by weight), with a pH of 7.

All solvents used in the chemical analysis were Mallinckrodt AR grade. Pentane and dichloromethane were distilled before use; other solvents were used directly from the original containers. The Baker 60–200 mesh silica gel was first washed with methanol, diethyl ether, and pentane, in that order. To avoid any laboratory contamination, all transfers were made with glass syringes. All glassware was washed with CrO_3–H_2SO_4, dilute NaOH, distilled H_2O, and CH_2Cl_2, in that order. Great care was taken to avoid the use of any substances containing plasticizers; copper tubing was used for N_2 gas in solvent removal. All vials were sealed with Teflon liners (Pierce). Because the syringe used in the field for obtaining the samples was plastic, an identical syringe was extracted as a control to find contaminants. The major contaminant was found to be dioctyl adipate, by gas chromatography and mass spectrometry (GC–MS). Several phthalates were also present (by GC–MS and gas chromatographic retention times). All extraction and fractionation procedures were carried out in parallel with blanks to identify any contaminants. Only phthalates were found in the blanks.

One milliliter of secretion was extracted with pentane–CH_2Cl_2 (5:1, vol/vol). The extract was blown to dryness with nitrogen (passed through molecular sieves) and loaded onto a column of silica gel in a 9-cm × 4 mm glass capillary pipette. Nine 8-ml fractions were collected.[4] The following solvent systems were used: #1, 100% pentane; #2, 1% ether in pentane (vol/vol); #3, 2% ether in pentane; #4, 3% ether in pentane; #5, 4%

[4] The fractionation procedure used was a modification of that of Fillerup and Mead (1953), as suggested by Dr. G.A. Dhopeshwarker.

ether in pentane; #6, 10% ether in pentane; #7, 30% ether in pentane; #8, 100% ether; and #9, 100% methanol.

A Hewlett-Packard model #5720A gas chromatograph with flame ionization detector and a 50-ft 0.020 in. IDSCOT (support-coated, open tubular) column (Perkin-Elmer) was used for analysis (37,500 theoretical plates with *n*-hexadecane). The following working conditions were standard: flow 10 ml/min N_2 with 10 ml/min make up N_2, column temperature programmed from 70° to 270° at 4°/min, injector 270°, and detector 270°. To separate cresol isomers, a 2m × 1/8 in. (3.2 mm) stainless steel column was packed with 0.1% SP-1000 on 80/100 Carbopack C (Supelco Inc.) and used under the following conditions: flow 20 ml/min N_2, column temperature 225°, injector 240°, and detector 240°. Mass spectra were obtained by interfacing the SCOT column with an AEI MS-902 mass spectrometer membrane separator operating under low-resolution conditions. Column conditions were the same as previously described, except that He was used as a carrier gas. Additional mass spectra were taken with a Hewlett-Packard 5992A GC–MS system, using a 6-m OV101 2-mm ID glass column and a membrane separator.

Fractions #1–5 were analyzed by GC–MS. Fraction #6 was blown down to 1 ml; 1 ml of CH_3OH and 1 ml of 13% BF_3 in CH_3OH were added to this in a 10-ml screw-cap vial (Farrington and Quinn, 1971). The vial was sealed and dropped into boiling water for 5 min. After cooling, 3 ml of H_2O was added and the top organic layer removed. The aqueous layer was extracted twice with 2-ml portions of CH_2Cl_2, and these were added to the organic layer. This mixture would be expected to contain the methyl esters of any free fatty acids present. The analysis of this portion is in progress.

RESULTS

The results indicated that the major volatile component (57% of GC response before fractionation, using the Hewlett-Packard 5380 GC integrator under standard conditions) was found in fractions #3 and #4. This component was found to be an isomer of cresol by mass spectral comparison to authentic samples. GC retention data on OV101, XE60, dioctyl phthalates, and Carbowax 30-m GC columns indicated that the isomer was either *p*- or *m*-cresol, which have identical retention times. The two isomers were separated on the SP-1000 column, and the presence of both the *m*- and *p*-isomers was confirmed by coinjection with known samples. To determine the relative quantity of the isomers present in the original secretion, a sample was acidified and extracted with dichloromethane. The extract was also shown to contain both *m*- and *p*-cresols by coinjection, and their amounts

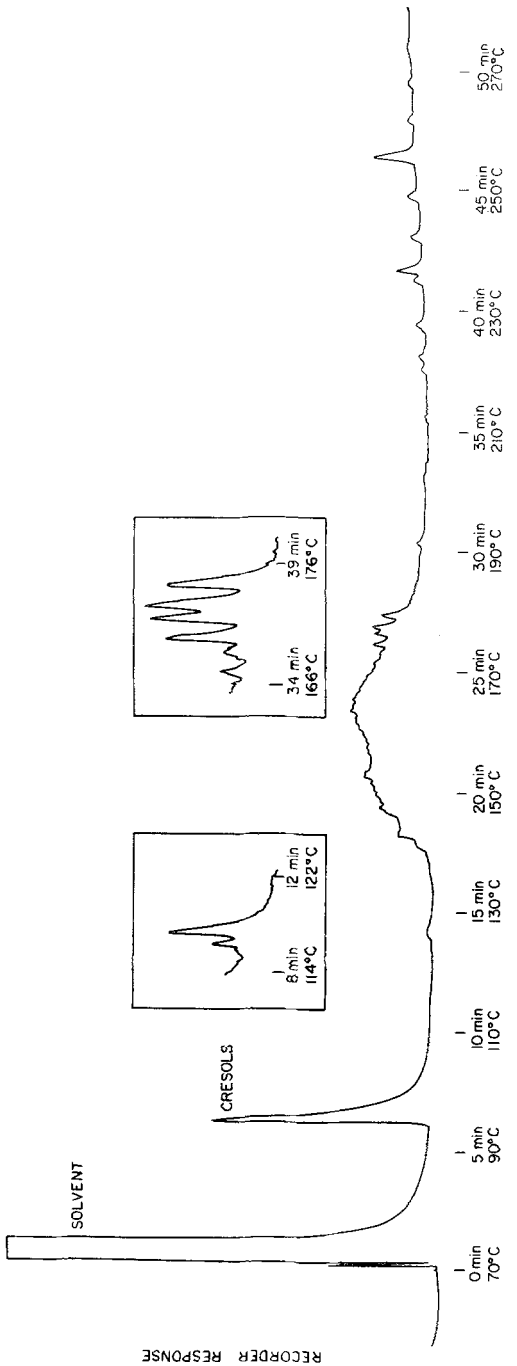

FIG. 4. Gas chromatograph of pentane extract of temporal gland secretion under standard conditions. Inserts: same conditions, but different column temperature (100–270° at 2°/min) and attenuation

were determined (peak height, half-height width corrected for response factors) to be 0.024% *p*-cresol and 0.0019% *m*-cresol by weight. Phenol (0.012%) was also found in these fractions and characterized by MS and retention data. Indole was tentatively identified by MS data, but no quantification was done. The next most abundant group of components (6% of total volatiles) was found in fraction #2; however, our GC–MS system was not sensitive enough for identification. More than 40 components were seen in all GC traces (Fig. 4). We are awaiting more sensitive GC–MS capabilities to continue the analysis.

DISCUSSION

Both *p*-cresol and phenol have previously been found in mammalian secretions such as urine. Hydroxybenzoic acid, a possible precursor of *m*-cresol, has been found in castoreum (Kingston, 1964). We had been concerned about the possibility that these phenolic substances were artifacts. This was made less likely by (1) the fact that similar ratios of phenols were found in secretions from two different elephants that had been collected in two different countries at different times, and (2) the fact that *o*-cresol was absent but *m*-cresol was present, whereas cresols of commercial origin might be expected to have *o*- and *p*-cresol but relatively little of the *m*-isomer.

The odor of the secretion was described by one of us as phenolic with mammalian overtones. The phenolic component seems to be accounted for quite well by the cresols and phenol, since an aqueous solution of the two cresols and phenol at the same concentration found in the natural secretion gave an approximate subjective match to the phenolic component of the odor.

Whether *p*- and *m*-cresols are the components of chemical communication among African elephants remains to be ascertained by behavioral studies. We have observed and photographed African elephants in the wild rubbing the area of the temporal gland on tree trunks and branches. Whether this behavior was intended as marking the environment could not be determined because other elephants did not appear to respond to it in any significant way. The rubbing of the gland area could have been a response to relieve itching or other unpleasant sensations, because we have frequently found pieces of wood, bark, and sand deeply embedded in glands that had been dissected for morphological and histological examination.

The function of the temporal gland in African elephants has not been clearly ascertained. We have observed temporal gland secretion on African elephants in captivity when they were subjected to stress and excitement. And, after more than 500 h of observation during a period of 1 year, we have

not observed the elephants rubbing the gland area on structures or other elephants in the elephant enclosure (Adams and Berg, 1977). When the temporal glands secreted on some elephants during stress and/or fear conditions, the presence of the secretion did not seem to affect the behavior of the other elephants in the compound. It is doubtful, at this stage of our thinking, that the temporal gland secretion in African elephants serves as a mammalian pheromone.

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Grateful appreciation is also expressed to the Department of National Parks and Wildlife Management, Rhodesia, for permission to collect elephant temporal gland secretions at Wankie National Park; and to Dr. Michael Kerr and Mr. R. Williams for their assistance in the collection procedure.

We also thank Dr. George Popjack for the use of the GC–MS system; and Dr. W. E. Reed and Dr. G. A. Dhopeshwarkar for their helpful suggestions.

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INSECT HYDROCARBONS
Corroboration of Structure by Synthesis and Mass Spectrometry of Mono- and Dimethylalkanes

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Abstract—Standard mono- and dimethyl-branched hydrocarbons were synthesized from substituted thiophenes for the purpose of comparing their mass spectra to those of hydrocarbons isolated from the tobacco hornworm, *Manduca sexta* L. The mass spectra of the standard compounds confirmed the structures of the partially characterized natural products.

Key Words: insect hydrocarbons, monomethylalkanes, dimethylalkanes, mass spectra, tobacco hornworm, *Manduca sexta* L.

INTRODUCTION

The assignment of structures to some mono-, di-, and trimethyl-substituted alkanes isolated from cuticular waxes of insects was based largely on the observed fragmentation pattern of these compounds when subjected to mass spectral (MS) analysis and gas-liquid chromatographic (GLC) retention times (Nelson and Sukkestad, 1970; Nelson et al., 1971, 1972; Nelson and Sukkestad, 1975). Final proof of structure has been based on the synthesis of some of these compounds (Martin and MacConnell, 1970; Jones et al., 1971; Pomonis and Nelson, 1973; Sonnet, 1976). In most cases, unambiguous comparison with synthetic samples was especially important because these hydrocarbons had been isolated as inseparable mixtures of isomers, which rendered interpretation of the MS difficult (Nelson, 1977).

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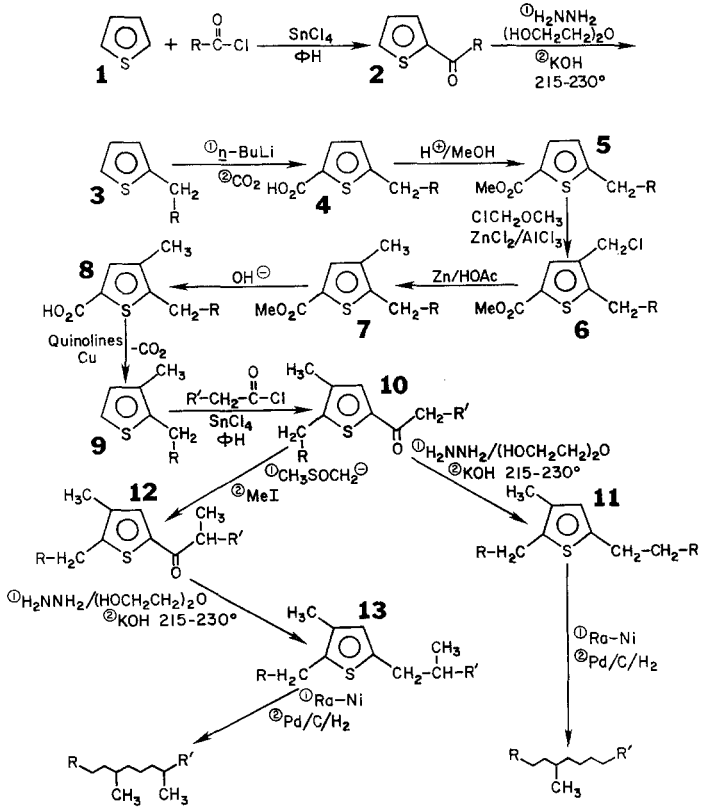
SYNTHESIS

The monomethyl- and dimethyl-branched hydrocarbons were synthesized from the appropriate trisubstituted thiophenes by Raney nickel desulfurization (Hauptmann and Walter, 1962) and additional reduction with hydrogen over palladium on carbon (Pomonis and Nelson, 1973; Pomonis et al., 1976a, b). Scheme 1 illustrates the generalized reaction sequence.



Scheme 1

This particular reaction sequence for synthesis of these compounds was selected for several reasons. The stepwise substitution of thiophene made use of classical reactions (Pomonis et al., 1976a), which resulted in products with the appropriate methyl groups in the desired position. Yields were good to excellent. Ambiguity in the position of the methyl groups in the hydrocarbons was avoided because the methyl groups, when substituted into the thiophene precursors, were regiospecific. Finally, a single thiophene with the appropriate

TABLE 1. SYNTHETIC MONO- AND DIMETHYL-SUBSTITUTED ALKANES

I	15-Methylpentatriacontane	$C_{36}H_{74}$
II	17-Methylpentatriacontane	$C_{36}H_{74}$
III	13-Methylheptatriacontane	$C_{38}H_{78}$
IV	15-Methylheptatriacontane	$C_{38}H_{78}$
V	17-Methylheptatriacontane	$C_{38}H_{78}$
VI	19-Methylheptatriacontane	$C_{38}H_{78}$
VII	9,13-dimethylheptacosane	$C_{29}H_{60}$
VIII	15,19-dimethylpentatriacontane	$C_{37}H_{76}$
IX	13,17-dimethylheptatriacontane	$C_{39}H_{80}$

substituents could be used as a synthetic precursor to several hydrocarbon homologs with the methyl substituent on the same numbered carbon atom (e.g., a homologous series of 9-methylalkanes). Hydrocarbons synthesized for this study are listed in Table 1.

MASS SPECTRA

Nelson and Sukkestad (1970, 1975) and Nelson et al. (1972) assigned structures to isolated mixtures of monomethylalkanes from the tobacco hornworm, *Manduca sexta* L., on the basis of empirical relationships first observed by McCarthy et al. (1968) in the MS of methylalkanes. These empirical relationships, which were based on the peaks observed from fragmentation about the branch point in the molecule, were amplified by Nelson and Sukkestad (1970) and Nelson et al. (1972) for the interpretation of spectra of mixtures of mono-, di-, and trimethyl-branched alkanes (Fig. 1). Because these empirical relationships were based on the intensity ratio of adjacent peaks of 1 amu difference as a result of a hydrogen loss or transfer to the neutral particle, a degree of ambiguity could have been introduced through instrumental parameters. To prove the structures and to determine whether the observed ratio of peaks was indeed strictly dependent on structural factors under controlled instrumental conditions, we synthesized a series of the postulated hydrocarbons and determined the MS (Fig. 2). The MS of the individual synthetic components and constructured mixtures of these component hydrocarbons are presented in Fig. 2*A–K*. The fragmentation of methyl-substituted alkanes adjacent to the branch point (from carbon position 4 inward) yielded fragments by pathways shown in Scheme 2. For 15-methylpentatriacontane (Fig. 2*A*, compound I), ions *a* and *a* − *1* appeared in the mass spectrum at *m/e* 225 and 224, respectively; the alternative pair of ions *a'* and *a'* − *1* appeared at *m/e* 309 and 308.

In addition to this fragmentation, other characteristically diagnostic

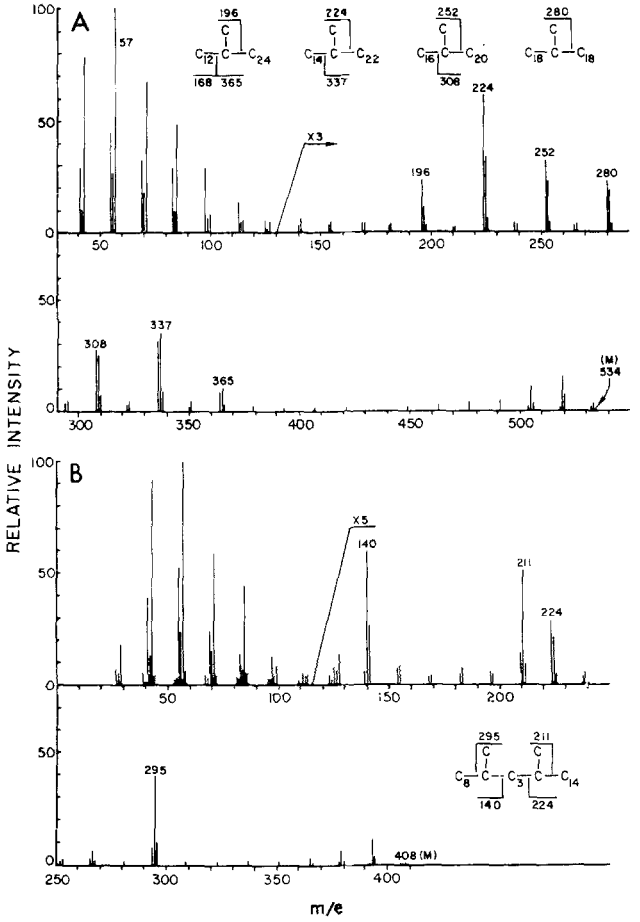
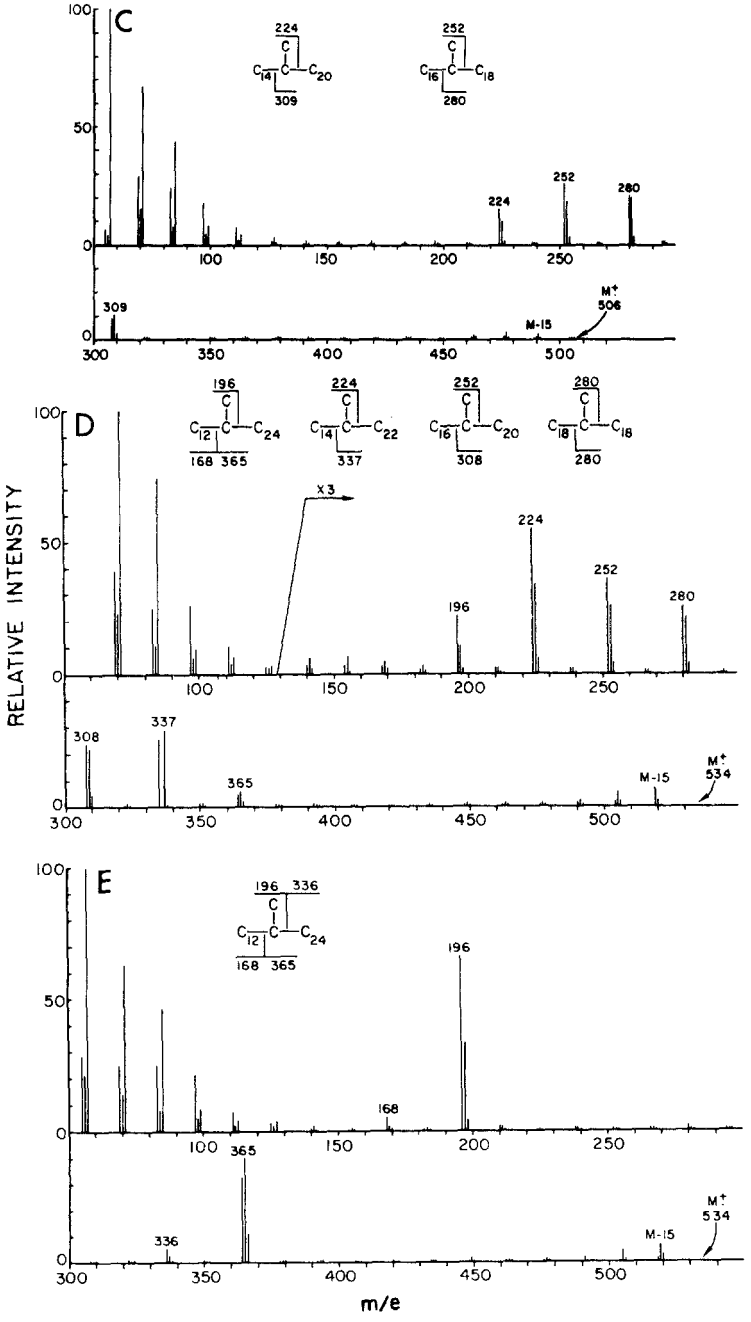


FIG. 1. (A and B, above; C–E, facing page) Mass spectra of naturally occurring hydrocarbons from the tobacco hornworm, *Manduca sexta* L. (Nelson and Sukkestad, 1970; Nelson et al., 1972). (A) Mixture of methylheptatriacontanes; (B) 9,13-dimethylheptacosane; (C) mixture of methylpentatriacontanes; (D) mixture of dimethylpentatriacontanes (E) mixture of trimethylpentatriacontanes.

ions of monomethylalkanes were pairs of peaks appearing at 28 amu less than *a*, $a-1$, and a', $a'-1$, *viz*, ions *c*, $c-1$, and c', $c'-1$ (Scheme 3). Ions *c*, $c-1$ and c', $c'-1$ appeared at *m/e* 197, 196 and at *m/e* 281, 280 for the compound shown in Fig. 2*A*. The intensities of these ions (*c*, $c-1$) were always less than those for *a*, $a-1$, etc., and are probably primary ions; those of the *a*, $a-1$ type are secondary ions. The differences in relative intensities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C16 C C18
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100
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M+
506
300
350
400
450
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D
196
C
C12 C C24
168 365
224
C
C14 C C22
337
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RELATIVE INTENSITY
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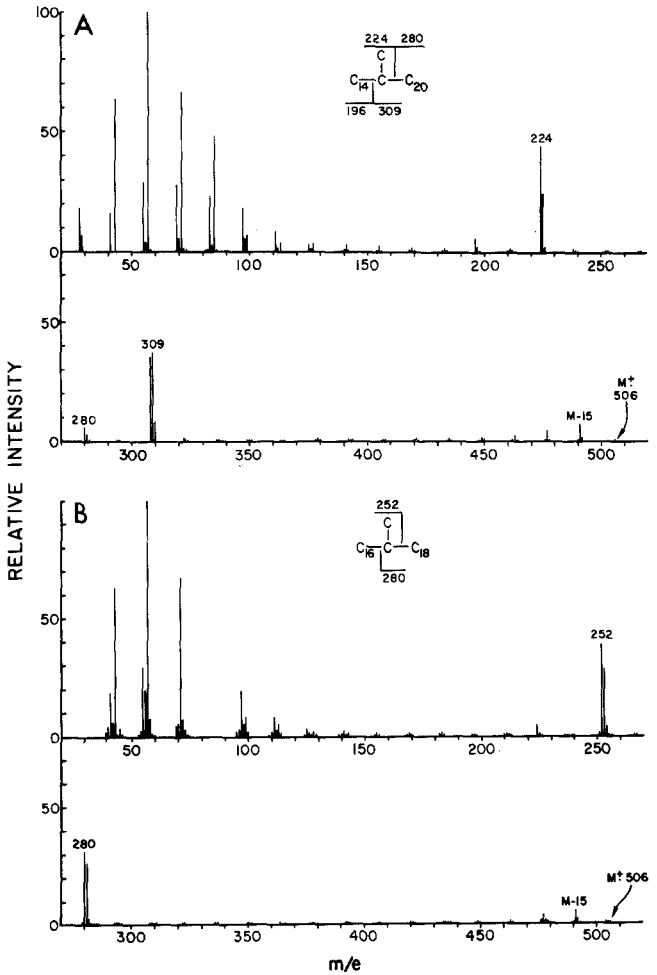
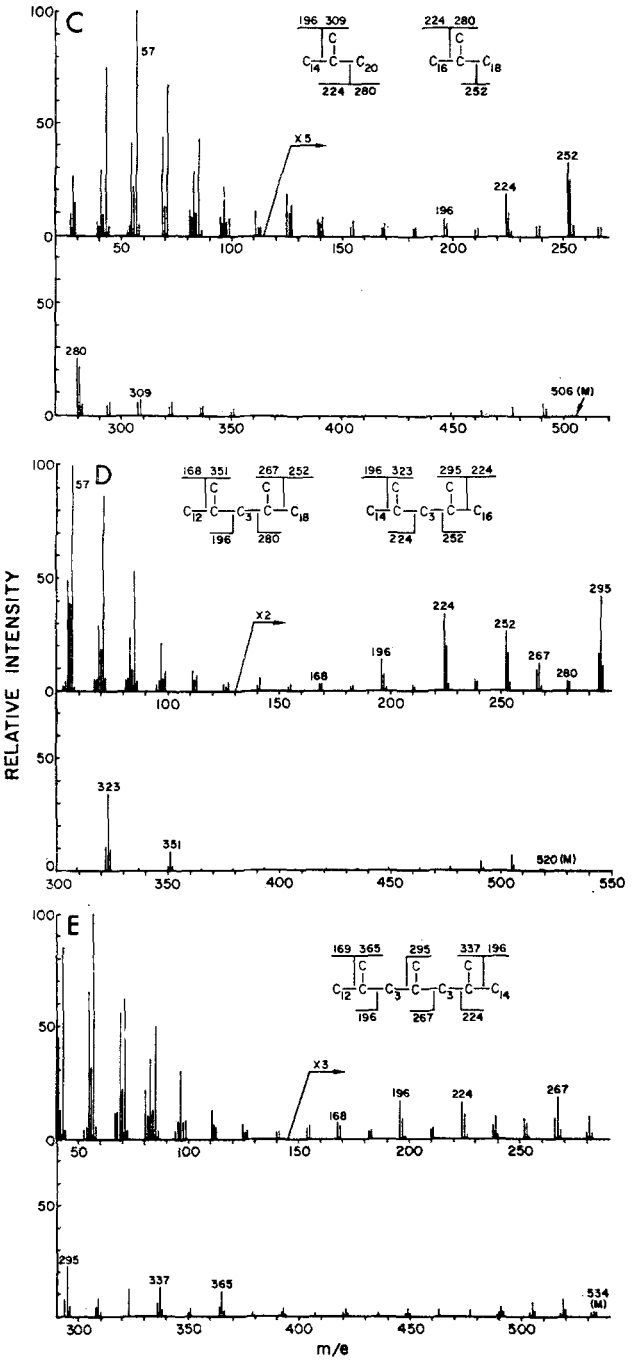


FIG. 2. (A and B, above) Mass spectra of synthetic 15-methyl- and 17-methylpentatriacontanes; (C, facing page) mass spectrum of a mixture of 2A and 2B. (D, facing page) Mass spectrum of a synthetic mixture of 2E through 2H; (E, facing page; F–H, page 34) mass spectra of synthetic 13-methyl-, 15-methyl-, 17-methyl-, and 19-methylheptatriacontanes. (I, page 35) Mass spectrum of 9,13-dimethylheptacosane; (J, page 35) mass spectrum of 15,19-dimethylpentatriacontane; (K), page 35) mass spectrum of 13,17-dimethylheptatriacontane.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RELATIVE INTENSITY
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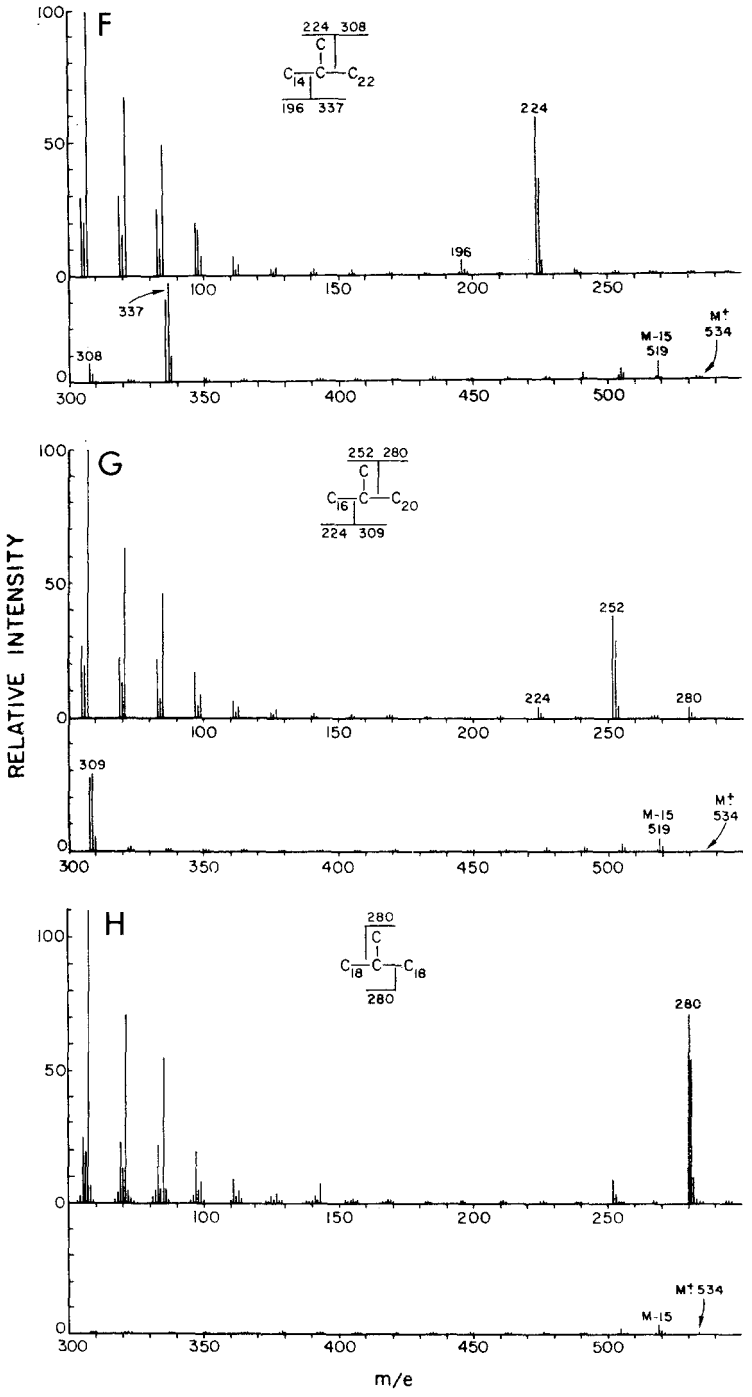
169 365
337 196

C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RELATIVE INTENSITY
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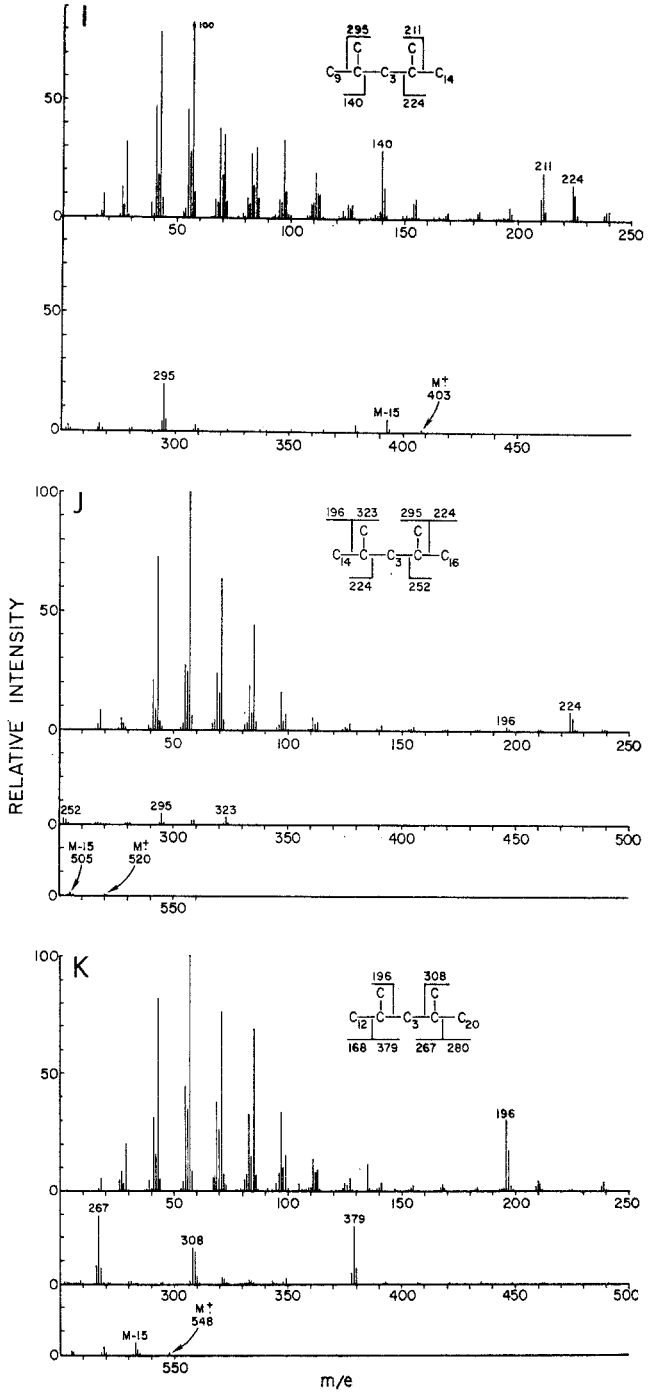
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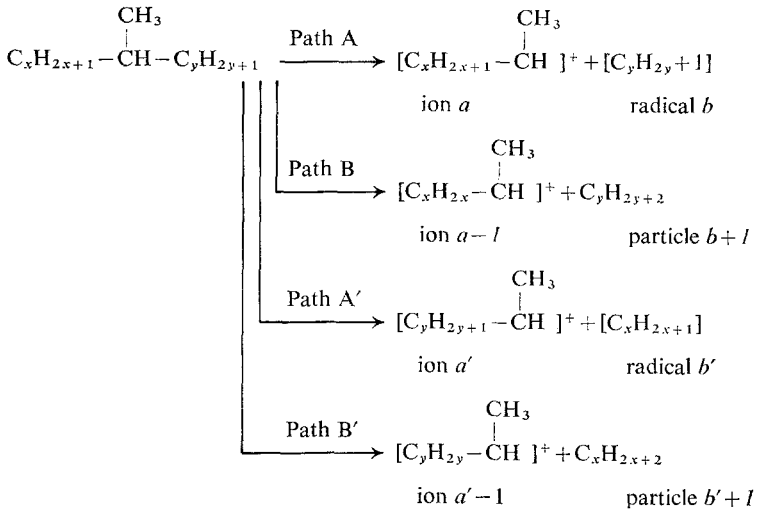
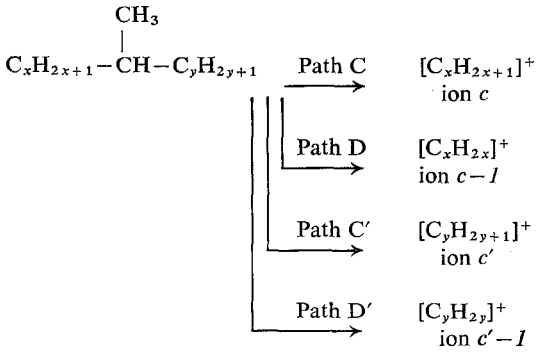


Scheme 2



Scheme 3

between these sets of pairs were probably the result of preferential formation of the more stable secondary ions over the primary ions.

The MS of an isomer of 15-methylpentatriacontane is shown in Fig. 2*B* (17-methylpentatriacontane, II). Peaks for the ions *a*, *a*−*1* and *a′*, *a′*−*1* appeared at *m/e* 253, 252 and 281, 280, respectively, while peaks for ions *c*, *c*−*1* appeared at *m/e* 225, 224, respectively. The peaks for ions *c′*, *c′*−*1* appeared superimposed on peaks *a*, *a*−*1* at *m/e* 253, 252, respectively, which distorted the relative intensities by increasing their value.

When the two isomers were mixed (compounds I plus II), a composite spectrum was obtained, as shown in Fig. 2*C*. This was done for the purpose of comparing the spectrum of the synthetic mixture to that of the natural product (compare Fig. 2*C* with Fig. 1*C*). In preparing all mixtures of the

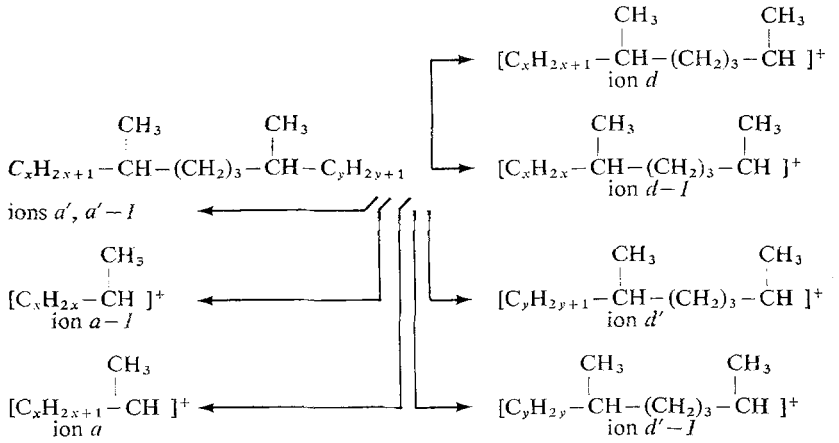
synthetic compounds in this study, the assumption was made that the relative intensity of peaks resulting from two or more compounds in the natural mixture was directly proportional to the concentration of these compounds, so that if two peaks were of the same intensity, the mixture of the two components was of equal proportions. Thus, in Fig. 1*C*, the ratio of *m/e* 252(II):224(I) is 1.8, or compound II is present in almost twice the amount of compound I. In the synthetic mixture, the spectrum (2*C*) is that of an equal amount of compounds I and II.

The significance of the ratio of intensities of adjacent peaks $a-1/a$ or $a'-1/a'$ has been discussed in detail elsewhere (Nelson et al., 1972, and references cited therein). However, an in-depth study using isomeric monomethyl-substituted alkanes in several homologous series has shown that the significance and cause of this effect are much more complex (Pomonis, to be published) than originally explained (McCarthy et al., 1968; Nelson et al., 1972). However, the observations made by Nelson et al. (1972) for the ratios $a-1/a$ etc. hold true for the compounds in this study because they are the synthetic counterparts of the naturally occurring compounds.

A more complex problem presented itself when the spectrum in Fig. 1*A* was originally analyzed (Nelson et al., 1972). It was proposed that the spectrum resulted from the fragmentation of a mixture of four natural isomeric methylheptatriacontanes. Thus, compounds III–VI were synthesized, and the individual spectra were determined as shown in Fig. 2*E–H*, respectively. The analyses of peaks for these compounds were performed by the method discussed above. The ratio of mixing for the synthetic compounds was 2:5:3:2 (III:IV:V:VI), based on the concentration determined by the peak intensities of the natural mixture shown in Fig. 1*A*. The MS for the mixture of synthetic isomers, shown in Fig. 2*D*, compared favorably with the spectrum of the natural mixture.

The spectrum of 9,13-dimethylpentacosane (compound VII and Fig. 2*I*) confirmed the identification of the alkane from the eggs of *M. sexta*, for its spectrum was identical to that of the natural product (Fig. 1*B*).

Other dimethylalkanes were synthesized (compounds VIII, IX) and the MS determined as shown in Fig. 2*J* and *K*, respectively. Although only one compound of each of the two mixtures was synthesized, enough information was obtained to give ample confidence in the original interpretation (Nelson and Sukkestad, 1970) of the natural mixtures (e.g., compare the spectra 1C with 2*J* and 1*D* with 2*K*). It is important to note that the presence of an additional methyl branch in the secondary ion *always* causes the ratio of the intensities of ions such as $d-1/d$ and $d'-1/d'$ to be less than unity (Scheme 4). However, the ratios of intensities of $a-1/a$ (Scheme 4) may be greater than unity, as discussed by McCarthy et al. (1968) and Nelson et al. (1972).



Scheme 4

The synthesis of trimethyl-branched alkanes such as those shown in Fig. 1*E* is currently underway. A more detailed study of the MS relationships of intensities of adjacent peaks to total structure will be reported later.

EXPERIMENTAL[1]

Compounds for MS analyses were trapped as single peaks from the GLPC effluent stream and were >99% pure. The GC was performed by means of a Beckman model GC-4 or a Hewlett-Packard model 5700A, with 365 cm × 0.318-cm stainless-steel columns packed with either 1.88% or 3% OV-101 on Gas Chrom Q, 100–120. The instruments were programmed at 4°/min from either 150–300° or 200–300°, and were equipped with a 10:1 stream splitter for collection and detection by flame ionization.

The MS were obtained with a Varian M-66 or a CH-5 DF mass spectrometer at 70 eV via the solid sample probe. A GLC mass spectrometer (CH-5-DF) interface fitted with a two-stage Watson-Biemann separator was used to obtain the spectra of some hydrocarbons.

Synthesis

Both the mono- (I-VI) and dimethyl-(VIII–IX)substituted alkanes were synthesized from the appropriately substituted trialkylthiophenes. The synthesis of 13,17-dimethylheptatriacontane (IX) will serve as an example.

A well-stirred slurry of 400 mg of 3-methyl-5-(2-methyltetradecyl)-2-nonadecylthiophene (Pomonis et al., 1976a) and 20 of activated Raney

[1] Mention of a proprietary product or company name does not constitute an endorsement of the product or company by the United States Department of Agriculture.

nickel catalyst in 60 ml of thiophene-free benzene was heated with vigorous stirring under reflux for 24 h. The spent catalyst was filtered from the solution, and the solvents were removed in vacuo. The viscous residue was dissolved in 150 ml of EtOH and 50 ml of benzene, then submitted to hydrogenation over 5% Pd/C at 55 psi for 68 h. When hydrogenation was complete, the catalyst was filtered, and the solvent was removed in vacuo. The residual wax was dissolved in hexane, and the resulting solution was percolated through a 1×30 cm column of commercially prepared silica gel impregnated with 25% $AgNO_3$. Evaporation of the solvent from the initial 50 ml of column eluant resulted in 175 mg of a waxy solid. The MS for $C_{39}H_{80}$:M^+ had *m/e* 548 (Fig. 2K). The GLPC of the purified mixture indicated that the product formed more than 90% of the mixture.

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CONTROLLED RELEASE PLASTIC STRIPS CONTAINING (*Z*)-9-DODECEN-1-OL ACETATE FOR ATTRACTING *Spodoptera frugiperda*[1,2]

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Abstract—Laminated plastic strips and polyethylene vials used for dispensing (*Z*)-9-dodecen-1-ol acetate attracted male *Spodoptera frugiperda* (J.E. Smith) in similar numbers for comparable periods. Addition of an antioxidant to the pheromone dispensed from the polyethylene vials did not significantly increase the period of effectiveness. The laminated plastic strips were acceptable dispensers of pheromone bait for attracting *S. frugiperda* males to sticky traps in the field.

Key Words—sex pheromone, *Spodoptera frugiperda*, fall armyworm, (*Z*)-9-dodecen-1-ol acetate, pheromone dispenser, trapping.

INTRODUCTION

Recently, there has been considerable interest in the use of insect pheromones, and now, with the development of synthetic pheromones, there is a need for effective dispensers for these chemicals. Beroza et al. (1974) reported that a three-layer plastic laminate bait dispenser was more effective than other dispensers of the pheromone of the cabbage looper, *Trichoplusia ni* (Hübner), and the gypsy moth, *Lymantria dispar* (L.).

Dispensers such as cotton wicks and polyethylene vials or caps have been used to disperse the pheromone of the fall armyworm, *Spodoptera*

[1] Lepidoptera: Noctuidae.

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

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frugiperda (J.E. Smith), in pheromone communication studies (Mitchell et al., 1974). However, trapping studies similar to those of Snow and Copeland (1969), in which virgin females were used as bait to determine the seasonal distribution of *S. frugiperda*, would be facilitated by a dispenser that would emit the attractant at a constant rate over an extended period. We therefore evaluated laminated plastic strips (Hercon®) containing the synthetic sex pheromone, (*Z*)-9-dodecen-1-ol acetate (*Z*-9-dda), of *S. frugiperda*.

METHODS AND MATERIALS

Laminated plastic strips containing *Z*-9-dda were used as bait in Pherocon® 1C sticky traps (Tingle and Mitchell, 1975) positioned ~30 m apart along the edges of corn or sorghum fields. The capture of male *S. frugiperda* in traps baited with the laminated strips was compared with that of traps baited with 1.25-ml polyethylene vials (Durham Aircraft Corp., Miami, Florida) containing 25 mg of *Z*-9-dda (>95% pure) that released the chemical at the rate of ~0.4 mg/day at 27°C and 0.4 m/s wind velocity. In test 1, rectangular strips with 12.9 cm^2 of surface area (on one side), each containing 24 or 122 mg of *Z*-9-dda/6.5 cm^2, and portions of these strips (6.5, 1.6, or 0.4 cm^2 of surface) were evaluated as trap baits for *S. frugiperda*. At 22°C, the strips containing 24 mg of *Z*-9-dda/6.5 cm^2 released the pheromone at the rate of ~0.2 mg/6.5 cm^2 per day, and the release rate of the strips containing 122 mg of *Z*-9-dda/6.5 cm^2 was ~1 mg/6.5 cm^2 per day.

In test 2, laminated strips containing 25 or 127 mg of *Z*-9-dda/6.5 cm^2 were tested at 0, 4, and 9 wk. Between testing, the vials and strips were aged outside in traps located in direct sunlight, and they were compared with fresh control vials and strips each time. The day and night temperatures averaged 32.1 and 20.4°C, respectively. The control vials were prepared immediately before testing; the control strips were held in a freezer at 0°C until needed.

In test 3, aged baits were tested for effectiveness at 0, 4, and 5 wk; temperatures averaged 32.6°C during the day and 22.2°C at night. In test 4, laminated strips aged at a constant temperature of ~26.8°C were evaluated in the field. In test 5, vials containing 25 or 100 mg of *Z*-9-dda without and with an antioxidant (5% UOP 688® to retard degradation of the pheromone) were used.

All tests except test 1 were conducted at Gainesville, Florida. Test 1 was conducted at Hastings, Florida. Each treatment was replicated 6–9 times. Data were analyzed for significance ($P = .05$) by Duncan's multiple range test.

RESULTS AND DISCUSSION

In test 1, catches of male *S. frugiperda* moths in traps baited with the larger Hercon strips (12.9 or 6.5 cm^2) of either dose or the 1.6-cm^2 strip containing the higher dose (122 mg/6.5 cm^2) did not differ significantly from catches in traps baited with a polyethylene vial containing 25 mg of *Z*-9-dda: Each sticky trap captured 15–20% of the total (1059) catch. The 1.6-cm^2 strip containing the lower dose (24 mg/6.5 cm^2) attracted only 11%. The difference was significant. The fewest moths (6%) were attracted by the 0.4-cm^2 strip (low dose); 10% were captured in the trap baited with the 0.4-cm^2 strip containing the higher dose.

Results of tests 2–4, in which *Z*-9-dda dispensed in Hercon strips (6.5 cm^2) and in vials was evaluated for longevity, are shown in Table 1. Strips containing either 25 or 127 mg/6.5 cm^2 and vials containing 25 mg of *Z*-9-dda remained effective at 4 wk; all were ineffective after 9 wk (test 2). However, in the third test, aged baits were ineffective at 4 wk, probably because the

TABLE 1. MEAN PERCENT CAPTURE OF MALE *Spodoptera frugiperda* ON PHEROCON 1C STICKY TRAPS BAITED WITH 6.5-CM^2 HERCON STRIPS OR POLYETHYLENE VIALS CONTAINING (*Z*)-9-DODECEN-1-OL ACETATE (*Z*-9-DDA), 9 REPLICATES EACH, AT GAINESVILLE, FLORIDA, 1975[a]

	Hercon strips				Vial	
	25 mg/6.5 cm^2		127 mg/6.5 cm^2		25 mg *Z*-9-dda	
Week	Aged	New	Aged	New	Aged	New
Test 2—Strips aged outside ($\bar{X}$ temperature = 32.1°C day, 20.4°C night)						
0		32.2 a		37.1 a		30.7 a
4	15.9 a	15.3 a	17.3 a	19.4 a	15.8 a	16.4 a
9	0.5 b	40.1 a	3.8 b	29.7 a	1.3 b	24.6 a
Test 3—Strips aged outside ($\bar{X}$ temperature = 32.6°C day, 22.2°C night)						
0[b]		42.1 a		32.4 a		24.4 a
4	6.4 d	23.9 a	13.0 c	20.6 ab	15.4 bc	20.7 ab
5	2.0 c	26.4 b	6.4 c	41.9 a	2.1 c	21.1 b
Test 4—Strips aged in laboratory at 26.8°C						
0		22.8 b		59.0 a		18.2 b
6	5.8 b	23.8 a	19.5 a	25.1 a		25.6 a
7			21.1 b	44.1 a		34.8 a

[a] Means in the same row followed by the same letter are homogeneous ($P = .05$, Duncan's multiple range test).
[b] Six replicates.

TABLE 2. MEAN PERCENT CAPTURE OF MALE *Spodoptera frugiperda* ON PHEROCON 1C STICKY TRAPS BAITED WITH POLYETHYLENE VIALS CONTAINING (*Z*)-9-DODECEN-1-OL ACETATE (*Z*-9-DDA) WITH OR WITHOUT ANTIOXIDANT (UOP 688 AT 5%), 9 REPLICATES EACH, GAINESVILLE, FLORIDA, 1975[a]

	25 mg *Z*-9-dda				100 mg *Z*-9-dda			
	Without antioxidant		With antioxidant		Without antioxidant		With antioxidant	
Week	Aged	New	Aged	New	Aged	New	Aged	New
0[b]		21.1 a		17.8 a		37.3 a		23.6 a
2[c]	19.9 a	20.2 a	18.4 a		19.0 a		22.5 a	
4	14.7 b	16.2 b	7.7 b		28.4 a		33.0 a	
5	5.2 c	9.8 bc	2.3 d	12.9 ab	15.1 ab	21.4 a	19.1 ab	14.2 ab
6	6.9 c	12.8 b	3.3 c	14.4 ab	17.8 a	15.7 ab	14.3 ab	14.7 ab
7	6.3 b	17.2 a	5.9 b	12.9 a	14.0 a	15.5 a	14.0 a	14.1 a
8		21.0 a			11.9 b	28.8 a	13.0 b	25.1 a

[a] Means in the same row followed by the same letter are homogeneous ($P = .05$ Duncan's multiple range test).
[b] Eight replicates.
[c] Six replicates.

temperatures were higher during the day and during the night than during the second test. In test 4, strips containing 25 mg/6.5 cm^2 that were aged in the laboratory at ~26.8°C, were not effective at 6 wk; strips containing 127 mg/6.5 cm^2 were effective at 6 wk, but not at 7 wk. Approximately 1800 moths were captured in each of these tests.

In all tests, the Hercon dispensers containing *Z*-9-dda were equal to the polyethylene vials in attraction of *S. frugiperda* males; also, they were effective for comparable periods at comparable conditions. The low dose (~25 mg/6.5 cm^2) of *Z*-9-dda seemed to be less effective than the higher dose (~125 mg/6.5 cm^2) in tests with strips and vials when moth populations were low.

The addition of an antioxidant (UOP 688) to *Z*-9-dda dispensed in polyethylene vials (test 5) did not significantly increase the length of their effectiveness (Table 2). When the amount of *Z*-9-dda/vial was increased fourfold (to 100 mg), the period of effectiveness was increased from 4 to 7 wk. Over 6000 moths were captured in this test.

In the field, the laminated plastic strip (Hercon) was therefore an effective dispenser for the pheromone of *S. frugiperda*. This dispenser lasted as long as the polyethylene vials dispenser and can therefore be used in monitoring *S. frugiperda* males over an extended period.

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CHRYSOMELIDIAL IN THE DEFENSIVE SECRETION OF THE LEAF BEETLE *Gastrophysa cyanea* MELSHEIMER

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Abstract—Larvae of the chrysomelid beetle *Gastrophysa cyanea* produce a defensive secretion in their eversible thoracic and abdominal glands that is an effective repellent for small predators such as fire ants. This secretion is composed primarily of chrysomelidial, 2-(2-formyl-3-methyl-2-cyclopentenyl)propanal, and a compound tentatively identified as its enol lactone. Adaptations that optimize the effectiveness of the larval defensive exudate are discussed.

Key Words—chrysomelidae, chrysomelidial, *Gastrophysa cyanea*, defensive secretion.

INTRODUCTION

The eversible glands of larval leaf beetles (Chrysomelidae) have proven to be a rich source of insect defensive products. A number of diverse compounds, including salicylaldehyde (Wain, 1943, Pavan, 1953; Wallace and Blum, 1969), benzaldehyde (Moore, 1967), and β-phenylethyl esters (Blum et al., 1972), have been identified in the secretions of the relatively few species of chrysomelid larvae that have been so far investigated. We have investigated the defensive secretion of *Gastrophysa cyanea* and have identified as its major constituent chrysomelidial, a novel cyclopentenoid monoterpene aldehyde.

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METHODS AND MATERIALS

The secretion was collected in 0.5-μl microcapillaries from the everted glands of tactually stimulated larvae, and stored in *n*-hexane or methylene chloride at $-10°$. These extracts were used directly for all chemical analyses.

The secretion was analyzed on the following gas-liquid chromatography columns: 10% Carbowax 20 M, 3% SP-1000, and 3% ECNSS-M. Combined gas chromatographic–mass spectrometric (GC–MS) analyses were carried out on an LKB 9000 instrument. Nuclear magnetic spectra were obtained on a Varian XL-100-15 spectrometer equipped with a Digilab Fourier transform system.

The deterrent value of the defensive secretion was examined by placing larvae on the foraging platform of a fire ant (*Solenopsis invicta*) colony and observing the subsequent confrontations. The repellency of chrysomelidial, collected by preparative gas chromatography, was examined by treating *Tenebrio* larvae with 10 μg of this compound and noting the reactions of fire ant workers that encountered these treated larvae placed on their foraging platform.

RESULTS

Identification of Chrysomelidial

Upon GC–MS, the extract showed two peaks of nearly equal height eluting at 10.6 and 15.5. min at 160° on a 2-m 10% Carbowax 20 M column. The first peak gave a molecular ion at *m/e* 164 (65), and important ions at *m/e* 136 (60), 121 (40), 107 (100), 93 (45), 91 (76), and 80 (82), whereas the second peak gave a molecular ion at *m/e* 166 (6) and important ions at *m/e* 151 (4), 148 (21), 138 (15), 137 (4), 133 (6), 123 (10), 109 (53), 108 (26), 81 (100), and 79 (37).

After isobutane chemical ionization, mass measurement of the two protonated molecular ions led to the formulas $C_{10}H_{12}O_2.H^+$ (found 165. 0925) and $C_{10}H_{14}O_2.H^+$ (found 167.1093), respectively. Neither compound reacted with silanizing agents, but the peak from the second compound disappeared after treatment with $NaBH_4$. Furthermore, the second compound reacts with 2,4-dinitrophenylhydrazine and Purpald (Dickinson and Jacobson 1970) (after collection from the gas chromatograph) and forms a dimethoxime exhibiting a molecular ion at *m/e* 224 (4) and other ions at *m/e* 193 (7), 178 (9), 138 (100), 107 (38), 106 (40), 87 (15), and 79 (23). The base peak at *m/e* 138 (M-86) and the rearrangement ion at *m/e* 87 in the mass spectrum of the methoxime, taken with the intense peak at *m/e* 109 (M-57, confirmed by a

metastable ion at 71.7) in the original compound, point to the structural feature $-CHCH_3CHO$ and indicate the presence of a γ-hydrogen atom.

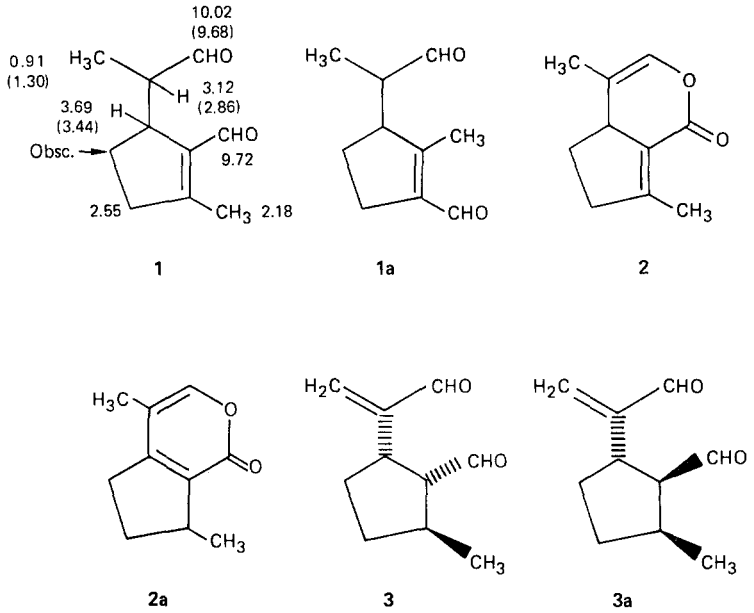
Preparative gas chromatography of this second peak provided enough material (~50 μg) for a Fourier-transform PMR (100 mHz, $CDCl_3$, 35,000 16K free induction decays). Two aldehydic protons at δ9.72 and 10.02, were clearly visible along with an olefin-bound methyl (δ2.18) weakly coupled to allylic protons ($\nu_{1/2}$ 6 Hz). In confirmation of the mass spectral results, a doublet was observed at δ0.91 (J~7 Hz) for the methyl protons of the $-CHCH_3CHO$ feature. The corresponding α proton (δ3.12) was additionally coupled to a downfield, presumably allylic hydrogen at δ3.69 (J~4 Hz). Finally, an allylic methylene triplet was observed at δ2.55, further broadened by long-range coupling. The remaining methylene group probably appeared in the δ1.5–1.7 region, but was obscured by impurities in the solvent. The lack of olefinic protons observed indicates a ring.

The presence of a tetrasubstituted, α,β-unsaturated aldehyde was suggested by the ultraviolet spectrum (λ_{max}^{Etoh} 256 nm). This absorption disappeared on treatment with $NaBH_4$ and shifted to 277 nm (285 nm infl.) on treatment with semicarbazide. There appear to be few models for a 2-substituted 1-cyclopentene carbaldehyde,[4] but the values for 2-methyl-1-cyclohexene carbaldehyde (λ_{max}^{Etoh} 242 nm, ε 11,000) (Braude and Timmons, 1955), 1-cyclohexene carbaldehyde (λ_{max}^{Etoh} 229 nm, ε 12,000) (Heilbron et al., 1949), and 1-cyclopentene carbaldehyde (λ_{max}^{Etoh} 237 nm, ε 12,000, semicarbazone 267 nm, ε 37,500, 277 nm infl., ε 25,500) (Heilbron et al., 1949) suggest that a value near 250 nm is reasonable.

These facts can be accommodated by structures 1 or 1a for chrysomelidial; the existence of the closely related dolichodial (3 and 3a) (Cavill and Hinterberger, 1961; Cavill and Whitfield, 1962; 1964; Cavill, 1969) and anisomorphal (3 or 3a) (Meinwald et al., 1962) leads us to favor the former, and we have assigned the resonances accordingly.[5] The lack of observable coupling in structure 1 between the aliphatic aldehyde and its adjacent H indicates that the methyl and carbonyl are essentially eclipsed, as expected for an α-substituted aldehyde (Karabatsos and Hsi, 1965). The downfield shift of the adjacent allylic hydrogen is unusual, and models suggest that it may be caused by the influence of the nearby unsaturated aldehyde. Its mass

[4] Carbaldehyde replaces carboxaldehyde according to IUPAC, nomenclature rules; c.f. J.H. Fletcher, O.C. Dermer, and R.B. Fox, *Nomenclature of organic compounds*, Advances in Chemistry Series #126, American Chemical Society, Washington, D.C., 1974, p. 161.

[5] After this work was completed, we learned that J. Meinwald had identified chrysomelidial in the secretion of another chrysomelid larva, a *Plagiodera* species. He has kindly compared a sample of chrysomelidial isolated from *G. cyanea* to an unambiguously synthesized sample of this compound and found them to be identical; cf. J. Meinwald, T.H. Jones, T. Eisner, and K. Hicks, *Proc. Natl. Acad. Sci.* 74:2189–2193 (1977).

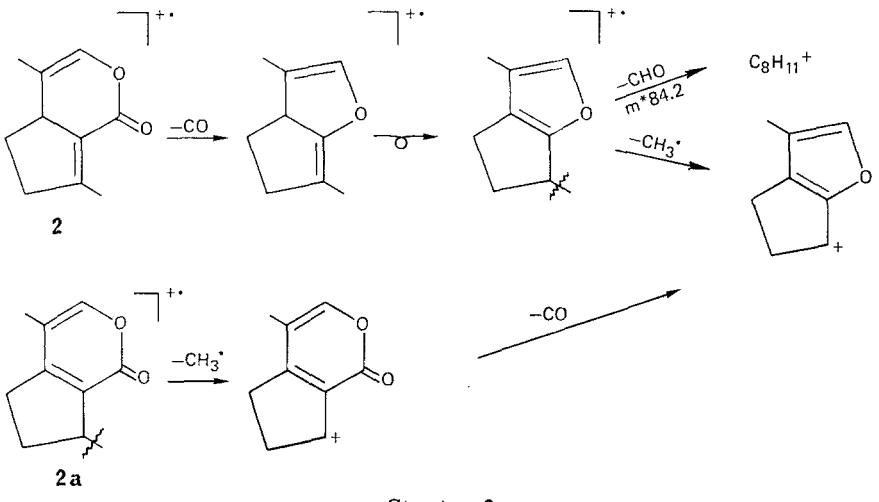


Structure 1

spectrum, involving loss of the sidechain, is, of course, markedly different from that of dolichodial. By analogy with dolichodial (Cavill and Hinterberger, 1961), hydrogenation of this compound (PtO_2–ethanol) might be expected to produce the known dihydro derivative, iridodial, or one of its isomers. Unfortunately, however, extensive hydrogenolysis occurs, giving at least seven compounds, none of which show a mass spectrum similar to that of irododial.

A second sample of chrysomelidial collected from a 3% SP-1000 GC liquid phase showed the presence of a second, very similar compound via a doubling of several major PMR peaks. When the sample was reexamined with a 3% ECNSS-M column, two GC peaks of similar intensity appeared at 14.2 and 15 min, giving nearly identical mass spectra. It is clear that the carbon alpha to the aliphatic aldehyde epimerized during GC in the presence of the somewhat acidic SP-1000 liquid phase. We have assigned to this epimer the NMR peaks shown in parentheses on structure 1.

With the later-eluting peak of the extract established as resulting from structure 1, the first peak, whose mass spectrum lacks two hydrogens compared with 1, may represent the corresponding enol lactone, 2. Its mass spectrum, characterized by loss of CO followed by loss of either methyl or CHO, may be explained as follows:



Structure 2

An old sample of the extract exhibited a trace of an isomer of 2 eluting just before 1 on 3% ECNSS-M and having an intense molecular ion at *m/e* 164 (100) and important ions at *m/e* 149 (64), 135 (45), 121 (53); i.e., loss of the same fragments occurred in the reverse order. We suggest this is the conjugated pyrone 2a, which would not require rearrangement prior to methyl loss as would 2.

Defensive Value of the G. cyanea *Secretion*

Mature larvae of *G. cyanea* that had been placed on the foraging platform of a fire ant colony were generally avoided by these normally aggressive ants. However, the occasional ant workers that attacked the larval beetles were totally disarmed by the chrysomelid secretion. Larvae that were tactually stimulated by their ant antagonists immediately evaginated their eversible glands, smearing their aggressors with the defensive secretion. Typically, a contaminated ant worker immediately withdrew from the scene of the encounter, dragging its head and antennae, and moving in a completely disoriented manner. The secretion-labeled ant was avoided by its sister workers. Furthermore, when a secretion-moistened larva fresh from an encounter moved near a group of ant workers feeding on a cockroach, the ants immediately abandoned their repast and moved rapidly from the area.

Tenebrio larvae treated with chrysomelidial were avoided by ant workers for 10 min or longer. On the other hand, untreated larvae were immediately overrun by ant workers.

DISCUSSION

The identification of chrysomelidial in the glandular exudate of *G. cyanea* further emphasizes the diversity of defensive compounds produced by larval Chrysomelinae. Salicylaldehyde dominates the secretions of species of *Phyllodecta* (Wain, 1943), *Melasoma* (Pavan, 1953), and some species of *Chrysomela* (Wallace and Blum, 1969). On the other hand, the defensive secretion of *Chrysomela interrupta* is comprised primarily of β-phenylethyl isobutyrate and β-phenylethyl 2-methylbutyrate (Blum et al., 1972). It would not prove surprising if the defensive exudates of species in other chrysomelid genera proved to be sources of interesting new insect natural products.

We consider the secretion of *G. cyanea* to be the most potent fire ant deterrent produced by any species of chrysomelid that we have examined. Several behavioral adaptations appear to optimize the effectiveness of the chrysomelidial-rich defensive exudate of *G. cyanea*. Early-instar larvae characteristically aggregate on the undersides of dock (*Rumex* sp.) leaves, where they are very inconspicuous. In a sense, the clumped larvae "pool" their limited defensive secretion. Disturbance of a larval aggregation results in the virtual simultaneous eversion of the glands of many larvae. In young larvae, only the thoracic glands are functional, whereas in older larvae, which are often solitary, the defensive secretion issues from glands through pairs of tubercles on the last two thoracic and first seven abdominal segments as has been reported for other species of Chrysomelinae (Garb, 1915).

Significantly, the pupae of *Gastrophysa*, unlike those of *Chrysomela* species, shed their pupal skin entirely, thus losing their defensive glands. *Chrysomela* pupae, which are exposed on the host plant, retain the larval integument that contains the salicylaldehyde-rich defensive glands. The glands can be discharged when the freehanging pupa is stimulated (Hinton, 1951; Wallace and Blum, 1969). Indeed, the freshly emerged adult, which is especially vulnerable to predators, is bathed in the salicylaldehyde derived from the retained larval defensive glands, and this aromatic bath renders the beetle highly repellent to invertebrate predators at a time when it is very inactive (Wallace and Blum, 1969). *G. cyanea*, on the other hand, discards its chrysomelidial-fortified glands when it pupates, and would seem to be especially susceptible to predation if exposed like the pupa of *Chrysomela*. However, the *Gastrophysa* larvae pupate in the ground litter near the host plant, and are very unobtrusive. These results illustrate the variety of defensive mechanisms evolved in the Chrysomelidae and emphasize the chemical and behavioral diversity manifested by the species in this large family.

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DETERRENCE OF REPEATED OVIPOSITION BY FRUIT-MARKING PHEROMONE IN *Ceratitis capitata* (DIPTERA: TEPHRITIDAE)

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Abstract—During ovipositor dragging on the fruit surface following egg laying in hawthorne fruit, *Ceratitis capitata* (Wiedemann) females deposit an unidentified pheromone that deters repeated oviposition attempts in that fruit. The pheromone proved water soluble and, when collected and sprayed in aqueous solution onto uninfested fruits in laboratory cages, effectively deterred boring attempts by *C. capitata* females of wild origin for at least 6 days (termination of test). A laboratory population of *C. capitata* cultured on artificial media for more than 200 generations deposited pheromone that proved equally as deterrent to wild fly oviposition as pheromone from wild flies. However, lab fly oviposition was not effectively deterred by the presence of pheromone. The ecological significance of the pheromone is discussed.

Key Words—pheromone, *Ceratitis capitata*, oviposition deterrent, laboratory insect quality, Tephritidae.

INTRODUCTION

Chemical mediation of intraspecific competition for oviposition, hence larval development sites, is a fascinating aspect of the trophic relations of certain frugivorous Tephritidae. For example, immediately following oviposition by an olive fruit fly, *Dacus oleae* (Gmelin), olive juice exuding from the oviposition puncture is spread via the female proboscis over the fruit surface. This marking juice then functions as a deterrent to repeated egg-laying attempts in that olive (Cirio, 1971). Analogously, immediately

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following oviposition by an apple maggot fly, *Rhagoletis pomenella* (Walsh), the female circles around the fruit dragging her extended ovipositor on the fruit surface. In so doing, she deposits a marking pheromone that deters repeated egg-laying attempts (Prokopy, 1972). Oviposition-deterring fruit-marking pheromones have since been discovered in eight other *Rhagoletis* species (Cirio, 1972; Katsoyannos, 1975; Prokopy, 1975; Prokopy et al., 1976) as well as in the Caribbean fruit fly, *Anastrepha suspensa* (Loew) (Prokopy et al., 1977). The amount of fruit surface area marked following a single oviposition is thought to be related to the amount of food or space required by one larva to grow to maturity. Small fruits ($\sim$10 mm in diam. or less) can normally support only one larva and normally receive only one egg, whereas larger fruits often can support more larvae and receive proportionately more eggs.

Ceratitis capitata (Wiedemann), the Mediterranean fruit fly, is in the same subfamily as *Rhagoletis* and *Anastrepha* (Trypetinae). It is widely distributed in southern temperate and subtropical regions of the earth, infests more than 200 species of fruits (Christenson and Foote, 1960) varying in size from $\sim$10 to more than 100 mm in diam., and causes more economic injury than perhaps any other tephritid. Like *Rhagoletis* and *Anastrepha* females, *C. capitata* also drag their ovipositors on the fruit surface after egg laying. But unlike most *Rhagoletis*, though like *R. completa* Cresson (whose only known hosts are walnuts and peaches, in which several larvae/fruit can mature), *C. capitata* often lays more than one egg per clutch, the average being 1.7–1.9 (Féron, 1957; Abasa, 1972).

Here, we report a positive relation between oviposition and ovipositor dragging in *C. capitata*, and demonstrate that the females do indeed deposit oviposition-deterring pheromone during dragging. We deal with the collection of this pheromone and its effect when reapplied to new oviposition sites. We also compare wild and lab-cultured *C. capitata* with respect to pheromone production and response.

METHODS AND MATERIALS

All studies were conducted in a laboratory under ambient temperature and humidity conditions of 27 ± 2°C, 50–70% RH. All flies designated as "wild" were either collected directly from host trees in nature or originated from pupae from infested host fruit collected in nature. They were maintained in $30\times30\times30$-cm cages containing water, sugar, and hydrolyzed protein, with about 20 females and 10 males per cage. All flies designated as "lab" had been cultured in laboratory cages at densities of 100,000 flies per 2.2 m^3 of cage volume for more than 200 generations. During this culturing, the

adult diet was the same as that used for both the wild and lab flies in this study, while the oviposition substrate consisted of five humidified 1-liter plastic containers per cage, each perforated with ~300 small holes into which the flies oviposited (Tanaka et al., 1970). The larval diet was artificial (Tanaka et al., 1969). When 5 days old, the lab flies assayed were placed in similar cages at similar densities of each sex as the wild flies. Both wild and lab females were assayed when mature, at 10 days or more of age.

All test fruits were fresh, unsprayed, uninfested fruits of hawthorne (*Crataegus*), ~15 mm in diam. They were readily accepted as oviposition and larval development sites by the wild and lab flies alike. We did not observe the egg-laying behavior of *C. capitata* in nature, but presume it was similar to that of *Rhagoletis* females, which normally leave the fruit after a single oviposition and ovipositor dragging. To obtain *C. capitata* females having what we hypothesized the equivalent of a "natural" threshold for oviposition, we (1) provisioned the cages with continuously available fruit, and (2) ~1 h or less before testing, allowed individual wild and lab females to oviposit in fruits until only one egg clutch per visit was laid, at which time the female was employed in a test.

The experimental procedure was nearly identical to that given by Prokopy et al. (1976). The only differences were (1) each test fruit marked with pheromone directly by flies received a standardized number of 25 ovipositor-dragging circles around the fruit (this number was slightly less than the largest number of dragging circles per fruit made by any individual wild or lab fly observed, and was obtained from the draggings of one to five females); (2) an unmarked control fruit (i.e., without previous exposure to tephritids) was presented only once per replicate; and (3) the same female was assayed on no more than two replicates (usually only one) of each treatment. Within an experiment, a replicate consisted of one fruit of each type of treatment, presented in random sequence.

In the experiment on collection and reapplication of marking pheromone, we exposed 50 coffee berries for 24 h to ~300 mature lab females. Each berry was then swished in 5 ml of distilled water (25°C) for 1 min to remove the pheromone and exuding fruit juices. The 5-ml solution was then sprayed as a mist with an atomizer onto five uninfested hawthorne fruits so as to completely cover each fruit. There was no runoff, but an unknown proportion of the solution did not strike the fruits. The controls were five uninfested hawthorne fruits sprayed in a similar manner with 5 ml of distilled water into which 50 coffee berries pricked with 25 pinholes each (to simulate oviposition holes with exuding fruit juices) had been swished for 1 min each.

Fruits tested six days after being fly-marked or sprayed with pheromone, as well as appropriate control fruits, were kept dry at ~25°C, 100% RH, until testing.

RESULTS

Both wild and lab *C. capitata* females showed a clearly positive relation between egg laying and subsequent dragging of the ovipositor: 26 of 26 wild females and 58 of 58 lab females that were found to have laid at least one egg in a hawthorne fruit subsequently dragged their ovipositors. Females were also offered a transparent plastic vial with small holes through its side for egg laying. Into this artificial egg receptacle, flies often bored without laying an egg. Wild and lab flies dragged only zero and one time, respectively, following 31 and 23 such unsuccessful oviposition attempts, whereas in 30 and 25 observations, respectively, ovipositor dragging always followed the laying of eggs.

Ovipositor dragging appeared random in direction, though in the first seconds it was greatest near the boring hole, later spreading over the entire fruit. It averaged 77 s in duration (range 4–267) and 4.8 circles around the fruit (range 1/4–26) ($n = 77$) for the wild flies, compared with 59 s in duration (range 9–217) and 5.1 circles around the fruit (range 1/2–27) ($n = 75$) for the lab flies. Dragging was not continuous in all cases, but was occasionally interrupted for several seconds for ovipositor cleaning. Wild females laid an average of 1.8 eggs per clutch (range 1–5) ($n = 35$), and lab females 4.2 eggs per clutch (range 1–9) ($n = 60$) in hawthorne fruit.

In our initial experiment (Table 1, Experiment 1), we found that fruits on which, by experimental manipulation (Prokopy, 1972), wild and lab females had not bored but had just dragged their ovipositors were highly deterrent to boring attempts by wild females. On the other hand, wild females bored with high frequency into control fruits and into fruits where other wild females had just bored but were allowed to drag only $\sim$1s before being flicked away, and where, in addition, each fruit was circled 25 times just before testing with fresh hawthorne juice applied with a small brush. Clearly, some substance of female origin, common to both wild and lab flies, is deposited on the fruit surface during ovipositor dragging, and it is this substance that deters repeated boring attempts by wild females.

Next (Table 1, Experiment 2), we found that nearly as great a proportion of lab females attempted boring into fruits where wild and other lab females had just dragged their ovipositors as into control fruits.

Finally (Table 1, Experiment 3), we found that fruits tested 6 days after being sprayed with aqueous marking pheromone–coffee juice solution were just as highly deterrent to boring attempts by wild assay females as fruits marked with pheromone directly by other females 0–6 days earlier. Conversely, fruits tested 6 days after being sprayed with the aqueous control–coffee juice solution were just as acceptable to the females as unsprayed control fruits. Wild females were also highly deterred from boring into fruits

TABLE 1. BORING FREQUENCY OF WILD (WF) AND LAB (LF) *C. capitata* ASSAY FEMALES INTO UNMARKED FRUITS (CONTROLS) AND INTO FRUITS THAT HAD EARLIER RECEIVED BORING ATTEMPTS (= + BORE), OVIPOSITOR DRAGGINGS IN THE AMOUNT OF 25 CIRCLES PER FRUIT (= + DRAG), OR OTHER TREATMENTS AS INDICATED

Experiment	Assay females	Days, treatment to testing	Treatment	No. of female arrivals on fruit	% attempting to bore[a]	% borers dragging ovipositor
1	Wild	0	No bore, +drag by WF	21	24 a	—
	Wild	0	No bore, +drag by LF	21	24 a	—
	Wild	0	+bore by WF, no drag, +juice	21	95 b	—
	Wild	—	Control	23	83 b	—
2	Lab	0	No bore, +drag by WF	36	78 a	—
	Lab	0	No bore, +drag by LF	36	69 a	—
	Lab	—	Control	47	92 a	—
3	Wild	0	No bore, +drag by WF	24	25 a	83 a
	Wild	6	No bore, +drag by WF	24	17 a	100 a
	Wild	6	+bore by WF, no drag, +juice	24	21 a	100 a
	Wild	6	Sprayed with aqueous pheromone solution	24	25 a	100 a
	Wild	6	Sprayed with aqueous control solution	24	88 b	100 a
	Wild	—	Control	43	91 b	100 a

[a] Any two values in each column in each experiment not followed by the same letter are significantly different at the 1% level according to chi-square test criterion.

where other wild females had bored 6 days earlier but had not dragged their ovipositors. Upon dissection of these fruits immediately after testing, all were found to contain first or second instar larvae, suggesting that the presence of growing larvae, or the 6-day-old hawthorne juice on the fruit surface, may also deter boring attempts. All but one female that attempted boring in this experiment subsequently dragged the ovipositor, indicative of egg deposition.

DISCUSSION

The findings presented here are clear evidence that *C. capitata* females deposit oviposition-deterring fruit-marking pheromone during ovipositor dragging after egg laying. *Ceratitis* is now the third genus of Tephritidae and the fifth genus of phytophagous insects (Oshima et al., 1973, Rothschild and Schoonhoven, 1977) in which such pheromones are known. The response of wild *C. capitata* females to the pheromone proved very similar to the way in which various *Rhagoletis* species and *A. suspensa* respond to their respective oviposition-deterring pheromones. The water solubility of *C. capitata* pheromone and its stability under dry conditions also proved very similar to the characteristics of *Rhagoletis* and *A. suspensa* pheromones. Fresh fruit juice, deterrent to *D. oleae*, did not deter wild *C. capitata*.

We hypothesize the ecological significance of this *C. capitata* marking pheromone to be mediation of uniformity in egg dispersion among available fruits. While we have been unable to find any literature on the pattern of *C. capitata* egg distribution in nature, there is good evidence that several other tephritid species, including *D. oleae* and several *Rhagoletis*, show a decided tendency toward uniformity in egg dispersion (Prokopy, 1976). Because there is a maximum number of larvae that can successfully complete development in one fruit, and because larvae are unable to move from one fruit to another, such uniformity would tend to ensure that a female would not overload an already infested fruit with the additional burden of her own progeny. The earliest-developing larva or larvae in a fruit can be expected to have a competitive advantage over subsequent larvae. Hence, it would be a competitive disadvantage for a female to oviposit in a fruit that already had received the number of eggs equivalent to its larval carrying capacity. Similarly, a distinct advantage accrues to a female that marks a fruit: rapid fruit reinfestation is unlikely.

The water solubility of *C. capitata* marking pheromone would seem to argue against its being an effective mediator of uniform egg distribution in areas or seasons with frequent rainfall. This may indeed turn out to be so, although, as suggested by Katsoyannos (1975) for *R. cerasi*, partial pro-

tection of fruit by leaves and rapid rainfall runoff from the waxy fruit surface may be tempering factors. Possibly any disadvantage to the flies stemming from water solubility is outweighed by the pheromone's being readily produced in large amount with little energy expenditure. Also, the pheromone may only need deter oviposition for a few days to give the first batch of eggs a decided competitive advantage over subsequent infestations. In any event, the high stability of the pheromone would strongly argue in favor of its being a residually effective mediator of uniform egg dispersion in dry conditions.

Results of Experiment 3 suggest that not only the presence of marking pheromone but also the presence of first or second instar larvae may deter *C. capitata* oviposition attempts. If this suggestion is confirmed, then this would constitute an additional means by which a female could recognize an already infested fruit. Marking pheromone would thus need be present in biologically active concentration only until early larval development (6 days or fewer after egg deposition) to mediate uniform egg distribution. This could be particularly advantageous under high-rainfall conditions. The presence in the fruit of first or second instar larvae was found to have no influence on the oviposition behavior of *R. fausta* (Osten Sacken) or *A. suspensa*, both of which deposit one egg per clutch (Prokopy, 1975; Prokopy et al., 1977). However, larval presence in walnuts proved deterrent to oviposition in *R. completa*, which, like *C. capitata*, deposits more than one egg per clutch (Cirio, 1972).

The pheromone deposited by lab *C. capitata* females elicited a degree of response from wild females essentially identical to pheromone deposited by wild females, indicating no difference in type or amount of pheromone produced by these two sorts of flies. On the other hand, the lab females were almost completely unresponsive to the same amount of pheromone (25 ovipositor-dragging circles) that effectively deterred boring by wild females. Nor, in an additional test, were the lab females responsive to even 75 ovipositor-dragging circles of pheromone. There are at least three possible explanations for this lack of lab fly responsiveness: (1) Selection has given rise to a population of lab flies whose threshold level of pheromonal response is very high, or perhaps even whose peripheral pheromone receptors or central nervous system are largely insensitive to the pheromone. (2) Selection has given rise to lab flies of high fecundity, whose threshold level for oviposition is very low. (3) A combination of 1 and 2 may occur. Some evidence in favor of the second explanation is the high degree of lab fly oviposition into artificial oviposition sites and onto cage walls lacking several stimuli (Sanders, 1962), which are necessary to elicit maximum oviposition from wild flies. In our studies, we observed that lab flies laid 6–10 times as many eggs on artificial egg receptacles as wild flies during the first 15 days of egg laying.

It should be noted that, at the high densities at which the lab flies are caged, they are often unable to complete more than 1–2 s of ovipositor dragging before they are disturbed by adjacent flies and cease dragging. The comparatively lesser amount of marking pheromone deposition would be conducive to continued oviposition.

The equivalent production of marking pheromone by wild and lab *C. capitata* flies, but lack of pheromonal response by lab flies, represents a very different situation than reported for *R. pomonella.* In that species, an uncrowded lab population cultured on apples for ~15 generations deposited an amount or type of marking pheromone less deterrent to oviposition than wild fly pheromone. However, the lab and wild flies were equally responsive to wild fly pheromone (Prokopy et al., 1976). Conceivably, some suboptimal adult dietary factor could have influenced pheromone production by the lab *R. pomonella* flies. Alternatively, this difference between *C. capitata* and *R. pomonella* may simply represent an example of selection operating in different directions under different circumstances.

Our findings here lead us to speculate that tephritids in genera other than *Rhagoletis, Anastrepha,* and *Ceratitis* might also deposit oviposition-deterring marking pheromone. One candidate might be the melon fly, *D. cucurbitae* Coquillett, which has been observed by the authors to drag its ovipositor.

If the oviposition-deterring pheromone of *C. capitata* could be identified and synthesized, it might provide a noninsecticidal means of managing this highly important pest in commercial plantations. The recent success of Katsoyannos and Boller (1976, and unpublished data) in effectively preventing oviposition by *R. cerasi* flies in nature with an aqueous marking pheromone solution sprayed on cherry trees demonstrates the potential usefulness of this management technique.

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HOST SELECTION BY *Hylemya antiqua* (MEIGEN)[1] Identification of Three New Attractants and Oviposition Stimulants[2]

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Abstract—Volatile components of *Allium cepa* L, trapped in and recovered from Porapak Q, were separated by gas-liquid and thin-layer chromatography. Fractions eliciting oviposition from *Hylemya antiqua* (Meigen) were analyzed by combined gas chromatography–mass spectroscopy (GC–MS) and were found to contain saturated and unsaturated disulfides. Authentic samples of the identified components were obtained by synthesis and tested for oviposition response by *H. antiqua*. Three new attractants and oviposition stimulants for *H. antiqua* are methyl propyl disulfide, *cis*-propenyl propyl disulfide, and *trans*-propenyl propyl disulfide. All were present in greater proportion in the volatiles from fresh onions than from stored onions. Dimethyl disulfide, methyl *cis*-propenyl disulfide, and methyl *trans*-propenyl disulfide did not elicit significant oviposition from *H. antiqua*.

Key Words: *Hylemya antiqua*, attractants and oviposition stimulants, *Allium cepa*, onion volatiles, Porapak Q, host selection, methyl propyl disulfide, *cis*-propenyl propyl disulfide, *trans*-propenyl propyl disulfide.

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INTRODUCTION

We recently described a sensitive bioassay for measurement of oviposition response of *Hylemya antiqua* (Meigen) to host volatiles and elaborated procedures for obtaining the latter by using Porapak Q as a trapping material (Vernon et al., 1977). The oviposition response of *H. antiqua* to Porapak Q–trapped onion volatiles was approximately 50% of the level induced by a onion slice, while propanethiol (*n*-propyl mercaptan) and dipropyl disulfide, two known attractants and oviposition stimulants for onion maggot (Matsumoto, 1970; Matsumoto and Thorsteinson, 1968), elicited low (17% each) oviposition responses. On the basis of these data, we hypothesized that onion volatiles contained additional host attractants and/or oviposition stimulants for *H. antiqua*, and we therefore initiated a search for these compounds. In this report, we describe the identification of three new attractants and oviposition stimulants for *H. antiqua*.

METHODS AND MATERIALS

Instrumentation

Varian 1200 and 2100 gas chromatographs equipped with flame ionization detectors were employed for analytical gas-liquid partition chromatography (GLPC). Columns used with the 1200 instrument were A (3.18 mm OD × 1.83 m) and B (3.18 mm OD × 3.05 m), which were stainless steel and packed with 5% Carbowax 20M on Chromosorb G, 70/80, AW-DMCS. Columns for the 2100 instrument were C (3 mm ID × 1.68 m, glass, 4% Carbowax 20M on Chromosorb G, 70/80, AW-DMCS) and D (3 mm ID × 3.66 m, glass, 25% Carbowax 20M on Chromosorb A, 60/80). Both columns were attached to a splitter and were also used for preparative separation of onion volatiles.

Four preparative separations by GLPC, Varian A700, and 1700 instruments were used. Preparative columns were E (6.35 mm OD × 3.05 m, stainless steel, 25% Carbowax 20M, terephthalic acid terminated, on Chromosorb A, 60/80) and F (6.35 mm OD × 1.52 m, stainless steel, 25% Carbowax 1540 on Chromosorb A 60/80).

Combined GC–MS was performed with a Hitachi-Perkin-Elmer RMU-6E mass spectrometer interfaced with a Varian 1400 gas chromatograph. Columns for analysis were G (2.2 mm ID × 3.66 m, glass, 5% Carbowax 20M, terephthalic acid terminated, on Gas Chrom Q, 100/120) and H (2.2 mm ID × 1.83 m, glass, 3%, SILAR-10C on Gas Chrom Q, 100/120). A temperature program of 6°C/min beginning at 60°C was used. For all gas

chromatographic analyses, helium was the carrier gas and, in general, injector and detector temperature were maintained below 225°C.

Nuclear magnetic resonance (NMR) spectra were recorded on a Varian A56/60A spectrometer. Samples were dissolved in $CDCl_3$ containing 1% tetramethylsilane as internal standard. Infrared IR spectra of neat samples between sodium chloride disks were obtained on a Perkin-Elmer 457 spectrometer.

Porapak Q Trapping and Isolation of Onion Volatiles

Apparatus for the aeration of onions and procedures for Porapak Q trapping and for recovery of trapped volatiles were as previously reported (Vernon et al., 1977).

Medium or large cooking onions obtained locally are referred to as stored onions. Fresh onions were harvested from Cloverdale, British Columbia, in August 1975. Volatiles were captured from 1–8 kg of quartered, stored onions and from 0.46 kg of the whole bulbs of fresh onions as described by Vernon et al. (1977). The volatiles were recovered from the Porapak Q by extraction with pentane in a Soxhlet extractor. The pentane extracts were concentrated to approximately 5 ml by distillation of the pentane through a 30-cm Dufton column. The concentrated extracts were diluted to a known volume and stored under refrigeration until used in bioassay or chemical analysis.

Identification of Onion Volatiles

Components in onion volatiles were identified by comparison of gas chromatographic retention times and mass spectra to those of authentic samples, whose mass spectra were obtained by GC–MS (columns H and G). No attempt was made at this time to identify the numerous (>30) peaks of small intensity in the gas chromatograms of onion volatiles. Several detailed analyses of the headspace and oil of onion have been previously reported (Boelens et al., 1971; Brodnitz et al., 1969).

Preparative Separation of Onion Volatiles by Gas Chromatography

The pentane extract of Porapak Q–trapped onion volatiles was concentrated by slow evaporation under a stream of nitrogen. Aliquots (50–100 μl) of the concentrate, an oil, were separated on columns C or D, and fractions were collected in glass spirals (2 mm ID $\times$ 8 cm), cooled with dry-ice jackets. The collected volatiles were recovered from the spirals by rinsing them with a known volume of pentane. The pentane solution of the separated

volatiles were stored in the freezer until bioassay and analysis by GLPC and GC–MS.

Separation of Onion Volatiles by Preparative Thin-Layer Chromatography

A concentrate of Porapak Q–trapped onion volatiles was applied to a 5×20 cm plate coated with a 1-mm-thick layer of silica gel HF-254+366. Development of the plate with benzene gave three bands of R_f 0.15, 0.4, and 0.67. The bands were removed from the plate and extracted with ether. After evaporation of solvent under a stream of nitrogen, small portions of the remaining oils were analyzed by GC, GC–MS, and IR. For bioassay the bands were redissolved in pentane to facilitate transfer and handling.

Bioassay

The standard bioassay procedure and apparatus employed for measurement of oviposition response of *H. antiqua* to fractionated onion volatiles and to compounds listed in Table 2 have been described in detail (Vernon et al., 1977). In this bioassay, gravid 12–45-day-old female *H. antiqua* are attracted to and induced to oviposit through small apertures at the base of an oviposition chamber through which the odor of onion or test compound(s) emanates. The oviposition chamber, an inverted 100-ml Nalgene plastic beaker with 10–15 small, regularly spaced nicks around the lip, was positioned on five 12.5 cm Whatman No. 1 circular filter papers stapled together and moistened. Individual compounds were tested as 1-μl aliquots applied in 100 μl of pentane to waxed dental cotton wicks suspended from the top of the oviposition chambers. Mixtures of stimulants were composed of 1 μl of each compound. All chemical stimuli were tested against a pentane control in the same cage of 15 gravid females in an environment chamber. In another environment chamber, flies from the same culture were offered a choice between onion slice–baited and blank control oviposition chambers. Five replicates were tested simultaneously in each environment chamber. Bioassays were conducted with one group of flies until the reserve supply was insufficient to maintain the required number of female flies per cage, or the rate of oviposition induced by onion stimulus was too low. Before each bioassay, the 10 cages were assigned new positions chosen at random in the environment chambers.

Materials

Dipropyl disulfide and dimethyl disulfide were synthesized by modification of the procedure for the synthesis of dibutyl disulfide (Stutz and Shriner,

1933). Methyl propyl disulfide was obtained by this same method by use of an equal molar mixture of methyl iodide and propyl bromide as alkylating agents. The product of reaction was a mixture of approximately 3% dimethyl disulfide, 30% methyl propyl disulfide, and 67% dipropyl disulfide. Pure methyl propyl disulfide was isolated from the mixture by preparative GLPC (column F, 110°C). Methyl propenyl disulfides and propenyl propyl disulfides were prepared by the methods of Wijers and co-workers (Wijers et al., 1969). The cis and trans isomers of the two compounds were separated by preparative GLPC (column E, 110°C, and column F, 110°C, respectively). All the above compounds were characterized by MS, NMR, and IR spectroscopy and the spectroscopic data were consistent with the proposed structures and literature data.

Propanethiol was obtained from the Eastman Kodak Company (Rochester, New York). Porapak Q (50/80 mesh), column packings, and liquid phases were purchased from Applied Science Laboratories, Inc. (State College, Pennsylvania). Silica gel HF 254+366 was a product of E. Merck (Darmstadt, West Germany).

RESULTS AND DISCUSSION

The concentrated pentane solution of onion volatiles was separated into six fractions by GLPC. Fractions 1 and 2 exhibited biological activity in the bioassay. A major component of fraction 1 was methyl propyl disulfide, and also detected in this fraction were trace amounts of methyl

TABLE 1. DISTRIBUTION OF SELECTED DISULFIDE CONSTITUENTS FROM FRESH AND STORED ONIONS IN GLPC FRACTIONS 1 AND 2

	Percent[a]	
	Fresh	Stored
Methyl propyl disulfide	38	5
Dipropyl disulfide	32	81
cis-Propenyl propyl disulfide	6	3
trans-Propenyl propyl disulfide	24	15

[a] Relative percentage distribution determined by GLPC analysis on column B for stored onions and on column D for fresh onions. The formula employed for computation of peak areas was: Area = height × width at half-height. The data are uncorrected for individual detector responses.

TABLE 2. OVIPOSITION RESPONSE OF *H. antiqua* TO SYNTHETIC COMPONENTS OF ONION VOLATILES OFFERED AS 1 μL IN 100 μL OF PENTANE ON WAXED DENTAL COTTON WICK

Test compound	No. of replicates	Number of eggs laid				% Activity relative to onion slice	
		Test compound(s)[b]	Pentane control	Onion slice	Blank control	Test compound	Pentane control
1. Propanethiol	10	832***	27	5677	0	14.6	0.6
2. Dimethyl disulfide	10	85 NS	91	5893	5	0.7	0.7
3. Methyl propyl disulfide	10	1639***	10	3205	12	50.9	0.3
4. Methyl *cis*-propenyl disulfide	5	156 NS	89	2124	2	7.3	4.2
5. Methyl *trans*-propenyl disulfide	5	38 NS	99	2714	0	1.4	3.6
6. Dipropyl disulfide	10	453***	24	2011[c]	2	12.1	0.6
7. *cis*-Propenyl propyl disulfide	5	512**	0	1675	2	30.6	0.0
8. *trans*-Propenyl propyl disulfide	5	242*	10	936[d]	0	15.5	0.6
9. Mixture of 1 μl each of 1, 3, 6–8	5	742***	32	2013	11	36.8	1.5

[b] Analysis of variance significance levels between mean numbers of eggs laid in response to test compound(s) and pentane controls as follows: ***) $P<.001$; **) $P<.01$; *) $P<.05$; NS, not significant.
[c] For onion slice, eight replicates.
[d] For onion slice, three replicates.

cis- and *trans*-propenyl disulfides. Fraction 2 contained mostly dipropyl disulfide and small amounts of *cis*- and *trans*-propenyl propyl disulfides. The later fractions 3–6 were biologically inactive. Separation of the onion volatiles by preparative TLC gave three major bands, 1–3. Only the least polar band, 3, exhibited biological activity in the bioassay. GLPC and GC–MS revealed dipropyl disulfide to be the major component in band 3.

The Porapak Q–trapped volatiles from fresh onions consisted essentially of four major disulfides, the composition differing markedly from that of the cut bulbs of stored onions. In Table 1, the relative distributions of the four components eluting in GLPC functions 1 and 2 are given for both types of onions. In particular, methyl propyl disulfide was present in the volatiles evolving from the surfaces of fresh onions to a relatively greater extent than from those of stored onions. The cis and trans isomers of propenyl propyl disulfide exhibited the same trend, but to a lesser extent. None of these compounds had previously been tested as ovipositional stimulants for *H. antiqua* (Matsumoto and Thorsteinson, 1968), probably because these minor constituents of stored onions (Wahlroos and Virtanen, 1965; Boelens et al., 1971) are not readily available. The volatiles of fresh uncut onions contained little of the oxygenated compounds (long-chain aliphatic ketones and 2,5-dialkyl-2,3-dihydrofuran-3-ones), which predominated in the volatiles from cut, stored onion (H.D. Pierce, Jr., unpublished data). Although fresh and stored onions contain substantial amounts of propanethiol (Freeman and Mossadeghi, 1970; Niegisch and Stahl, 1956), its presence in Porapak Q–trapped volatiles could not be determined directly by analysis by GLPC because a massive solvent peak and several solvent impurity peaks obscured the early portion of the chromatograms.

The biologically active fractions contained mostly aliphatic and olefinic disulfides. Accordingly, two previously tested compounds and five new candidate ovipositional stimulants were obtained by synthesis and tested for ovipositional response in the bioassay.

Three new attractants and oviposition stimulants for *H. antiqua* are indicated from the data in Table 2. Possessing half the activity of an onion slice, methyl propyl disulfide was the most biologically active of the synthetic onion volatiles tested. It was superior at this dosage to both propanethiol and dipropyl disulfide, already known to be active (Matsumoto and Thorsteinson, 1968; Vernon et al., 1977). In addition, both the cis and trans isomers of propenyl propyl disulfide were attractants and oviposition stimulants of moderate activity. All three of the new active compounds were more prevalent in the volatiles of fresh than of stored onions (Table 1). This observation is consistent with the pronounced response of *H. antiqua* to developing onions in the field.

Dimethyl disulfide and methyl *cis*- and *trans*-propenyl disulfides did not

elicit significantly more oviposition from *H. antiqua* than pentane controls, indicating that they are not ovipositional stimulants. Matsumoto and Thorsteinson (1968) concluded that dimethyl disulfide was "not favorable for oviposition," and, in field trapping tests, Matsumoto (1970) found it was "not attractive."

No single compound induced an oviposition response equivalent to that of an onion slice, but only one concentration level for each compound has been tested so far. Extensive testing would be required to determine the optimal concentration of each stimulant for a maximum response. A mixture of 1-μl aliquots of the five active compounds produced only 36.8% of the response to onion slices (Table 2), far below the expected value of 124%. This result demonstrates that the individual responses are not directly additive. Presumably, a mixture of the more active compounds, in the ratio and at a concentration emitted by a growing onion, would elicit an optimum level of response.

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MATING STIMULANT PHEROMONE AND CUTICULAR LIPID CONSTITUENTS OF *Fannia pusio* (WIEDEMANN) (DIPTERA: MUSCIDAE)[1,2]

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Abstract—Chromatograms of the cuticular lipids washed from newly emerged male and female *Fannia pusio* were nearly identical. By the time the flies were 1 day old, the chromatographic profiles for the sexes were different. Mature females contained more C_{31}- and C_{33}-hydrocarbons than the males. The double bonds of the female monoolefins were mostly at the eleventh and thirteenth carbons, but those of the males were predominantly at the ninth carbon. Most active in stimulating copulation by males were the unbranched monoolefins with 31 and 33 carbons from the females. When they were synthesized and tested, the most active compound was (*Z*)-11-hentriacontene.

Key Words: *Fannia pusio* (Wiedemann), pheromone, mating stimulant pheromone, (*Z*)-11-hentriacontene, cuticular lipid, fly.

INTRODUCTION

Fannia pusio (Wiedemann) is a species of fly generally associated with poultry because chicken manure serves as a breeding medium for the larvae. The

[1] 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.

[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 or the University of Maryland.

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recorded geographical distribution of this species is the eastern and southeastern United States (Chillcot, 1960), Guam (Bohart and Gressitt, 1951), and Hawaii (Sherman and Herrick, 1973).

The presence of mating stimulants in the cuticular lipids of several species of Muscidae is now well documented (Carlson et al., 1971; Uebel et al., 1975a, b). More recently, we (Uebel et al., 1977) have investigated the pheromone of the female little house fly, *Fannia canicularis* (L.), that stimulates the mating response and identified it as (*Z*)-9-pentacosene. We report here the isolation and identification of the mating stimulant of *F. pusio* and the results of analysis of the hydrocarbons occurring in both the males and the females of this species. The components of the mating stimulant and cuticular lipids of a closely related fly, *F. femoralis* (Stein), are reported in an accompanying paper (Uebel et al., 1978).

METHODS AND MATERIALS

The starter colony of *F. pusio* was obtained from a colony maintained by the Department of Entomology at the University of Hawaii. The rearing procedure was that described previously for *F. canicularis* (Uebel et al., 1977). Also, the procedures used in collecting the cuticular lipids, identifying the components, and isolating active materials were the same as those reported previously by Uebel et al. (1977). Briefly, cuticular materials were washed from the flies with petroleum ether (b.p. 30–60° C), and the hydrocarbons were separated from the nonhydrocarbons by open-column chromatography on Florisil® (60–100 mesh) with hexane, 50% benzene in hexane, and 2% acetic acid in benzene. Initially, the hydrocarbon fraction was separated into its saturated and unsaturated components by chromatography on 20% silver nitrate–impregnated Florisil (Uebel et al., 1977). Later, this fractionation scheme was modified for separation of alkanes, monoolefins, and polyolefins by elutions with hexane, 15% benzene in hexane, and benzene, respectively.

The presence of monoolefins and dienes and the geometry of the double bond of the monoolefins were determined by comparing the R_f values of these materials with those of standards on 20% silver nitrate–impregnated silica gel G thin-layer plates (Ag TLC). Cuticular lipids and hydrocarbon fractions were examined by gas-liquid chromatography (GLC) on a column of 5% SE-30 on Gas Chrom Q (temperature programmed from 180 to 280°C at a rate of 1°/min). Straight-chain monoolefins and *n*-alkanes were identified by GLC by coinjections with standards.

Monoolefins of the same chain length were separated from those of other chain lengths by trapping from the gas-liquid chromatograph. The

positions of the double bond in the monoolefins were located by ozonolysis of the trapped monoolefins (Beroza and Bierl, 1967), and the resulting aldehydes were identified by GLC through coinjections with aldehyde standards,

The synthetic *cis*-monoolefins were prepared according to the procedure of Schwarz and Waters (1972); (*Z*)-11-hentriacontene and (*Z*)-11-tritriacontene were also prepared as described by Sonnet et al. (1975). The olefins had a minimum cis content of 95%.

Materials were tested for their ability to elicit copulatory responses from male flies by the pseudofly bioassay (Uebel et al., 1976, 1977). Knots of black yarn were impaled on pins and treated with 100 or 200 μg of the test material; usually, four or five materials were tested concurrently. The knots were inserted into quart jars (1 knot per jar) in which 10 male flies were confined. The copulatory attempts with the treated knots made by the males for a 5-min period were counted. Each knot was tested for 5 min in five separate jars.

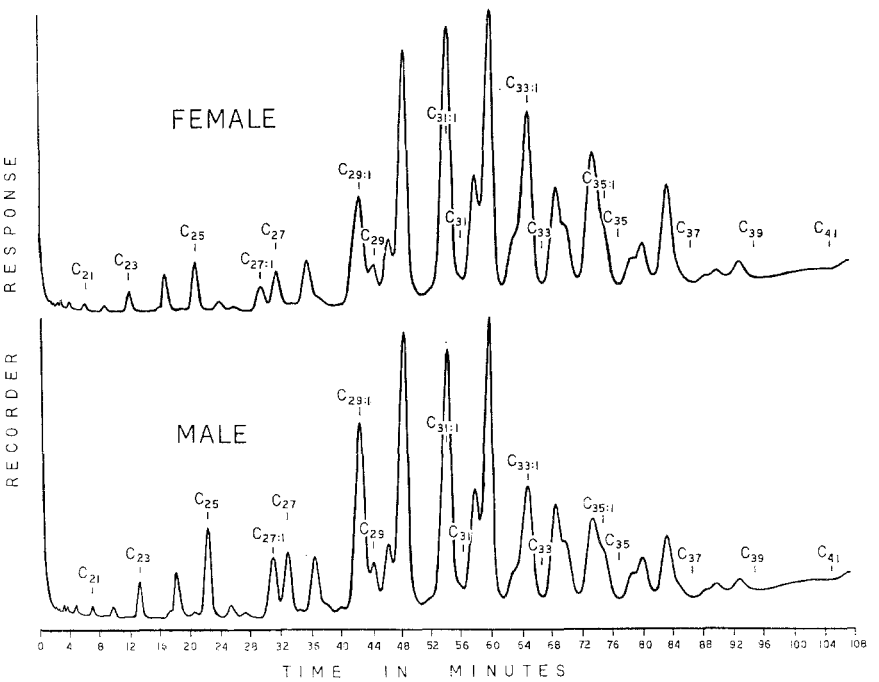

FIG. 1. Gas-liquid chromatograms of the cuticular lipids from newly emerged female and male *F. pusio*. The letter C with a subscript indicates the retention time of straight-chain saturated hydrocarbon and straight-chain monoolefin standards.

RESULTS

Cuticular Lipids from Female and Male F. pusio

The amounts of cuticular lipids obtained from newly emerged flies (< 24-h-old) were ~4.9 μg/female and 3.7 μg/male, and the chromatograms were nearly identical. Peaks that did not match the *n*-alkane or monoolefin standards constituted the majority of the materials present on these young flies (Fig. 1). However, the percentages of major (>5%) alkanes and monoolefins had changed considerably by the time the flies were 1 day old and showed distinctive female and male patterns that were relatively unchanged throughout the next 15 days (Table 1).

Approximately 6.5 μg of total lipid was obtained from each 5-day-old female and 7.1 μg from each 5-day-old male. All major peaks in gas chromatograms of the cuticular lipids (Fig. 2) from 5-day-old females and males represented hydorcarbons, and most of the saturated hydrocarbons from both sexes consisted of odd-numbered, straight-chain alkanes. The unsaturated hydrocarbons from both sexes consisted mainly of straight-chain, odd-numbered monoolefins, but Ag TLC revealed that the female unsaturated hydrocarbons also contained some C_{31}, C_{33}, and C_{35} diolefins.

TABLE 1. PERCENTAGES OF MAJOR ALKANES AND MONOOLEFINS IN THE CUTICULAR LIPID FROM *F. pusio* FEMALES AND MALES OF DIFFERENT AGES

Materials	% in lipid from flies of indicated ages				
	0 days	1 day	3 days	5 days	15 days
♀ C_{23}	1.0	1.7	3.1	0.1	0.8
♂ C_{23}	1.4	6.8	11.6	12.4	11.4
♀ $C_{25:1}$	0.2	0.6	1.7	0.7	0.9
♂ $C_{25:1}$	0.2	4.3	10.4	11.3	8.8
♀ C_{25}	2.4	8.1	9.9	12.6	9.4
♂ C_{25}	3.8	11.9	11.4	12.6	15.3
♀ $C_{27:1}$	1.3	2.8	4.7	2.3	1.8
♂ $C_{27:1}$	2.7	33.7	38.1	34.9	32.3
♀ C_{27}	1.9	3.6	3.1	5.9	5.1
♂ C_{27}	2.8	3.2	2.5	2.6	4.9
♀ $C_{29:1}$	5.0	11.9	17.8	13.5	11.6
♂ $C_{29:1}$	8.4	19.3	15.0	16.4	16.1
♀ $C_{31:1}$	12.0	23.5	27.0	25.8	25.7
♂ $C_{31:1}$	11.2	3.2	1.4	1.6	2.1
♀ $C_{33:1}$	7.9	15.1	15.4	19.4	22.2
♂ $C_{33:1}$	5.1	1.1	0.5	0.5	0.5

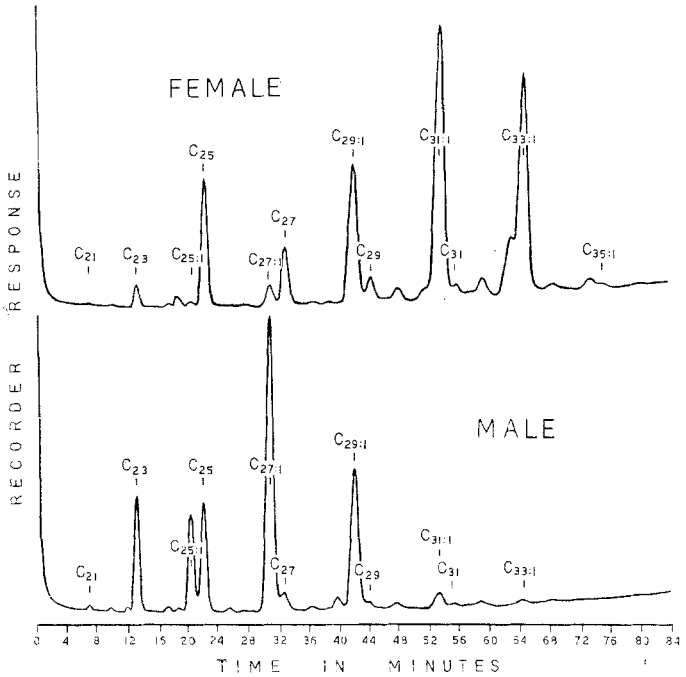

FIG. 2. Gas-liquid chromatograms of the cuticular lipids from 5-day-old female and male *F. pusio*. The letter C with a subscript identifies the straight-chain saturated hydrocarbons and straight-chain monoolefins.

These appear as shoulders on the front on the monoolefin peaks in Fig. 2. No diolefins were found in the male unsaturated hydrocarbons.

Ag TLC demonstrated that the double bonds in the female and male monoolefins were only of the cis configuration. Ozonolysis of individually trapped monoolefins revealed that most of the double bonds of the monoolefins from males were located at the ninth carbon; the majority of the double bonds of the longer-chain monoolefins from the female were at the eleventh and thirteenth carbons (Table 2).

Calculations from area of peak measurements showed that the female cuticular lipid contained 30% saturated hydrocarbons, 63% monoolefins, and 7% dienes. The male cuticular lipid contained 33% saturated hydrocarbons and 67% monoolefins.

Identification of the Mating Stimulant Pheromone of F. pusio

Male *F. pusio* responded readily to the pseudoflies treated with female cuticular lipid and hydrocarbon but did not respond to pseudoflies treated

TABLE 2. PERCENTAGES OF ISOMERS IN THE MONOOLEFINS OF THE CUTICULAR LIPIDS OF 5-DAY-OLD FEMALE AND MALE *F. Pusio*

	Percentages of monoolefin with a double bond at the indicated carbon positions												
Monoalkenes	3	5	7	8	9	10	11	12	13	14	15	16	17
♂ $C_{23:1}$	—	—	6	11	83	—	—	—					
♀ $C_{25:1}$	7	6	12	—	20	18	37	—					
♂ $C_{25:1}$	—	—	—	—	100	—	—	—					
♀ $C_{27:1}$	8	2	6	—	52	5	20	—	7				
♂ $C_{27:1}$	—	—	—	—	100	—	—	—	—				
♀ $C_{29:1}$	—	—	—	—	—	—	53	2	36	9			
♂ $C_{29:1}$	—	—	—	—	94	—	—	6	—	—			
♀ $C_{31:1}$	—	—	3	1	4	3	58	—	21	4	6		
♂ $C_{31:1}$	—	—	—	—	84	—	8	—	4	2	2		
♀ $C_{33:1}$	—	—	—	—	—	—	66	—	34	—	—	—	
♂ $C_{33:1}$	—	—	—	—	44	—	32	—	8	6	6	4	
♀ $C_{35:1}$	—	—	—	—	—	—	51	—	30	—	15	—	4

TABLE 3. NUMBER OF ATTEMPTS BY *F. Pusio* MALES TO COPULATE WITH PSEUDOFLIES TREATED WITH CUTICULAR LIPID OR WITH HYDROCARBON FRACTIONS FROM 5-DAY-OLD FEMALE AND MALE FLIES

	Total number of copulatory attempts[a,b]	
Test materials	100 μg/pseudofly	200 μg/pseudofly
♀ Cuticular lipid	39 b	50 ab
♂ Cuticular lipid	0 c	0 c
♀ Hydrocarbon	68 ab	74 a
♂ Hydrocarbon	0 c	0 c
♀ Saturated hydrocarbon	1 c	1 c
♂ Saturated hydrocarbon	0 c	0 c
♀ Monoolefins	74 a	52 ab
♂ Monoolefins	0 c	0 c
♀ Diolefins	12 c	12 c
♀ First nonhydrocarbon fraction[c]	10 c	6 c
♂ First nonhydrocarbon fraction	7 c	6 c

[a] Totals are for 20 replicates each of 5-min duration.
[b] Values followed by the same letter are not significantly different at the 5% level by the square root of $\chi+0.5$ transformation, the one-way analysis of variance, and the Student–Newman–Keul's test.
[c] 50% benzene–hexane fraction of the Florisil column.

with the cuticular lipid or with hydrocarbon fractions from the males (Table 3). Once the female hydrocarbons were separated into fractions according to their degree of unsaturation, the monoolefins elicited the greatest activity; only a few attempts to mate were produced by the diolefins and the saturated hydrocarbons. Materials present in the first nonhydrocarbon fractions from the Florisil fractionation of the female and male cuticular lipids stimulated the males to make a few copulatory attempts; no responses were made to the materials in the second nonhydrocarbon fractions from either sex.

The monoolefins of different chain lengths were separated by trapping from the gas-liquid chromatograph. The amounts of trapped materials were determined by reinjection and comparison with standards.

When the individual monoolefin fractions from the female cuticular lipid were tested, the C_{31} and C_{33} monoolefins elicited the most response from the *F. pusio* males; the C_{25}, C_{27}, and C_{29} olefins were essentially inactive.

The three most abundant isomers of the female C_{31} monoolefins and the two C_{33} monoolefins (Table 2) were synthesized and tested for biological activity. The results (Table 4) show that the (*Z*)-11-hentriacontene elicited the greatest response; (*Z*)-13-hentriacontene and (*Z*)-13-tritriacontene were less active.

DISCUSSION

To obtain an adequate response from the males, we found it necessary to use a relatively high concentration of material per pseudofly. Thus, the

TABLE 4. NUMBER OF ATTEMPTS BY *F. Pusio* MALES TO COPULATE WITH PSEUDOFLIES TREATED WITH SYNTHETIC *Cis*-MONOOLEFINS

	Total number of copulatory attempts[a,b] at pseudoflies treated with indicated amounts	
Test materials	100 μg	200 μg
(*Z*)-11-Hentriacontene	83 ab	109 a
(*Z*)-13-Hentriacontene	29 d	43 cd
(*Z*)-15-Hentriacontene	1 e	0 e
(*Z*)-11-Tritriacontene	6 de	8 de
(*Z*)-13-Tritriacontene	18 de	62 bc

[a] Totals are for 20 replicates each of 5-min duration.

[b] Values followed by the same letter are not significantly different at the 5% level by the square root of $\chi+0.5$ transformation, the one-way analysis of variance, and the Student–Newman–Keul's test.

100-μg amount represents $\sim$15 female equivalents of total cuticular lipid and $\sim$100 female equivalents of (*Z*)-11-hentriacontene. The need for such large amounts of material is consistent with the data obtained for other species of flies that we have investigated. We assume that it indicates that these hydrocarbons are short-range sex stimulants rather than sex attractants.

The differences in the components of the cuticular lipid of *F. pusio* and *F. canicularis*, which we previously studied, are marked. (*Z*)-9-Pentacosene, the compound that stimulates male *F. canicularis* to initiate sexual activity, made up 66% of the cuticular lipid of the female; the only other monoolefin present in a significant amount was (*Z*)-9-heptacosene. These same compounds were found in the cuticular lipid of female *F. pusio*, but the amounts were relatively small, and the position of the double bond was not exclusively at the ninth carbon. Moreover, longer-chain monoolefins were more abundant in the female *F. pusio*, and the compounds that caused the most mating strikes had a double bond at the eleventh or thirteenth carbon.

Inspection of Fig. 2 shows that there are minor differences in the saturated hydrocarbons in lipid from female and male *F. pusio;* for instance, tricosane is more abundant on the males; heptacosane occurs on the females in larger quantities. However, the differences in the unsaturated hydrocarbon in lipid from females and males are much greater: the males have more pentacosene and heptacosene than the females, and the females have much more hentriacontene and tritriacontene than the males. Both contain nearly equal amounts of nonacosene, but the position of the double bond is entirely different: 94% of the male C_{29} olefin has a double bond at the ninth carbon, while 53 and 36% of the female C_{29} olefin contains a double bond at the eleventh and thirteenth carbons, respectively. And this difference in the positions of unsaturation is characteristic of the other dominant male and female monoolefins. In the pentacosene and heptacosene from the males, the unsaturation is nearly exclusively at the ninth carbon, but in the hentriacontene and tritriacontene from the females, the unsaturation is mainly at the eleventh, and to a lesser extent, the thirteenth carbons.

Since the females contain large amounts of hentriacontenes and tritriacontenes that are nearly absent in the males, it is not surprising that mating stimulation is associated with these olefins. The most active compound, (*Z*)-11-hentriacontene, which is the major constituent of the female C_{31} monoolefin fraction, is only a minor constituent of the male C_{31} olefins.

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MATING STIMULANT PHEROMONE AND CUTICULAR LIPID CONSTITUENTS OF *Fannia femoralis* (STEIN) (DIPTERA: MUSCIDAE)[1,2]

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Abstract—The cuticular lipids of male and female *Fannia femoralis* were similar for recently emerged insects but soon began to develop chromatographic patterns characteristic of each sex. Mature females contained more C_{31} and C_{33} monoolefin in the cuticular lipid than males. Also, the double bonds in the monoolefins of the female lipid were situated predominantly at the eleventh and thirteenth carbons, while most of those from the males were centrally located in the molecule or at the ninth carbon.

The female C_{31} monoolefin stimulated copulation by the males, but more mating activity occurred when the saturated hydrocarbons present in the female cuticular lipids were added. The synthetic monoolefin most active as a mating stimulant pheromone was (*Z*)-11-hentriacontene, but the addition of female alkanes or of synthetic *n*-alkanes to (*Z*)-11-hentriacontene increased the activity of the synthetic pheromone.

Key Words: *Fannia femoralis* (Stein), pheromone, mating stimulant pheromone, (*Z*)-11-hentriacontene, cuticular lipid, fly.

[1] 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.

[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 or the University of Maryland.

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INTRODUCTION

Fannia femoralis (Stein) occurs in the eastern, southern, and western United States (Chillcott, 1960); its biology and behavior were studied by Tauber (1968). Sometimes called "the coastal fly" (Peck and Anderson, 1970), the species is of economic importance in California, where flies often become abundant around poultry ranches (Anderson and Poorbaugh, 1964; Georghiou 1967).

The constituents of the cuticular lipids of three species of *Fannia*, small flies that often breed in poultry manure, have been investigated in an effort to identify the mating stimulant pheromones. The material isolated from female *F. canicularis* (L.) that stimulated mating by the males was (*Z*)-9-pentacosene (Uebel et al., 1977); the most active compound isolated from female *F. pusio* (Wiedemann) was (*Z*)-11-hentriacontene (Uebel et al., 1978). The mating stimulant pheromone and the major components in the cuticular lipids of the females and males of a third species, *F. femoralis*, are described here.

METHODS AND MATERIALS

A colony identified as *F. femoralis* by the Systematic Entomology Laboratory, ARS, USDA, was established from larvae collected in chicken manure at the Beltsville Agricultural Research Center, ARS, USDA. The colony was maintained in the same manner as the *F. canicularis* colony (Uebel et al., 1977). Also, the methods of isolation and identification of the components of the *F. femoralis* cuticular lipid were the same as for the other species of *Fannia* previously investigated (Uebel et al., 1977, 1978). Briefly, external lipids were washed from 0-, 1-, 3-, 5-, or 15-day-old unmated females and males with petroleum ether (b.p. 30–60° C). The cuticular lipids from 5-day-old female and male flies were fractionated on Florisil® (60–100 mesh) to separate hydrocarbons from nonhydrocarbons. Initially, the hydrocarbons were fractionated by liquid chromatography on an open column of 20% silver nitrate–impregnated Florisil (Ag LC). The saturated hydrocarbons were eluted with hexane, and the unsaturated hydrocarbons with benzene. However, when it was observed that both monoolefins and diolefins were present, the elution scheme through the silver nitrate column was modified so that the alkanes were eluted with hexane, the monoolefins with 15% benzene in hexane, and the diolefins with benzene. The presence of monoolefins and diolefins was determined by comparing the R_f values of the isolated unsaturated hydrocarbons with the R_f values of authentic standards on silver nitrate–impregnated silica gel thin-layer plates (Ag TLC). Ag

TLC was also used to determine the geometric configuration of the double bond of the monoolefin. Gas-liquid chromatography of the cuticular lipid and hydrocarbon fractions was performed on a column of 5% SE-30 with the temperature programmed from 180 to 280°C at a rate of 1°C/min. The positions of the double bonds in the monoolefins were located by ozonolysis and identification of the resulting aldehydes (Beroza and Bierl, 1967). The synthetic monoolefins were prepared as described in the accompanying paper (Uebel et al., 1978).

Fractions and synthetic monoolefins were assayed for activity in eliciting copulatory responses from male flies by the pseudofly bioassay (Uebel et al., 1977). Thus, small knots constructed from black yarn were impaled on pins and treated with 100 or 200 μg of the test material. These treated pseudoflies were then inserted into jars (one pseudofly per jar) containing 10 *F. femoralis* males. Four or five such jars were observed concurrently for 5 min, and the number of attempted copulations by the males with the pseudoflies was counted. Five consecutive 5-min observations were made with the same treated pseudoflies.

RESULTS

Cuticular Lipids from Female and Male F. femoralis

The amounts of cuticular lipids obtained from 24-h-old female and male flies were ~3.2 μg and 2.3 μg, respectively. Gas chromatograms of the cuticular lipids washed from these newly emerged females and males were nearly identical and resembled the chromatograms of lipids from newly emerged *F. pusio* (Uebel et al., 1978). However, the relative proportions of the major (>5%) alkanes and monoolefins in the cuticular lipids of both sexes changed dramatically during the ensuing 3 days and then continued to change more slowly through 15 days (Table 1).

The more detailed study of 5-day-old flies revealed that the weight of the cuticular lipids from these flies ranged from 5.8 to 6.8 μg for females and from 4.6 to 5.6 μg for males. Also, from the chromatograms, all the major peaks for these cuticular lipids were found in the first fraction from the Florisil column, an indication that they were hydrocarbons.

The saturated hydrocarbon content of female and male lipids was very similar (Fig. 1). Calculations from areas of peaks on the chromatograms showed that the saturated hydrocarbons, monoolefins, and diolefins made up 44, 52, and 4%, respectively, of the cuticular lipid of 5-day-old female flies, and 52, 44, and 4%, respectively, of the cuticular lipid from 5-day-old male flies.

TABLE 1. PERCENTAGES OF MAJOR ALKANES AND MONOOLEFINS IN THE CUTICULAR LIPID FROM *F. femoralis* FEMALES AND MALES OF DIFFERENT AGES

Materials	% in lipid from flies of indicated ages				
	0 days	1 day	3 days	5 days	15 days
♀ C_{23}	0.6	3.0	4.9	8.5	11.3
♂ C_{23}	0.6	4.4	7.3	11.6	18.6
♀ C_{25}	2.1	8.6	13.9	19.2	24.0
♂ C_{25}	1.9	9.1	16.2	21.2	28.0
♀ $C_{27:1}$	0.8	1.9	1.9	2.2	2.0
♂ $C_{27:1}$	1.8	6.3	7.6	6.8	5.6
♀ C_{27}	1.8	3.7	5.9	8.1	8.6
♂ C_{27}	2.8	5.8	9.3	11.7	11.5
♀ $C_{29:1}$	9.4	25.3	26.6	19.9	19.3
♂ $C_{29:1}$	14.2	30.9	30.0	23.1	17.8
♀ $C_{31:1}$	12.4	19.7	18.3	14.4	10.5
♂ $C_{31:1}$	13.7	11.2	9.6	7.9	4.1
♀ $C_{33:1}$	4.1	6.0	10.0	10.0	8.2
♂ $C_{33:1}$	3.1	1.2	0.8	0.7	0.3

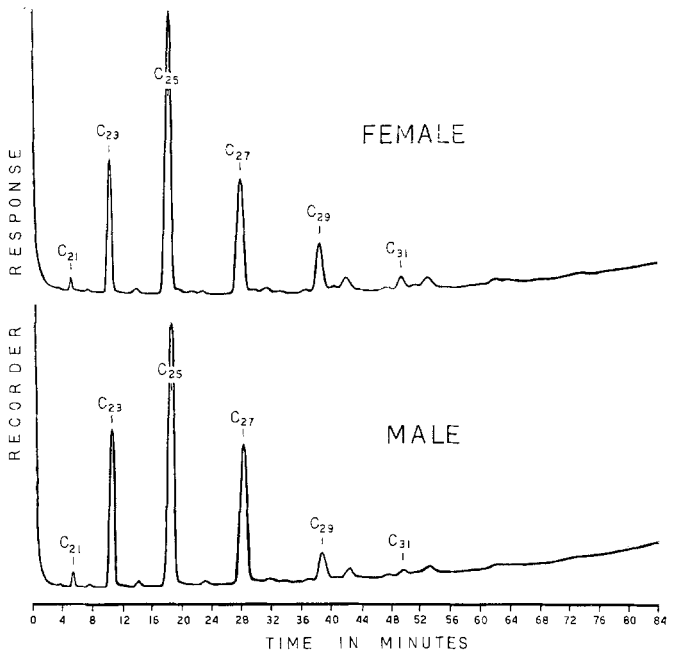

FIG. 1. Gas-liquid chromatograms of the saturated hydrocarbons from 5-day-old female and male *F. femoralis*. The letter C with a subscript identifies the straight chain saturated hydrocarbons.

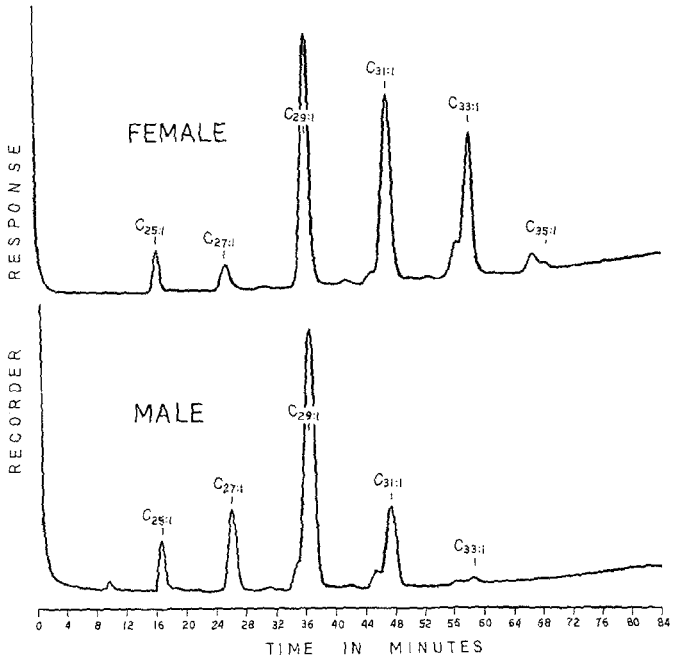

FIG. 2. Gas-liquid chromatograms of the unsaturated hydrocarbons from 5-day-old female and male *F. femoralis*. The letter C with a subscript identifies the straight chain monoolefins.

Chromatograms of the unsaturated hydrocarbons (Fig. 2), unlike those for the saturated hydrocarbons, showed a definite difference between sexes; the 5-day-old females contained more C_{31} and especially more C_{33} olefins than did the males. When the unsaturated hydrocarbon fraction was analyzed by Ag TLC, a small amount of material was found that had the same R_f value as a diene; therefore, the monoolefins and diolefins were then separated by Ag LC. Once the monoolefins were freed of the other unsaturates, chromatograms demonstrated that the shoulders on the front of the longer-chain olefin peaks (Fig. 2) were diolefins. Also, Ag TLC of the monoolefins from 5-day-old flies were those of cis isomers; no trans isomers were detected.

A comparison of the positions of the double bonds in the C_{27}, C_{29}, and C_{31} monoolefins from female and male flies (Table 2) also showed definite differences between sexes. The monoolefins from females contained a higher percentage of the double bond at the eleventh and thirteenth carbons than the monoolefins from the males; the male olefins were usually represented by the compound with the double bond at the ninth carbon.

TABLE 2. PERCENTAGES OF ISOMERS IN THE MONOOLEFINS OF THE CUTICULAR LIPIDS OF 5-DAY-OLD FEMALE AND MALE *F. femoralis*

	Percentages of monoolefin with a double bond at the indicated carbon positions											
Monoalkenes	5	6	7	8	9	10	11	12	13	14	15	16
♀ $C_{25:1}$	—	—	14	—	81	5	—	—				
♂ $C_{25:1}$	—	—	15	—	82	3	—	—				
♀ $C_{27:1}$	—	—	16	—	21	5	17	6	35			
♂ $C_{27:1}$	—	—	30	—	58	—	—	—	12			
♀ $C_{29:1}$	—	—	—	—	2	2	30	3	43	20		
♂ $C_{29:1}$	—	—	2	—	30	—	2	4	19	43		
♀ $C_{31:1}$	—	—	3	—	2	3	38	3	26	8	17	
♂ $C_{31:1}$	1	1	3	—	51	1	3	2	6	10	22	
♀ $C_{33:1}$	—	—	—	—	2	3	79	1	5	1	2	7
♀ $C_{35:1}$	—	—	2	2	1	17	56	2	2	4	2	12

TABLE 3. NUMBER OF ATTEMPTS BY *F. femoralis* MALES TO COPULATE WITH PSEUDOFLIES TREATED WITH CUTICULAR LIPID OR WITH HYDROCARBON FRACTIONS FRCM 5-DAY-OLD FEMALE AND MALE FLIES

Test materials	Total number of copulatory attempts at pseudoflies treated with indicated amount of test material[a,b]	
	100 μg/pseudofly	200 μg/pseudofly
♀ Cuticular lipid	3 bc	12 bc
♂ Cuticular lipid	0 c	0 c
♀ Hydrocarbon	17 b	31 a
♂ Hydrocarbon	0 c	0 c
♀ Saturated hydrocarbon	0 c	0 c
♂ Saturated hydrocarbon	0 c	0 c
♀ Monoolefins	10 bc	7 bc
♂ Monoolefins	0 c	1 c
♀ Diolefins	0 c	0 c
♂ Diolefins	0 c	0 c
♀ Saturated hydrocarbon and monoolefin[c]	15 b	45 a

[a] Totals are for 20 replicates each of 5-min duration.
[b] Values followed by the same letter are not significantly different at the 5% level by the square root of $\chi+0.5$ transformation, the one-way analysis of variance, and the Student–Newman-Keul's test.
[c] A 50:50 mixture of female saturated hydrocarbon and female monoolefins.

Identification of the Mating Stimulant Pheromone of F. femoralis

Only a few copulatory responses were elicited from males by pseudoflies treated with 100 or 200 μg of the cuticular lipid from 5-day-old females (Table 3). However, the hydrocarbon fraction of the female cuticular lipid eluted from the Florisil column stimulated greater activity from the males; the nonhydrocarbon fractions produced no activity. When the female hydrocarbon was separated into its components (alkane, monoolefin, and diolefin), only the monoolefins stimulated a sexual response from the males, but the response, especially to 200 μg, was considerably less than the response to the unfractionated hydrocarbon. Recombination of the female monoolefins and alkanes as a 1:1 mixture (Table 3) produced activity equivalent to that of the hydrocarbon fraction, but recombination of the female diene (50 or 75%) with the female saturated hydrocarbon produced no activity (200 μg/pseudofly). The cuticular lipid from 5-day-old males and its different hydrocarbon fractions were inactive.

TABLE 4. NUMBER OF ATTEMPTS BY *F. femoralis* MALES TO COPULATE WITH PSEUDOFLIES TREATED WITH GLC TRAPPED FEMALE MONOOLEFINS COMBINED WITH FEMALE SATURATED HYDROCARBONS[a]

Test materials	Total number of copulatory attempts[b,c] at pseudoflies treated with 200 μg of mixture with indicated ratio	
	50% Monoolefins and 50% saturated hydrocarbons	75% Monoolefins and 25% saturated hydrocarbons
♀ C_{25} Monoolefins + ♀ saturated hydrocarbons	0 c	—
♀ C_{27} Monoolefins + ♀ saturated hydrocarbons	5 bc	0 c
♀ C_{29} Monoolefins + ♀ saturated hydrocarbons	3 bc	2 bc
♀ C_{31} Monoolefins + ♀ saturated hydrocarbons	14 b	39 a
♀ C_{33} Monoolefins + ♀ saturated hydrocarbons	0 c	5 bc
♀ C_{35} Monoolefins + ♀ saturated hydrocarbons[d]	0 c	—

[a] The monoolefins and saturated hydrocarbons were obtained from 5-day-old virgin females.

[b] Totals are for 20 replicates each of 5-min duration.

[c] Values followed by the same letter are not significantly different at the 5% level by the square root of $\chi+0.5$ transformation, the one-way analysis of variance, and the Student–Newman–Keul's test.

[d] Only 137 μg of the C_{35} and female saturated hydrocarbon (50:50) mixture were applied to the pseudofly.

The monoolefins from 5-day-old females were, therefore, separated according to their chain length by trapping from the gas-liquid chromatograph. The amounts of the recovered olefins were determined, and the olefins were combined with the female saturated hydrocarbon in mixtures containing 50 and 75% monoolefins. When these were tested at 200 μg/pseudofly (Table 4), the mixture containing 75% of the C_{31} monoolefin was the most active.

The three most abundant isomers (Table 2) of the female C_{31} monoolefins were determined to be (*Z*)-11-, (*Z*)-13-, and (*Z*)-15-hentriacontenes, which constituted 38, 26, and 17%, respectively, of the mixture. These three compounds were synthesized and compared with (*Z*)-11- and (*Z*)-13-tritriacontene; the (*Z*)-11-hentriacontene tested alone and as a mixture with 25% of the female saturated hydrocarbon was more active than the other synthetic monoolefins (Table 5). Also, the mixture of monoolefin and female alkane was considerably more active than the olefin alone.

Inspection of the chromatogram of the female-borne saturated hydrocarbons (Fig. 1) showed that the major components are odd-numbered carbon alkanes containing only small amounts of branched hydrocarbon. To confirm that the *n*-alkanes in the female saturated hydrocarbon were responsible for increasing the activity of (*Z*)-11-hentriacontene, we combined synthetic odd-numbered carbon *n*-alkanes from C_{23} through C_{31} in the same proportions as found on the female. When synthetic alkane was added to (*Z*)-11-hentriacontene to form 25, 50, and 75% admixtures, the

TABLE 5. NUMBER OF ATTEMPTS BY *F. femoralis* MALES TO COPULATE WITH PSEUDOFLIES TREATED WITH SYNTHETIC *cis*-MONOOLEFINS OR WITH MONOOLEFINS PLUS FEMALE SATURATED HYDROCARBONS

Test materials	Total number of copulatory attempts[a,b] at pseudoflies treated with 200 μg of indicated materials	
	Monoolefin	75% monoolefin and 25% ♀ saturated hydrocarbons
(*Z*)-11-Hentriacontene	22 b	44 a
(*Z*)-13-Hentriacontene	3 c	10 c
(*Z*)-15-Hentriacontene	0 c	3 c
(*Z*)-11-Tritriacontene	0 c	3 c
(*Z*)-13-Tritriacontene	0 c	0 c

[a] Totals are for 20 replicates each of 5-min duration.
[b] Values followed by the same letter are not significantly different at the 5% level by the square root of $\chi+0.5$ transformation, the one-way analysis of variance, and the Student–Newman–Keul's test.

TABLE 6. NUMBER OF ATTEMPTS BY *F. femoralis* MALES TO COPULATE WITH PSEUDOFLIES TREATED WITH (*Z*)-11-HENTRIACONTENE PLUS FEMALE ALKANES OR SYNTHETIC *n*-ALAKANES[a,b]

Test material	Total number of copulatory attempts at pseudoflies treated with 200 μg of test material[c,d]
(*Z*)-11-Hentriacontene	19
(*Z*)-11-Hentriacontene+ ♀ alkanes (75:25)	24
(*Z*)-11-Hentriacontene+ synthetic alkanes (75:25)	32
(*Z*)-11-Hentriacontene+ ♀ alkanes (50:50)	33
(*Z*)-11-Hentriacontene+ synthetic alkanes (50:50)	37
(*Z*)-11-Hentriacontene+ ♀ alkanes (25:75)	20
(*Z*)-11-Hentriacontene+ synthetic alkanes (25:75)	23

[a] The female alkanes were isolated from 5-day-old virgin females.
[b] The synthetic alkanes were a mixture of C_{23}, C_{25}, C_{27}, C_{29}, and C_{31} *n*-alkanes containing the same percentages as found on 5-day-old females.
[c] Totals are for 20 replicates each of 5-min duration.
[d] Values are not significantly different at the 5% level by the one-way analysis of variance.

activities of the mixtures (Table 6) were similar to those of mixtures of the natural (female) saturated hydrocarbon with (*Z*)-11-hentriacontene.

We then identified the alkanes in the synthetic mixture that boosted the activity of (*Z*)-11-hentriacontene by combining synthetic odd-numbered carbon saturated hydrocarbons with (*Z*)-11-hentriacontene in mixtures containing 25, 50 and 75% alkanes (Table 7). In the 25% alkane–olefin mixture, tricosane produced the most activity; in the 50% alkane mixture, pentacosane was the most active; and in the 75% mixture, tricosane and pentacosane were the two most active alkanes.

DISCUSSION

In *F. canicularis*, another *Fannia* species previously investigated by us (Uebel et al., 1977), the mating stimulant (*Z*)-9-pentacosene constitutes the major portion of the female cuticular lipid. In *F. pusio* and *F. femoralis*, the most active olefin is (*Z*)-11-hentriacontene, but it constitutes only about 15% of the total female cuticular lipid of *F. pusio*, and only 6% of that of *F. femoralis*. Also in *F. pusio* and *F. femoralis*, we found some of the homo-

TABLE 7. NUMBER OF ATTEMPTS BY *F. femoralis* MALES TO COPULATE WITH PSEUDOFLIES TREATED WITH DIFFERENT MIXTURES OF (*Z*)-11-HENTRIACONTENE PLUS *n*-ALKANE[a]

Test material	Total number of copulatory attempts at pseudoflies treated with 200 μg of mixture with indicated ratio[b,c]		
	75% Hentriacontene and 25% n-alkane	50% Hentriacontene and 50% n-alkane	25% Hentriacontene and 75% n-alkane
(*Z*)-11-Hentriacontene (alone)	23 bc	29 d	26 fg
(*Z*)-11-Hentriacontene + n-alkane mixture[d]	42 b	44 d	34 f
(*Z*)-11-Hentriacontene + tricosane	62 a	19 de	20 fg
(*Z*)-11-Hentriacontene + pentacosane	33 b	31 d	19 fg
(*Z*)-11-Hentriacontene + heptacosane	30b	22 de	7 g
(*Z*)-11-Hentriacontene + nonacosane	17 bc	25 d	4 g
(*Z*)-11-Hentriacontene + hentriacontane	4 c	2 e	6 g

The (*Z*)-11-hentriacontene and *n*-alkanes were synthetic compounds.

[b] Totals are for 20 replicates each of 5-min duration.

[c] Values in the same column followed by the same letter are not significantly different at the 5% level by the one-way analysis of variance and the Student–Newman–Keul's test.

[d] The synthetic alkanes were a mixture of C_{23}, C_{25}, C_{27}, C_{29}, and C_{31} *n*-alkanes containing the same percentages as found on 5-day-old females.

logues of (Z)-11-hentriacontene that had activity; in *F. canicularis*, (Z)-9-pentacosene is the specific excitant.

It is interesting that both *F. femoralis* and *F. pusio* (Uebel et al., 1978), which are taxonomically close and have overlapping habitats, have similar hydrocarbon profiles. This similarity even extends to the fact that the same compound, (Z)-11-hentriacontene, is the most active monoolefin in stimulating the males. However, there were definite behavioral differences between the two species: the male *F. pusio* made many more copulatory attacks on treated pseudoflies or conspecific females than did the *F. femoralis* males. Also, the alkane content of the cuticular lipids of male and female *F. femoralis* are nearly identical but, in *F. pusio*, tricosane is more abundant in the male lipid. On the other hand, female *F. femoralis*, like the female *F. pusio*, have more hentriacontene and tritriacontene than the males. The predominant position of the double bond in the monoolefins from *F. femoralis* and *F. pusio* males is at the ninth carbon; the usual position in the monoolefins from the females is at the eleventh carbon.

The major difference in the makeup of the pheromone of the two species, therefore, appears to be the synergistic action in *F. femoralis* of the female alkanes. This effect is similar to the synergistic activity we found when methyl- and dimethyl-branched alkanes of the female house fly, *Musca domestica* L., were combined with (*Z*)-9-tricosene (Uebel et al., 1976). However, the synergistic alkanes of the house fly were found on the females only, while both the female and male *F. femoralis* bear similar alkanes.

In the present tests, assays with different monoolefins showed that (*Z*)-11-hentriacontene was consistently the most active. However, in tests with different alkanes, no one was the best synergist for the olefin. In fact, in at least one mixture with (*Z*)-11-hentriacontene, the C_{23}, C_{25}, and C_{27} alkanes each stimulated more copulatory attempts than did the olefin alone.

Fannia pusio and *F. femoralis*, therefore, have the same pheromone [(*Z*)-11-hentriacontene] and, if the males of one species are presented with a female of the opposite species in a confined environment, they will attempt copulation. Since the geographical ranges of these species overlap, reproductive isolation must occur by some mechanism other than dissimilar male mating stimulants.

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OLFACTORY BEHAVIOR AND RECEPTOR POTENTIALS OF THE KHAPRA BEETLE *Trogoderma granarium* (COLEOPTERA: DERMESTIDAE) INDUCED BY THE MAJOR COMPONENTS OF ITS SEX PHEROMONE, CERTAIN ANALOGUES, AND FATTY ACID ESTERS

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Abstract—On the basis of the antennal receptor potentials and the extent of attraction and copulation induced in unmated male khapra beetles, (*Z*)- and (*E*)-14-methyl-8-hexadecenal were recognized as the most important components of the pheromone system of female *Trogoderma granarium* (Everts), and were named (*Z*)- and (*E*)-trogodermal. Air blown over 10^{-5} to 10^{-4} μg of (*Z*)-trogodermal produced receptor potentials equivalent to that elicited by one virgin female *T. granarium*, while $\sim 10^{-2}$ μg of (*Z*)-trogodermal was required to cause complete attraction and copulation of unmated males. (*Z*)-Trogodermal was about 10 times more active than (*E*)-trogodermal. (*Z*)-8-Hexadecenal was $\sim 10^2$ times less effective than (*Z*)-trogodermal in causing attraction and 10^4 time less active in stimulating copulation. (*Z*)- and (*E*)-14-methyl-8-hexadecen-1-ol and methyl (*Z*)- and (*E*)-14-methyl-8-hexadecenoate displayed a relatively low activity for unmated male khapra beetles. Methyl and ethyl oleate, ethyl linoleate, ethyl palmitate, and ethyl stearate were less effective than (*Z*)-trogodermal by 6–8 orders of magnitude and are nonspecific attractants. The intensity of response to a particular compound was consistent when assessed by the essential components of mating behavior: receptor potentials, attraction, and copulation.

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Key Words—olfaction, behavior, electroantennograms, sex pheromone components, (*Z*)- and (*E*)-trogodermal, fatty acid esters, *Trogoderma granarium* (khapra beetle), Dermestidae, Coleoptera.

INTRODUCTION

Because the khapra beetle *Trogoderma granarium* (Everts) is a serious storage pest in warm, dry countries and is a continual object of quarantine surveillance in temperate regions, its pheromone biology has been the subject of several investigations (Levinson, 1971; 1975). The ultimate aim of those studies was the design of effective survey and control methods for the khapra beetle and other harmful species of the genus *Trogoderma;* a comprehensive review of this topic was presented by Burkholder (1976).

Female khapra beetles release a pheromone (Bar Ilan et al., 1965) that, at levels of 10^{-5} to 10^{-3} female equivalents, causes arousal and subsequent orientation to the odor source and precopulatory excitation of the males (Levinson and Bar Ilan, 1967, 1970a), and, at considerably higher levels, aggregation of the females (Levinson and Levinson, 1973). Male khapra beetles release neither a sex pheromone nor an assembling scent (Levinson and Bar Ilan, 1970c). Detailed descriptions have been given of the pheromone-induced responsiveness, orientation, and precopulatory behavior of males (Levinson and Bar Ilan, 1970a–c; Karnavar, 1972) and of the pheromone emission by females under the influence of age and mating (Levinson and Bar Ilan, 1967; Adeesan et al., 1969; Levinson and Levinson, 1973). Unmated females assemble and cease moving in response to pheromone levels of about one female equivalent, the aggregations being maintained by intense stimulation of the numerous mechanoreceptors on the antennae of female khapra beetles (Levinson and Bar Ilan 1970a, b). Continuous exposure for 5 days of unmated males to a pheromone level of approximately 25 female equivalents disrupts the responsiveness of the males, probably because of either olfactory and central nervous adaptation or motor fatigue (Rahalkar et al., 1972; Levinson and Levinson, 1973).

The method of Byrne et al. (1975), based on continuous aeration of insects and collection of the volatiles on an absorbent (Porapak Q), was used for the isolation of the pheromone components emitted by unmated female *T. granarium* beetles throughout their adult stage, ~30 days at 24°C. (*Z*)-14-Methyl-8-hexadecenal and (*E*)-14-methyl-8-hexadecenal were identified as the major pheromone constituents of *T. granarium*, being released at a *Z*–*E* ratio of 92:8 (Cross et al., 1976). Additional compounds excreted by female khapra beetles include *n*-hexanoic acid, γ-caprolactone, $p-1,8$-menthadiene, methyl 14-methyl-8-hexadecenoate, and methyl-7-hexadecenoate (Greenblatt et al., 1977).

To identify the sex pheromone components, we investigated the behavior patterns and antennal receptor potentials of unmated male khapra beetles in response to various amounts of the above isolates as well as synthesized compounds. These parameters were also tested for certain fatty acid esters, which had been described as constituents of the aggregation pheromone of *T. granarium* (Ikan et al., 1969; Yinon et al., 1971).

METHODS AND MATERIALS

Test Insects

A laboratory colony of *T. granarium* originating from the Pest Infestation Control Laboratory Slough, Berkshire (England) was maintained at $30 \pm 0.5°C$ and $50 \pm 10\%$ RH on a mixture of equal parts of crushed wheat, rye, barley, oats, millet, and bran supplemented with 2% of brewer's yeast. Pupation invariably occurred below the exocuticle of the last larval instar. Unmated males were used for testing 3–6 days after pupal-adult ecdysis. Sex determination was usually carried out by comparing the pupal size as well as the antennal clubs of adults (Hinton, 1945); in doubtful cases, we observed the evagination of the aedeagus after brief exposure to carbon dioxide (Levinson and Bar Ilan, 1970c).

Olfactory Responses

Quantitative bioassays were performed by the olfactometer technique of Levinson and Bar Ilan (1970a) at a constant light intensity (~ 500 lux) between 1 and 5 p.m. Test compounds applied to felt wicks in amounts ranging from 10^{-5} to 10^{3} μg (dissolved in 10 μl of hexane) were vaporized at $30 \pm 0.1°C$ to provide concentric scent gradients (Fig. 1). Ten unmated males were released at the periphery of the olfactometer arena (diam. 55 mm), and the number attracted to the scent outlet was recorded at 60-s intervals for 10 min. Each experiment, involving 10 male beetles for each compound and amount, was repeated six to eight times with different insects. Parallel controls involving single virgin females (3–4 days after emergence) were run. The degree of attraction was calculated by dividing the average number of males attracted to a compound by the average number of males attracted to one virgin female within 10 min. With this parameter, dosage–response curves were established for each test compound. The behavior pattern of single males was also recorded.

The electrophysiological technique devised by Schneider et al. (1967) was employed for recording of receptor potentials (electroantennograms)

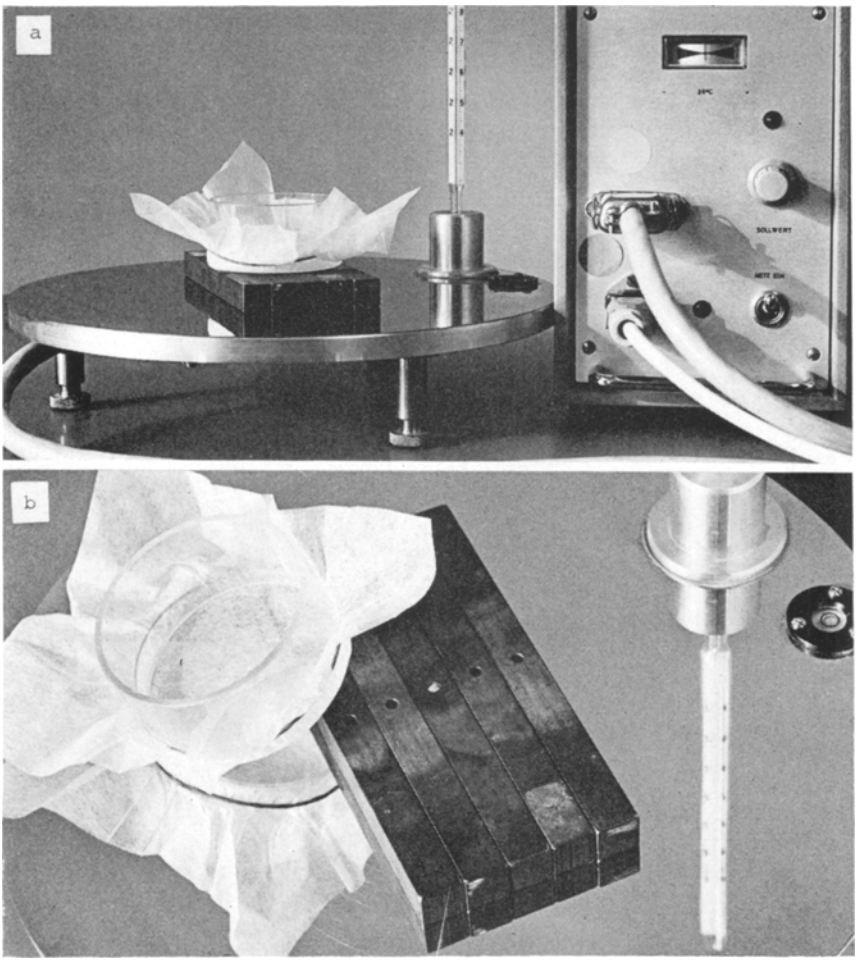

FIG. 1. Olfactometer for the recognition of pheromone components of dermestid beetles (modified from Levinson and Bar Ilan 1970). (a) Total view of the apparatus; (b) olfactometer arena being placed on the five perforated brass bars. The apparatus consists of a chromium-plated hot stage (diameter, 245 mm) with a built-in precision thermometer, maintained at $30 \pm 0.1°C$ by an electronic regulator. Five quadrangular brass bars ($123 \times 12 \times 9$ mm), each provided with a central perforation (bore, 3 mm), are arranged on the hot stage so as to adjoin each other by their long sides. One layer of porous paper (replaced for each experiment) is stretched by a rubber band over the lower opening of a glass tube (diameter, 55 mm; height, 30 mm); the glass tube is put on top of the bars, so that the five perforations are situated along the diameter of the paper arena. The distance between adjacent perforations is 12 mm, and the position of the third one is precisely below the centre of the test arena. Felt wicks (previously hexane-extracted), impregnated with 10 μl of hexane solution of graded amounts of the odorant are singly inserted in the central perforation prior to testing (see "Methods and Materials" section. Photograph by H. Kacher, Seewiesen.)

from the antennae of unmated male khapra beetles. Olfactory stimuli were produced by passing an air current of 40 ml/s over cotton plugs (10 mg) impregnated with various amounts of the test compounds.

The beetles were mounted dorsally in soft wax, with the ventral side of their antennae exposed. The recording electrode was inserted into the middle of the terminal antennal segment, and the indifferent electrode was inserted through the intersegmental cuticle between the head and the thorax. Recordings were obtained from 10–12 different antennae for each concentration and compound.

Compounds Tested

Several constituents of the volatile secretion of unmated female *T. granarium* (Cross et al., 1976; Greenblatt et al., 1977) were synthesized and rigorously purified by GLC:

(*Z*)-14-methyl-8-hexadecenal (Cross et al., 1976),
(*E*)-14-methyl-8-hexadecenal (Cross et al., 1976),
methyl (*Z*)-14-methyl-8-hexadecenoate (Yarger et al., 1975),
n-hexanoic (caproic) acid (Yarger et al., 1975),
γ-caprolactone (Yarger et al., 1975),
1,8-menthadiene (Cassidy, unpublished).

The following analogues of the above aldehydes were also prepared:

(*Z*)-14-methyl-8-hexadecen-1-ol[3] (Yarger et al., 1975),
(*E*)-14-methyl-8-hexadecen-1-ol[3] (Yarger et al., 1975),
(*Z*)-8-hexadecenal[4] (Cassidy, unpublished).

The esters of the following fatty acids were highly purified preparations provided by Sigma Chemical Co., St. Louis, Missouri; they have been isolated from homogenized khapra beetles of both sexes and erroneously described as their pheromone components (Ikan et al., 1969; Yinon et al., 1971):

methyl oleate ($bp_{10} = 190.5°$, $D^{20} = 0.8739$),
ethyl oleate ($bp_{13} = 207°$, $D^{25} = 0.868$),
ethyl linoleate ($bp_{6} = 193°$, $D^{25} = 0.8776$),
ethyl palmitate (mp = 24.4°, $bp_{10} = 185.5°$, $D^{25} = 0.8577$),
ethyl stearate (mp = 33.9°, $bp_{4} = 180°$, $D^{36} = 0.848$).

RESULTS

The levels of odorants mentioned in this section merely represent the amount of a pure compound initially deposited on either felt wicks or

[3] Found in other *Trogoderma* species (Greenblatt et al., 1977).
[4] Not found in *Trogoderma* species.

cotton plugs before starting the experiments; the actual amounts of odorants taken up by the corresponding chemoreceptors are unknown.

Behavioral Responses

Figure 2 reveals the responsiveness of unmated male khapra beetles to the major airborne compounds emitted by female *T. granarium* as well as to the analogues. A threshold amount of 10^{-5} μg of (*Z*)-14-methyl-8-hexadecenal induced arousal of hitherto immobile males, successive antennal sampling of the air currents, directed movement, and location of the scent outlet by an average of 25% of the beetles. An amount of 10^{-3} μg attracted an average of 50%, while a level of 2×10^{-2} μg lured 100% of the males released. Approximately 25 ng of (*Z*)-14-methyl-8-hexadecenal was thus equivalent in bioassay attraction to one virgin female *T. granarium*, whereas about 1 μg of this compound was at least twice as attractive as one virgin female. Up to a level of 10^{-2} μg, (*E*)-14-methyl-8-hexadecenal was approximately 10 times less attractive to male khapra beetles than the *Z*-isomer. This gap decreased at higher levels and almost disappeared beyond a dosage of

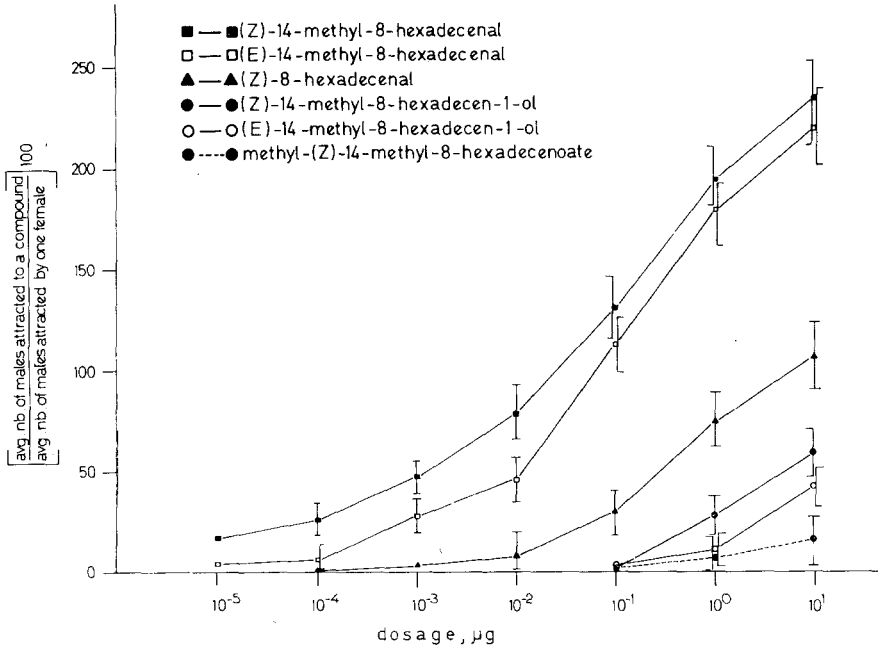

FIG. 2. Attractiveness of some volatile compounds released by females of *T. granarium* and of synthetic analogues. Each point represents the responses of 60-80 unmated male khapra beetles.

10^{-1} μg. Because of their marked pheromone activity, we propose to name these isomers (Z)-trogodermal and (E)-trogodermal.

It also became evident (Fig. 2) that the unbranched (Z)-8-hexadecenal was about $(2–6) \times 10^2$ times less attractive for male khapra beetles than (Z)-trogodermal. On the other hand, the activity of (Z)-14-methyl-8-hexadecen-1-ol was $(5–8) \times 10^3$ times lower than that of the corresponding aldehyde, and the activity of (E)-14-methyl-8-hexadecen-1-ol was $(1.5–2.5) \times 10^4$ times lower than that of (E)-trogodermal; exceedingly large amounts, such as 10 μg, of these branched alcohols induced only incomplete attraction of male *T. granarium*. Methyl (Z)-14-methyl-8-hexadecenoate was $\sim 2.5 \times 10^5$ times less effective than (Z)-trogodermal. No significant behavioral responses resulted upon exposure of male khapra beetles to caproic acid, γ-caprolactone, methyl (Z)-7-hexadecenoate, or 1,8-menthadiene, in spite of their being emitted by females of *T. granarium*. The activity of mixtures between the above compounds and trogodermal has not been tested.

(Z)- or (E)-Trogodermal was found to elicit in males the complete behavior sequence triggered by pheromone-emitting females of *T. granarium:* arousal, attraction, excitation, and copulatory movements. Upon exposure to levels of either 10^{-4} μg of (Z)- or 10^{-3} μg of (E)-trogodermal, male khapra beetles frequently touch other males with their palpi and occasionally push their heads against them. Amounts of 10^{-3} μg of (Z)- or 10^{-2} μg of (E)-trogodermal induce 50% of the male beetles to explore the dorsal posterior abdomen of other males with their protruded aedeagus and to perform copulatory movements. The unnatural (Z)-8-hexadecenal was found to

TABLE 1. EFFICIENCY OF SYNTHESIZED PHEROMONE COMPONENTS AND DERIVATIVES FOR ATTRACTION AND COPULATORY BEHAVIOR IN MALE *Trogoderma granarium*

Compound	Amount (μg) inducing male			
	Attraction		Copulation	
	50%	100%	50%	100%
(Z)-14-Methyl-8-hexadecenal	10^{-3}	2×10^{-2}	10^{-3}	10^{-2}
(E)-14-Methyl-8-hexadecenal	10^{-2}	6×10^{-2}	10^{-2}	10^{-1}
(Z)-8-Hexadecenal	3×10^{-1}	6×10^{0}	10^{1}	*d*
(Z)-14-Methyl-8-hexadecen-1-ol	5×10^{0}	*b*	10^{2}	*d*
(E)-14-Methyl-8-hexadecen-1-ol	2×10^{1}	*b*	*c*	
Methyl (Z)-14-methyl-8-hexadecenoate	*a*	*b*	*c*	

[a] 2×10^1 μg caused 25% of the males to respond.
[b] Levels of up to 10^1 μg induced less than 100% attraction.
[c] 10^2 μg failed to induce copulation in 50% of the beetles.
[d] 10^2 μg failed to induce copulation in 100% of the beetles.

exert almost the same behavior sequence when provided at a level 10^4 times higher than that of (*Z*)-trogodermal, and it required a level of (*Z*)-14-methyl-8-hexadecen-1-ol that was 10^5 times greater than that of (*Z*)-trogodermal to elicit the same responses. (*Z*)- and (*E*)-Trogodermal were the only compounds that caused attraction as well as copulation in 100% of the males exposed (Table 1).

A comparison between the attractivity of alkyl esters of long-chain fatty acids and (*Z*)-trogodermal (Fig. 3) revealed methyl oleate to be about

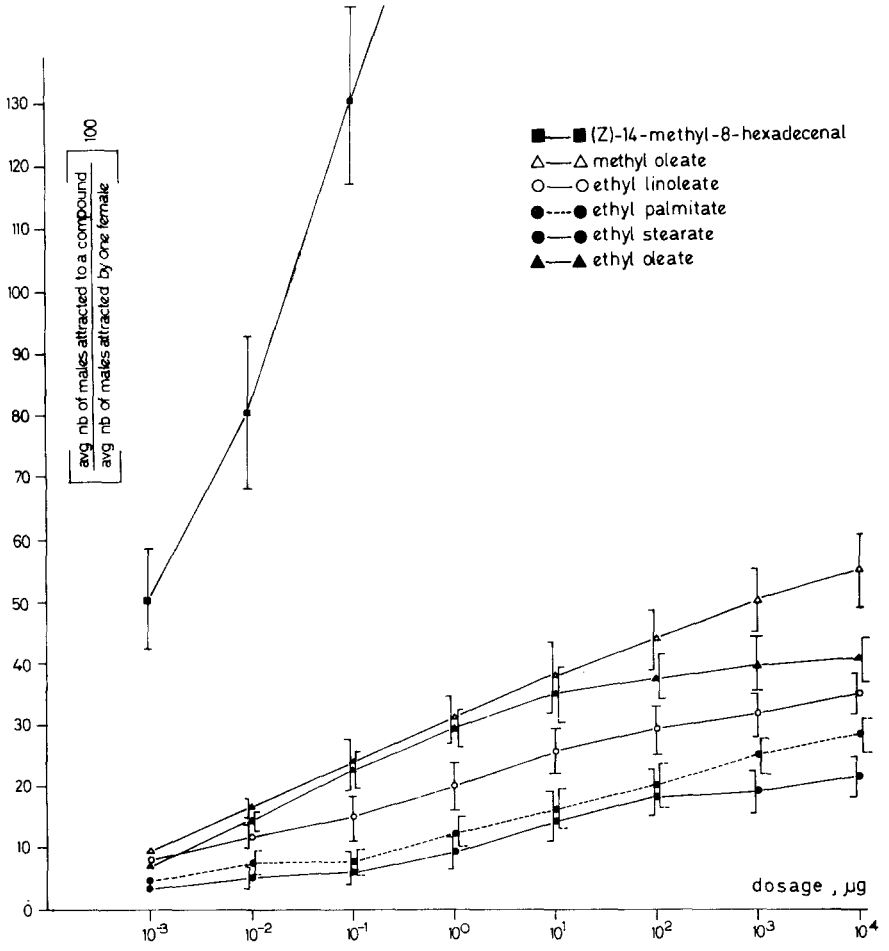

FIG. 3. Attractiveness of certain alkyl esters of long-chain fatty acids and of (*Z*)-14-methyl-8-hexadecenal (*Z*-trogodermal) for unmated male khapra beetles. Each point represents the responses of 60–80 males.

10^6 times less attractive than the aldehyde, while the activity of the remaining esters is definitely lower than that of methyl oleate. The maximal percentages of male attraction recorded were approximately 55 for methyl oleate, 40 for ethyl oleate, 35 for ethyl linoleate, 25 for ethyl palmitate, and 20 for ethyl stearate, when administered in 10-mg levels. As those esters did not induce the males to perform the behavior sequence triggered by the pheromone of unmated female *T. granarium*, they should be regarded as nonspecific and weak attractants (Levinson and Levinson, 1973).

Receptor Potentials

The receptor potentials recorded from the antennae (EAGs) of male khapra beetles following stimulation at various odorant levels (Fig. 4) showed (*Z*)-trogodermal to be the most active compound tested; it elicited

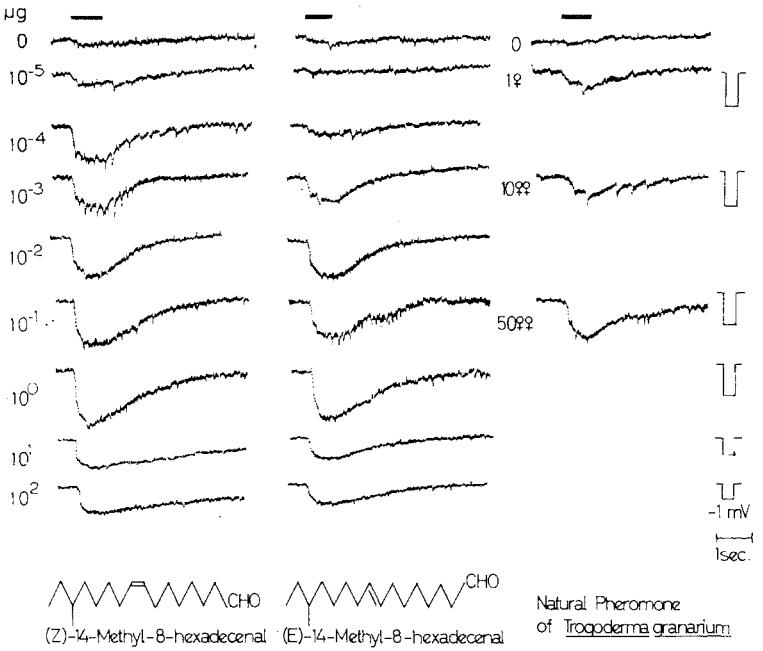

FIG. 4. Receptor potentials recorded from an antenna of male *Trogoderma granarium* induced by various amounts of (*Z*)-14-methyl-8-hexadecenal (*Z*-trogodermal), (*E*)-14-methyl-8-hexadecenal (*E*-trogodermal) and the pheromone emitted by female khapra beetles. Extracellular DC potentials from the terminal antennal segment were measured with glass microcapillaries. The bars indicate stimuli lasting 0.8–1.0 s.

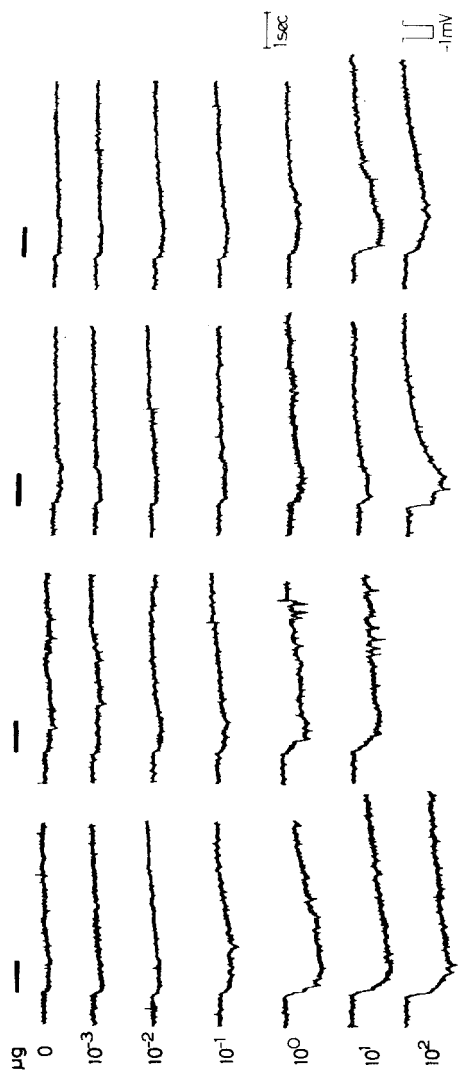

FIG. 5. Receptor potentials recorded from an antenna of male *Trogoderma granarium* induced by graded levels of (*Z*)-14-methyl-8-hexadecen-1-ol, (*E*)-14-methyl-8-hexadecen-1-ol, (*Z*)-8-hexadecenal, and methyl (*Z*)-14-methyl-8-hexadecenoate. The experimental details are given in Fig. 4.

amplitudes of approximately 0.3 mV at a threshold amount of 10^{-5} μg and 1.0 mV at 10^{-4} μg. (*E*)-Trogodermal, being at least 10 times less active than its *Z*-isomer, produced amplitudes of 0.3 mV at the threshold dosage of 10^{-4} μg and 1.0 mV at the level of 10^{-2} μg. An air current blown over a single virgin female produces a receptor potential of about 0.5 mV, which corresponds to the EAGs elicited at levels between 10^{-5} and 10^{-4} μg of (*Z*)-trogodermal or between 10^{-4} and 10^{-3} μg of (*E*)-trogodermal.

As is evident from Fig. 5, the EAGs elicited by (*Z*)- or (*E*)-14-methyl-8-hexadecen-1-ol, (*Z*)-8-hexadecenal, or methyl (*Z*)-14-methyl-8-hexadecenoate showed far lower amplitudes than those obtained from (*Z*)- or (*E*)-trogodermal. At a level of 1.0 μg, (*Z*)-14-methyl-8-hexadecen-1-ol caused an amplitude of 1.0 mV, whereas its *E*-isomer induced one of only 0.7 mV. A dosage of 10^2 μg of (*Z*)-8-hexadecenal was necessary to produce an amplitude of 1.2 mV. Methyl (*Z*)-methyl-8-hexadecenoate gave an amplitude of 0.7 mV at a level of 10^2 μg.

DISCUSSION

A rather good agreement among three essential components of the mating behavior is evident. Thus, olfactory perception, degree of attraction, and extent of copulation of male khapra beetles following exposure to either highly or weakly active compounds have virtually the same trend. (*Z*)-Trogodermal was found to induce the highest levels of receptor potentials, attraction, and copulation, while 10 times as much (*E*)-trogodermal was required to produce a comparable response. The less active compounds, except for (*Z*)-8-hexadecenal, stimulated correspondingly lower levels of receptor potential, attraction, and copulation. Since only (*Z*)-trogodermal or (*E*)-trogodermal, among the various attractants tested, induces successful copulation of male *T. granarium* (cf. Table 1), we conclude that the central nervous system is capable of separating those key stimuli from nonspecific stimuli (Levinson, 1975).

In addition to the two isomers of trogodermal, other compounds, as yet unidentified, emitted by female khapra beetles may well have pheromone function. The difference in level of activity between the aldehyde fraction and the Porapak eluate as well as the attractiveness of the residual fractions A and B derived from the volatiles of female *T. granarium* (Fig. 6) imply the existence of a *pheromone system* in this species. This would be unexceptional, as sex pheromones consisting of two or more components, occasionally with different functions, also occur in other insect species (Silverstein and Young, 1976). Unmated male khapra beetles were found to be indifferent to dosages of up to 10 μg of caproic (*n*-hexanoic) acid. According to Cohen et al.

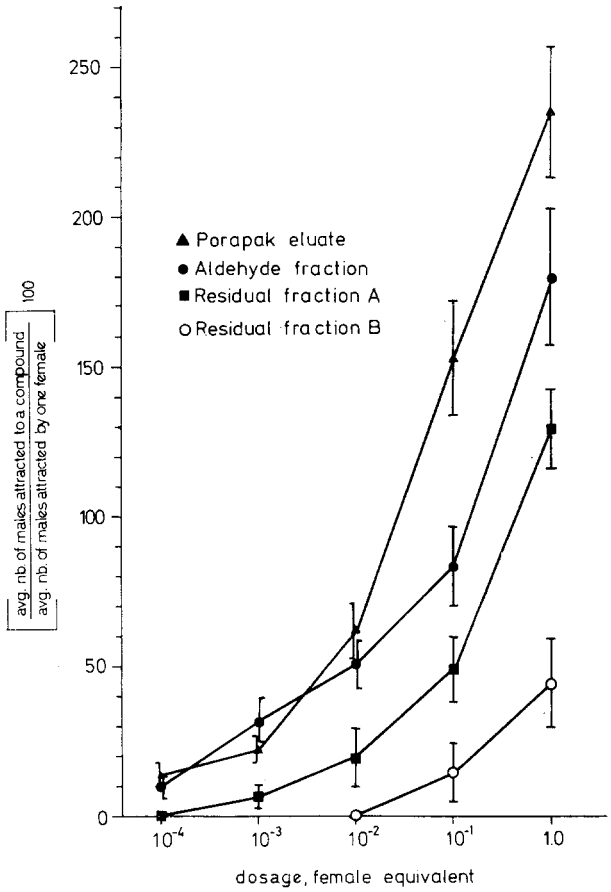

FIG. 6. Attractiveness of several pheromone fractions derived from the total volatiles of unmated female khapra beetles, absorbed on Porapak Q. Each point represents the responses of 60–80 unmated male *Trogoderma granarium*.

(1974), *n*-fatty acids with a chain length of 5 to 8 carbon atoms discourage aggregation of female *T. granarium*. The females were also found to emit 1,8-menthadiene and methyl 7-hexadecenoate (Greenblatt et al., 1977), for which no behavioral rôle has been discovered so far. (*Z*)- and (*E*)-14-Methyl-8-hexadecen-1-ol are at least 3–4 orders of magnitude less active than (*Z*)- and (*E*)-trogodermal, respectively, when compared by the three parameters mentioned above. They were not detectable in the volatiles of unmated female *T. granarium*, but were extracted from homogenized females

of other *Trogoderma* species (Rodin et al., 1969; Yarger et al., 1975). The alcohols may perhaps be converted to trogodermal by a glandular alcohol dehydrogenase, before pheromone emission. The relatively high attractiveness for male khapra beetles previously attributed to (*Z*)-14-methyl-8-hexadecen-1-ol (Levinson and Levinson, 1974) may have been caused by a small amount of (*Z*)-trogodermal as an impurity in this preparation. We do not know whether these alcohols are present in the intact khapra beetle; they were not detected in the volatiles or in the filter paper on which beetles were held during aeration. The studies described show clearly that methyl oleate, ethyl oleate, ethyl linoleate, ethyl stearate, and ethyl palmitate induce neither copulatory behavior nor significant attraction when offered to unmated male *T. granarium* in amounts known to exert pheromone activity (cf. Figs. 2 and 3 and Table 1). These esters thus seem unlikely to be components of the aggregation pheromone of female khapra beetles (Levinson and Levinson, 1973).

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SOME SOLID-PHASE SYNTHESES OF THE SEX ATTRACTANT OF THE SPRUCE BUDWORM—*trans*-11-TETRADECENAL[1]

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Abstract—The sex attractant of the spruce budworm, *Choristoneura fumiferana*, *trans*-11-tetradecenal (I), was synthesized on solid phases by three different routes from inexpensive 1,10-decanediol. The preparation of the *trans*-alkene I directly on the polymer support was not completely stereoselective.

Key Words—solid-phase synthesis, *trans*-11-tetradecenal, spruce budworm, *Choristoneura fumiferana*, *trans*-11-tetradecenol and acetate, 11-tetradecyne-1,10-diol.

It has been demonstrated that insoluble polymer supports can be used as monoprotecting agents of symmetrical diols (Wong and Leznoff, 1973; Fyles and Leznoff, 1976; Fréchet and Nuyens, 1976). Inexpensive symmetrical diols were then used as precursors in the solid phase syntheses of cis insect sex attractants via an alkyne-coupling route, a Wittig route, a "reverse" Wittig route (Leznoff and Fyles, 1976; Leznoff, Fyles, and Weatherston, 1977), and a two-step alkyne-coupling route (Fyles, Leznoff, and Weatherston 1977).

The synthesis of an isomerically pure trans insect attractant on solid phases presents a particularly difficult problem, as shown herein. Although the sex attractant, *trans*-11-tetradecenal (I), of the spruce budworm, *Choristo-*

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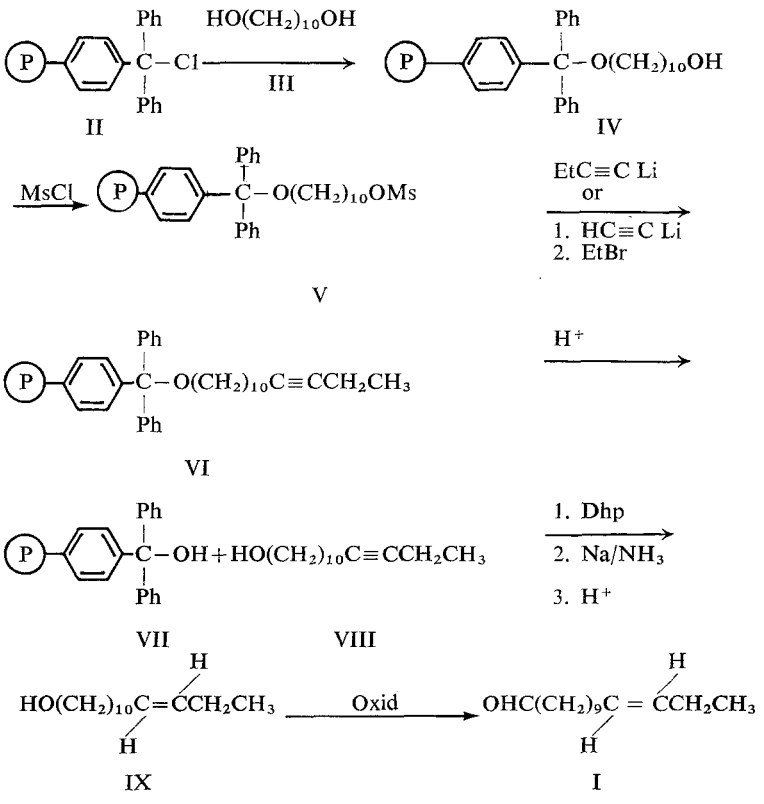
neura fumiferana, was isolated, characterized, and synthesized some time ago (Weatherston and Percy, 1970; Weatherston et al., 1971) and is even commercially available (Chemical Samples Co.), details of its synthesis and its spectroscopic characteristics have never been published.

In this paper, we describe the preparation of I on solid phases by three different routes. The synthesis of I was accomplished according to Scheme A. This method produces 11-tetradecyn-1-ol (VIII), previously prepared on solid phases (Leznoff and Fyles, 1976) by a conversion of 58% from III. Attempts to reduce the polymer-bound alkyne VI directly to a *trans*-alkene by the standard procedure (Campbell and Eby, 1941; Warthen and Jacobson, 1973) of using sodium in liquid ammonia predictably failed, as liquid ammonia is a notoriously bad solvent for performing chemical reactions on cross-linked polystyrene beads because of the inability of ammonia to swell the polymer beads. Hence, VIII, prepared on solid phases, was stereoselectively reduced in solution (Warthen and Jacobson, 1973) with sodium in liquid ammonia to give *trans*-11-tetradecen-1-ol (IX), which was analyzed by high-pressure

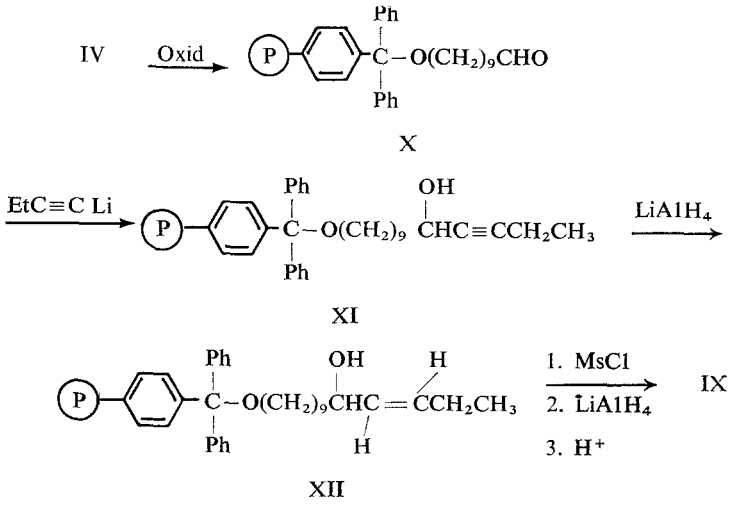
Scheme A



liquid chromatography (HPLC) and vapor-phase chromatography (VPC) as its acetate (Warthen, 1975). Oxidation of IX with a mixture of chromyl chloride, *tert*-butanol, and pyridine in CH_2Cl_2 (Sharpless and Akashi, 1975) gave I in 76% overall yield from VIII.

Although the synthesis of I was thus achieved partly on solid phases and partly in solution, we wished to accomplish the *total* synthesis of I on solid phases for adaptation to automation (Merrifield et al., 1966). An attempt to achieve this goal is outlined in Scheme B. It is known that propargylic alcohols

Scheme B

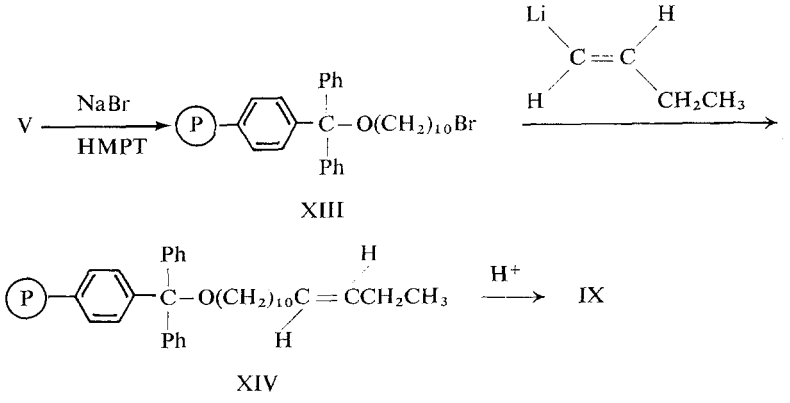


can be reduced stereoselectively to *trans*-allylic alcohols (Bharucha and Weedon, 1953; Raphael, 1955), and we hoped that this procedure could be applied to the synthesis of a polymer-bound *trans*-alkene required for the synthesis of I. The polymer-bound diol IV was oxidized to the polymer-bound aldehyde X (Sharpless and Akashi, 1975; Leznoff et al., 1977). Treatment of X with 1-lithio-1-butyne gave the polymer-bound alkynol XI, which, on acid cleavage, yielded 11-tetradecyne-1,10-diol. Reduction of XI with $LiAlH_4$ or $NaAlH_2(OCH_2CH_2OCH_3)_2$ and subsequent elaboration according to Scheme B gave only VIII and IX in 28% and 15% conversions, respectively. The isolation of VIII can be readily rationalized if one assumes that the reduction of XI was incomplete. This sequence was particularly discouraging because I was contaminated by VIII and did not exhibit a sufficiently high cis–trans ratio (1:9 in the best instance) to warrant further development.

A third route to I is shown in Scheme C. In this approach, a polymer-bound bromide XIII, prepared from V, reacted with the preformed *trans*-1-

lithio-1-butene (Neumann and Seebach, 1976) to give a polymer-bound alkene XIV, which, on acid treatment and oxidation as described before, gave IX in 37% yield and 67% conversion from starting diol III. The synthesis of I according to Scheme C proceeded well, but the cis–trans ratio (1:3)

Scheme C



was not as high as the analogous reaction in solution (Neumann and Seebach, 1976). Perhaps the necessity of using slightly higher temperatures and hexamethylphosphoric triamide (HMPT) as solvent in the coupling step contributed to the diminished selectivity on the solid phase.

The synthesis of a trans insect sex attractant, *trans*-11-tetradecenal (I), has been achieved partially on solid phases using inexpensive symmetrical diols. The synthesis of I totally on solid phases has been accomplished, but further work must be done to give I having a cis–trans ratio of 4:96, comparable to that of the natural attractant (Sanders and Weatherston, 1976).

METHODS AND MATERIALS

Instruments and Standard Procedures

A Bausch and Lomb Abbé 3L refractometer was used to record the refractive indices (n_D). Infrared spectra (IR) were recorded on a Unicam SP1000 IR spectrophotometer as neat films between NaCl disks unless otherwise specified. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM360 spectrometer, with deuteriochloroform as solvent and tetramethylsilane as internal standard. Mass spectra (MS) were recorded on a Perkin-Elmer-Hitachi RMU6E mass spectrometer. Silica gel was used for all thin and preparative layer chromatography. Fractions were extracted with ether in a Soxhlet extractor. Filtration was

done under vacuum through sintered glass Büchner funnels. Filtration under an inert atmosphere was done as previously described (Fyles and Leznoff, 1976). Microanalyses were performed by G. Gygli of Toronto.

Vapor-phase chromatograms were run with a Perkin-Elmer 990 instrument using a 17 ft × 1/8 in. column of 10% Silar 10C on Gaschrom Q(60/80) at a temperature of 175° and a nitrogen flow rate of 15 ml/min. High-pressure liquid chromatograms were run with a Waters Associates Model 440 instrument, with an R-400 refractive index detector. A 30 × 0.4 cm reverse phase μ Bondapak C-18 column with 30% water in acetonitrile was used at a flow rate of 0.8–1.0 ml/min. Spectral and analytical data are given for all new compounds and for known compounds where data are unreported.

trans-11-Tetradecen-1-ol(IX) and Its Acetate

Compound IX was prepared from VIII by the method of Warthen and Jacobson (1973) in 90% yield.

n_D: 1.4597.
IR: γ 3350, -OH; 3020 and 960 *trans*-CH=CH.
NMR: δ 5.4 (m, 2H); 3.6 (t, 2H, J = 8 Hz); 3.0 (s, broad, 1H); 2.3–1.9 (m, 4H); 1.8–1.2 (m, 16H); 0.95 (t, 3H, J = 7 Hz).
Anal. calc. for $C_{14}H_{28}O$: C 79.18, H 13.29; found: C 79.20, H 13.18.

The acetate of IX was prepared by treatment of IX with acetic anhydride in pyridine. Analysis of the acetate by HPLC revealed less than 0.5% contamination by the cis isomer.

n_D: 1.4531.
IR: γ 1740, $CH_3C=O$; 1240; C–O; 960, *trans* CH=CH.
NMR: δ 5.4 (m, 2H); 4.2 (t, 2H, J = 8 Hz); 2.3–1.9 (m, 7H); 1.8–1.2 (m, 16H); 0.95 (t, 3H).
MS: *m/e* (16 eV) 254 (0.6) (M); 194 (100) (M–HOAc).
Anal. calc. for $C_{16}H_{30}O_2$: C 75.54, H 11.89; found: C 75.41, H 11.80.

trans-11-Tetradecenal (I)

Oxidation of IX by the method of Sharpless and Akashi (1975) and subsequent purification by preparative TLC (eluant, benzene) yielded I in 85% isolated yield. Analysis of I by HPLC and VPC showed more than 98% of I was in the trans form.

n_D: 1.4495.
IR: 2720, O=C–H; 1720, C=O; 960, trans CH=CH.

NMR: δ 9.7 (t, 1H, J = 0.5 Hz); 5.4 (m, 2H); 2.4 (m, 2H); 2.1–1.8 (m, 4H); 1.8–1.2 (m, 14H).
MS: *m/e* 210 (4.3) (M); 192 (16.2); 44 (100).
Anal: calc. for $C_{14}H_{26}O$: C 79.94, H 12.46; found; C 80.09, H 12.31.

Polymer-Bound Propargylic Alcohol XI)

To a solution of 50 mmol of 1-lithio-1-butyne in 80 ml of tetrahydrofuran (THF) was added 10 g of polymer-bound aldehyde X, derived from IV containing 0.60 mmol/g of III, and 80 ml of hexamethylphosphoric triamide (HMPT). The mixture was stirred under an argon atmosphere overnight, hydrolyzed with water, filtered, washed, and dried as previously described (Leznoff et al., 1977). The IR spectrum of XI did not exhibit an absorption band at 1720 cm^{-1} caused by unreacted X.

11-Tetradecyne-1,10-diol

Acid hydrolysis (Leznoff et al., 1977) of 1.0 g of XI and purification of the product by preparative TLC (eluant, ether–benzene, 1:1) gave 54 mg (0.31 mmol) of 1,10-decanediol (III) (R_f, 0.25) and 30 mg (0.13 mmol) of 11-tetradecyne-1,10-diol (R_f, 0.70) in 21% yield, or 44% conversion from III.

n_D: 1.4450.
IR: γ 3400, –OH; 2320, C≡C.
NMR: δ 4.2 (m, 1H); 3.6 (t, 2H, J = 8 Hz); 3.0 (s, broad, 2H); 2.4–1.9 (m, 4H); 1.8–1.2 (m, 14H); 0.95 (t, 3H, J = 8 Hz).
MS: *m/e* 226 (1) (M); 225 (4); 224 (5); 208 (14) (M-H_2O); 43 (100).
Anal: calc. for $C_{14}H_{26}O_2$; C 74.29, H 11.58; found C 74.36, H 11.46.

Polymer-Bound trans-Allylic Alcohol XII

In a typical experiment, 2 g of XI was added to a solution of 25 mmol of $LiAlH_4$ or $NaAlH_2(OCH_2CH_2OCH_3)_2$ in 40 ml of THF or benzene, respectively. The mixture was heated under reflux under an argon atmosphere overnight, cooled, filtered under anhydrous conditions (Fyles and Leznoff, 1976), and washed with three 25-ml portions of dry solvent. Polymer XII was transferred to a Büchner funnel, washed, and dried (Leznoff et al., 1977). The IR spectrum of XII was uninformative.

Since acidic cleavage of XII yielded a complex mixture of products, XII was directly converted to a mesylate (Leznoff et al., 1977), reduced to polymer-bound *trans*-alkene as described for the formation of XII, and

cleaved with acid to yield, upon purification by TLC, some recovered III, alkynol VIII in 28% yield, and the desired IX in 15% yield. Analysis of IX as its acetate by HPLC and VPC, revealed a cis–trans ratio of only 1:9 in the best instance.

Polymer-Bound trans-Alkene XIV

To a solution of 5 mmol of *trans*-1-lithio-1-butene at −80° was added 1.2 g of XIII (Leznoff et al., 1977), containing 0.3 mmol/g of Br, and 30 ml of HMPT. The resulting slurry was allowed to warm to room temperature over 1 h, then warmed to 60° and stirred overnight. The polymer was filtered, washed (Leznoff et al., 1977), and air dried. The IR spectrum of XIV was uninformative.

Acid cleavage of XIV yielded IX, as before (Leznoff et al., 1977). Analysis of IX, as its acetate, by HPLC and VPC revealed a cis–trans ratio of 1:3.

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THE ROLE OF PHYTOECDYSTEROIDS IN BRACKEN FERN, *Pteridium aquilinum* (L.) KUHN AS A DEFENSE AGAINST PHYTOPHAGOUS INSECT ATTACK

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Abstract—Analysis of green bracken fronds collected during July, August, and October, 1975, for phytoecdysteroids showed that these compounds occur in only trace amounts (0.25–0.53 μg/kg fresh weight [FW]). The effect of ecdysteroids on the feeding behavior of seven species of insect showed that four species were deterred at ecdysteroid concentrations at or above 60 mg/kg FW diet; one species of insect at 6 mg/kg or above, and two species which were not affected at the higher concentrations. It was concluded that the levels of phytoecdysteroids in bracken would not deter insects from feeding on the plant. The previously published data relevant to the possible role of phytoecdysteroids as defense compounds are also discussed.

Key Words—Bracken fern, *Pteridium aquilinum*, phytoecdysteroids, insect feeding deterrents.

INTRODUCTION

Since the isolation of the first insect molting hormone from a plant (Nakanishi et al., 1966), 37 different phytoecdysteroids have been found in 83 different families of plants (Hikino and Takemoto, 1974). However, the role of phytoecdysteroids in plants is still unknown. Reports that ecdysteroids possessed physiological activity in plants similar to the endogenous plant growth regulators, the gibberellins (Carlisle et al., 1963; Matsuoka et al., 1969), have not been substantiated by work with pure ecdysteroids (Hendrix and Jones,

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1972); nor have ecdysteroids been shown to influence flowering (Jacobs and Suthers, 1971) or spore germination (McMorris and Voeller, 1971).

It is well known that ecdysteroids have marked physiological effects in insects. Consequently it has been suggested, not unreasonably, that phytoecdysteroids may play a part in the defense of the plant against attack by phytophagous insects (Galbraith and Horn, 1966; Staal, 1967). The present communication examines this hypothesis for phytoecdysteroids of the common bracken fern [*Pteridium aquilinum* (L.) Kuhn] (Kaplanis et al., 1967; Takemoto et al., 1968; Hikino et al., 1969; McMorris and Voeller, 1971). The possible defensive role of phytoecdysteroids as feeding deterrents (Ma, 1972) is also examined, and the role of phytoecdysteroids as "antiherbivore" compounds is discussed in more general terms.

If phytoecdysteroids are to be effective antiherbivore compounds it must be demonstrated that insects encountering the plant could either ingest a physiologically significant dose or be significantly deterred from feeding. A comparison of phytoecdysteroid levels in bracken with the efficacy of ecdysteroids in disrupting development, reproduction, survivorship, and feeding behavior of insects permits an evaluation of the role of phytoecdysteroids in this plant. This work has been carried out in our laboratory as part of a study on the chemical defenses of bracken against insect attack (Lawton, 1976).

METHODS AND MATERIALS

Ecdysteroid Standards

Ecdysone and ecdysterone were obtained from Simes S.P.A., Milan; Sigma Ltd; and kindly given by Dr. H.H. Rees, Liverpool University. Tritiated ecdysterone was obtained from N.E.M. Gmbh. and was purified before use by thin-layer chromatography on Merck silica gel HF_{254} run in toluene/methanol (80:20). The ecdysteroids were eluted from the silica gel with methanol.

Solvents and Reagents

All solvents and reagents were analytical grade commercial. Trimethyl silyl immidazole (TSIM) was obtained from Pierce Chemicals and was distilled and stored in small sealed vials under nitrogen.

Ecdysteroid Extraction of Bracken

The extraction was a modification of the method of Morgan and Poole (1976). Freeze-dried bracken fronds (60 g) were homogenized in a blender with cold methanol (1.2 liters) and then extracted by stirring for 24 hr at 4°C.

The homogenate was filtered through glass-fiber paper, the filtrate divided into six equal samples (200 ml) and rotary-evaporated to dryness at 40°C. To two samples was added 200 μl of a 0.1-mg/ml solution of ecdysterone in methanol as the first internal standard. Each of the six samples was then partitioned between a saturated mixture of methanol/water (25:75) (100 ml) and petroleum ether (60–80) (100 ml), and then back-extracted with a further 50 ml of each solvent. The aqueous methanolic layer was retained, rotary-evaporated to dryness at 40°C, and the residue partitioned between a saturated mixture of butanol (50 ml)/water (50 ml) and then back-extracted with a further 25 ml of each solvent. The butanolic layer was retained, rotary evaporated to dryness at 40°C, the residue partitioned between a saturated mixture of ethyl acetate (15 ml)/water (15 ml), and back-extracted with a further 10 ml of each solvent. The aqueous layer was retained and rotary evaporated to dryness at 40°C. To a further two samples was added 200 μl of a 0.1 mg/ml solution of ecdysterone in methanol as the second internal standard. Three samples, one without internal standards and one each of first and second internal standards, were then retained for analysis by radioimmunoassay (RIA). Each of the remaining 3 samples was reacted with TSIM (200 μl) for 6 hr at 100°C in sealed reaction vials. The derivatized extracts were then diluted with toluene (300 μl) and run through a 2.0×0.6-cm diameter Biosil column (100–200 mesh) using ethyl acetate/toluene (30:70) as eluant (1 ml). The eluant was collected, blown down to dryness under nitrogen, and resuspended in toluene (200 μl) for gas–liquid chromatography.

Gas–Liquid Chromatography (GLC)

GLC was carried out on a Pye series 104 chromatograph fitted with a Pye GCV (nickel^{63}) electron capture (EC) detector. A 1 m 0V-17 (1.5%) column of theoretical plate efficiency 1550 plates/meter was used (column was 3% filter coated 0V-17 in toluene on Gaschrom Q 100–120 mesh).

Settings were as follows: EC amplifier 9 mV, detector oven 300°C, column oven 278°C, column head 290°C, carrier gas nitrogen 85 ml/min, injection volume 1 μl. Detector response, from calibrations using standard ecdysterone and ecdysone, was linear over the range used. The EC GLC was connected to a Varian CDS 101 integrator. Calculations of ecdysteroid content and recoveries were determined using internal and external ecdysterone standards.

The use of dual internal standards permitted the accurate measurement and reproducibility for any one sample, although recoveries between different monthly samples varied considerably due to differing chemical content over the growing season.

Radioimmunoassay (RIA)

RIA was kindly carried out for us by Dr. D. Whitehead, University of Bristol, using antisera made at Bristol. The method was that of Borst and O'Connor (1972). Levels of ecdysterone and ecdysone as total ecdysteroids were determined using internal and external standards.

Rearing and Collection of Insects

The insects chosen for study embraced representatives of three major orders (*Lepidoptera*, *Orthoptera*, and *Coleoptera*) and showed different degrees of foodplant specificity. They also varied in the extent to which they were likely to encounter bracken in their natural habitat. The ease of rearing or collecting each species was also an important criterion of selection since experiments required a large stock of insects. All experimental insects were reared or maintained on their natural food-plants.

Schistocerca gregaria (Forsk.) (Orthoptera) (polyphagous feeder on Graminae and other plants, unlikely to encounter bracken in its natural environment). Seven-day-old adults were obtained from breeding stock maintained at York on 4-week-old *Zea mays* (var. Kelvedon Glory). The adults were maintained in cages for a further 7 days on *Zea mays* at $28 \pm 3°C$, $45 \pm 10\%$ relative humidity on a 14 hr/10 hr light/dark cycle. Fourteen-day-old mixed-sex populations were used for experiments. *Zea mays* was grown in the laboratory by standard procedures.

Chilo partellus (Swinhoe) (Lepidoptera) (relatively oligophagous feeder on rice, maize, and related species, unlikely to encounter bracken in its natural environment). Eggs were kindly provided by Dr. R. Roome, Centre for Overseas Pest Research, London. Breeding stock were hatched out onto 7-day-old *Zea mays* and maintained on the maize for 7 days before transfer to an artificial diet based on the diet of Keaster and Harrendorf (1965), modified by Roome (1975), until pupation. Pupae were transferred to cages. Adults were kept in cages and fed on 10% aqueous sucrose solution in small vials stoppered with cotton wool. Eggs were laid on maize leaves or on the greaseproof paper lining of the cage. Experimental stock was reared from egg to 5th instar on *Zea mays* in moist perforated sandwich boxes. Rearing conditions were $26 \pm 3°C$, $60 \pm 10\%$ relative humidity on a 14 hr/10 hr light/dark cycle. Second-day 5th instar larvae were used for experiments.

Spodoptera littoralis (Boisduval) (Lepidoptera) (polyphagous on a wide variety of herbaceous plants, unlikely to encounter bracken in its natural environment). Eggs were kindly provided by Dr. P. Ellis, Centre for Overseas Pest Research, London. Eggs were hatched on *Phaseolus vulgaris* (var. Canadian Wonder) in moist sandwich boxes and maintained on fresh leaves until

pupation. Pupae were transferred to cages. Adults were fed on 10% aqueous sucrose/honey solution in small vials stoppered with cotton wool. Eggs were laid on bean leaves. Rearing conditions were 26±3°C, 60±10% relative humidity on a 14 hr/10 hr light/dark cycle. Second-day 5th instar larvae were used for experiments. *Phaseolus vulgaris* was grown under laboratory conditions by standard procedures.

Pieris brassicae (L.) (Lepidoptera) (cruciferous feeder, likely to occasionally encounter bracken in its natural environment). Eggs were obtained from Philip Harris Biological Ltd. and hatched out on *Brassica oleracea* (Winter White or Spring varieties, depending on time of year). Larvae were maintained in moist sandwich boxes until pupation. Pupae were transferred to cages. Adults were fed on 10% aqueous sucrose solution in artificial flowers made from small vials and blue paper. Adult females oviposited on cabbage leaves. Rearing conditions were 23±3°C on a 16 hr/10 hr light/dark cycle at 50±10% relative humidity. Second-day 5th instar larvae were used for experiments. *Brassica oleracea* was obtained locally.

Hyponomeuta euonymella (L.) (Lepidoptera) (monophagous on *Prunus padus*, bird cherry; occurs in environment commonly associated with bracken). Early 5th instars were collected from *Prunus padus* trees recently defoliated by heavy infestation of the larvae. The trees were growing adjacent to bracken on hills close to Huddersfield, Yorkshire (SK 039171), and many of the larvae had left the defoliated trees and moved on to adjacent vegetation. There was no evidence that any of the many species of surrounding vegetation, including bracken, had been partially eaten, with the exception of *Vaccinium myrtilis* which had some larval damage. Larvae were maintained on bird cherry leaves at 23±3°C on a 14 hr/10 hr light/dark cycle at 40% relative humidity. Late 5th instar larvae were used for experiments.

Phyllobius argentatus (L.) (Coleoptera) and *Phyllobius pyri* (L.) (Coleoptera) (oligophagous on *Betula pubescens*; occurs in environments often closely associated with bracken). The weevils were collected as adults of indeterminate age by beating of *Betula pubescens* trees adjacent to a bracken stand on Skipwith Common (SE 657377) and maintained on birch leaves at 23±3°C on a 14 hr/10 hr light/dark cycle at 40% relative humidity. Indeterminate age adults of both sexes were used for experiments.

Collection of Plant Material

Pteridium aquilinum (L. Kuhn) var. *aquilinum*. Green fronds were collected monthly throughout the growing season (May–October 1975) from Skipwith Common, 15 km south of York (SE 657377) adjacent to the study area used by Lawton (1976). Primary pinnae (fronds) were removed from the rachis and stored in the deep freeze before freeze-drying.

Feeding Trials

These were carried out using petri dishes 4.8 cm in diameter for all species but *Schistocerca gregaria*, where small perforated sandwich boxes were used (17.5 × 11.2 × 5.8 cm). Each feeding arena contained a moist filter paper and a single piece of food material, either treated or control. One insect larva or adult, depending on species, was introduced to each arena. Food material was dipped into methanol (5 ml) control or methanolic solutions of ecdysteroids (5 ml) for 1 min and then air-dried. The following food materials were used for each species:

Pieris brassicae. 1.4-cm-diam disks of *Brassica oleracea* (Winter or Spring varieties) of uniform thickness punched from intervein areas of the leaves.

Chilo partellus. 1.0-cm-diam disks of *Zea mays* (Kelvedon Glory) punched from the center of 28-day-old leaves.

Schistocerca gregaria. 5 × 1.2–1.4-cm squares of 28-day-old *Zea mays* (Kelvedon Glory) cut from the whole leaf. (For convenience, this square is referred to in the same way as the other food material, as a disk.)

Phyllobius argentatus and *Phyllobius pyri.* 1.4-cm-diam disks of *Betula pubescens* of uniform thickness punched from the edges of the leaves.

Hyponomeuta euonymella. 1.4-cm-diam disks of *Prunus padus* of uniform thickness punched from the edges of the leaves.

Spodoptera littoralis. 1.4-cm-diam disks of *Phaseolus vulgaris* of uniform thickness punched from the edges of the leaves.

Feeding trials were carried out at the rearing temperature of the insect and at 16 klx illumination. All feeding trials were commenced at the same time of day (9:00 AM). Control and treated disks were each replicated 20 times. Larvae or adults were only used once for each experiment. The cumulative percentage eaten of each replicated control and treated disk was then recorded at hourly intervals up to 8 or 24 hr, depending on the species. Visual assessment of the amount eaten was found to be accurate to $\pm 5\%$ by using photographic enlargements of a random selection of disks measured on mm^2 grid paper. The amount of ecdysteroid introduced onto the surface of the treated leaf disks was calculated by weighing them and by measurement of the incorporation of tritiated ecdysterone.

Data Treatment

The cumulative percentages eaten from each disk were arcsined, and the means and 95% confidence intervals for controls and treatments plotted against the time since the start of the experiment. The area under a "treatment" curve compared with that under its appropriate control then provided a

simple measure of the effect of the treatment. This effect was expressed as the C/E ratio, which is defined as the area under the cumulative mean curve for the control disks, divided by that for the experimental (treated) disks. Obviously if the treatment had no effect, the C/E ratio $= 1$; if it stimulated feeding, $C/E < 1$, and if it inhibited feeding, $C/E > 1$. The problem is to decide whether inhibition (or stimulation) is statistically significant. This is not simple, because cumulative amounts eaten yield a sequence of measures in which later data points are not independent of earlier ones, and there are no standard procedures available in the literature for dealing with this problem. We adopted the following method. Suppose a treatment appeared to inhibit feeding ($C/E > 1$); this inhibition was taken to be significant when the cumulative area defined by the upper 95% confidence intervals of the experimental curve no longer overlapped the cumulative area defined by the lower 95% confidence intervals of the control curve. (Upper and lower bounds would be reversed if stimulation was suspected.) This, like all statistical tests of significance, was arbitrary; it had the advantage that it provided a simple standard criterion against which to judge the effects of a treatment and was, if anything, conservative in its recognition of significant results. The derived C/E ratio itself was independent of the duration of the different experiments, the different feeding rates of controls in the different experiments, the different feeding rates of each species in the different experiments, and the different areas of plant material provided initially for these species. The method has the advantage over single-time measurements of differences in amounts eaten because it measures the kinetics of the feeding behavior. Consequently the C/E ratio provides a comparative index for measuring the effects of chemicals, in this case ecdysteroids, on the feeding behavior of insects.

RESULTS

The analysis of the major ecdysteroids in bracken, ecdysone[2] and ecdysterone, proved difficult due to the low levels which occurred in fronds harvested in July and August 1975. The levels of these compounds were below the limit of the EC GLC quantitation (< 0.25 μg/kg ecdysterone fresh weight at 100% recovery) and subsequent RIA analysis confirmed these low values (Table 2). In fronds harvested in October 1975, ecdysterone was detectable by EC GLC at 53 μg/kg fresh weight, this value again being confirmed by RIA analysis (Table 2). It was evident from these results that insects ingesting fronds of Skipwith bracken in summer 1975 were unlikely to be exposed to levels of phytoecdysteroids that would significantly affect their

[2] For IUPAC nomenclature see Table 1.

TABLE 1. IUPAC NOMENCLATURE OF ECDYSTEROIDS REFERRED TO IN THIS PAPER

Ecdysone (α-ecdysone):
2β, 3β, 14α, 22α, 25, pentahydroxy-5β-cholest-7-en-6-one
Ecdysterone (β-ecdysone, crustecdysone, 20-hydroxyecdysone, isoinokosterone, polypodine A, commisterone):
2β, 3β, 14α, 20β, 22α, 25, hexahydroxy-5β-cholest-7-en-6-one
Inokosterone (callinecdysone A):
2β, 3β, 14α, 20β, 22α, 26, hexahydroxy-5β-cholest-7-en-6-one
Cyasterone:
2β, 3β, 14α, 20β, 22α, pentahydroxy-5β-stigmast-7-en-6-one-26-oic-acid-γ-lactone
Ponasteroside A:
2β, 14α, 20β, 22α, tetrahydroxy-5β-cholest-7-en-6-one-3β, D-glucose
Makisterone A (callinecdysone B):
2β, 3β, 14α, 20β, 22α, 25, hexahydroxy-5β-ergost-7-en-6-one
Makisterone B:
2β, 3β, 14α, 20β, 22α, 27, hexahydroxy-5β-ergost-7-en-6-one
Podecdysone A (makisterone C, lemmasterone):
2β, 3β, 14α, 20β, 22α, 25, hexahydroxy-5β-stigmast-7-en-6-one
Makisterone D:
2β, 3β, 14α, 20β, 22α, 29, hexahydroxy-5β-stigmast-7-en-6-one
Polypodine B (5β-hydroxyecdysterone):
2β, 3β, 5β, 14α, 20β, 22α, 25, heptahydroxycholest-7-en-6-one
Deoxycrustecdysone (deoxyecdysterone):
3β, 14α, 20β, 22α, 25, pentahydroxy-5β-cholest-7-en-6-one
Posterone:
2β, 3β, 14α, trihydroxy-5β-pregna-7-en-20-en-6-one
Ponasterone A:
2β, 3β, 14α, 20β, 22α, pentahydroxy-5β-cholest-7-en-6-one
Ponasterone B:
2α, 3α, 14α, 20β, 22α, pentahydroxy-5β-cholest-7-en-6-one
Ponasterone C:
2β, 3β, 5β, 14α, 20β, 22α, 24, heptahydroxycholest-7-en-6-one
Ponasterone D:
Not yet identified; probably lacks enone (Nakanishi et al., 1974)
Viticosterone E:
2β, 3β, 14α, 20β, 22α, pentahydroxy-5β-cholest-7-en-6-one-25 acetoxyl
Ajugasterone C:
2β, 3β, 11α, 14α, 20β, 22, hexahydroxycholest-7-en-6-one

development, reproduction, or survivorship (see Discussion). The low levels of the major ecdysteroids of bracken did not encourage us to analyze the minor ecdysteroids of the plant (Hikino et al., 1969; Kaplanis et al., 1967).

There was, however, a possibility that the higher levels of ecdysteroids found in October fronds were sufficient to act as feeding deterrents to insects, as ecdysteroids have been shown to act in this manner at lower concentration

TABLE 2. LEVELS OF ECDYSTEROIDS DETERMINED IN GREEN SKIPWITH BRACKEN FRONDS[a]

Month (1975)	Ecdysteroid	Conc. (EC–GLC) (μg/kg FW)	Ecdysteroid	Conc. (RIA) (μg/kg FW)
July	Total	<0.25	Total	0.07
August	Total	<0.25	Total	0.22
October	Ecdysterone	53	Total	28–105

[a] Weights expressed are per kg fresh weight. RIA levels are total ecdysteroids as ecdysone and ecdysterone

than those required for developmental effects (Ma, 1972; Schoonhoven and Derksen-Koppers, 1973). Thus, to investigate the possibility of phytoecdysteroids in bracken acting as feeding deterrents, the effect of ecdysterone and ecdysone on the feeding behavior of several phytophagous insects was studied (Table 3). Ecdysterone had no deterrent effect on the feeding of *Schistocerca gregaria*, *Hyponomeuta euonymella*, or *Spodoptera littoralis* at concentrations equivalent to 50–70 mg/kg fresh weight diet. *Pieris brassicae* was deterred from feeding at concentrations above 5 mg ecdysterone or ecdysone/kg fresh weight diet. *Chilo partellus*, *Phyllobius pyri*, and *Phyllobius argentatus* were deterred from feeding at ecdysterone concentrations, in the diet, exceeding 50–70 mg/kg fresh weight. Thus it was unlikely that the levels of ecdysterone found in Skipwith bracken in summer 1975 could influence the feeding behavior of the seven insects studied (see Discussion).

DISCUSSION

A number of phytochemicals induce physiological and biochemical responses in other organisms, and it is tempting to predict a role for such phytochemicals on the basis of their known physiological or biochemical activity. However, such a prediction is only realistic if it can be clearly shown that the chemicals occur in the plant in amounts sufficient to provide a physiologically active dose.

The literature on the occurrence of phytoecdysteroids in plants is extensive. The summary in Table 4 shows that the concentration of these compounds in plants varies considerably in the range 1–2000 mg/kg fresh weight, as does the type of ecdysteroid, although ecdysterone predominates. Some authors have often omitted the season of sampling of the plant, a crucial factor in determining the involvement of these compounds in defense. The more

TABLE 3. FEEDING DETERRENT ACTIVITY OF ECDYSTEROIDS[a]

Insect species	Food material	Ecdysteroid	Concentration (mg/kg FW)	C/E ratio	95% significance
Pieris brassicae	*Brassica oleracea*	Ecdysone	55	169.5	S
			5.5	15.1	S
			0.55	1.01	NS
		Ecdysterone	61	16.6	S
			6.1	5.5	S
			0.61	0.89	NS
Chilo partellus	*Zea mays*	Ecdysterone	60	3.16	S
			6	1.21	NS
Phyllobius pyri	*Betula pubescens*	Ecdysterone	59.7	2.65	S
			5.97	1.3	NS
Phyllobius argentatus	*Betula pubescens*	Ecdysterone	59.7	2.72	S
			5.97	1.01	NS
Schistocerca gregaria	*Zea mays*	Ecdysterone	52	1.51	NS
Spodoptera littoralis	*Phaseolus vulgaris*	Ecdysterone	60	0.70	NS
Hyponomeuta euonymella	*Prunus padus*	Ecdysterone	68	0.92	NS

[a] Concentrations of ecdysteroids are expressed in mg/kg fresh weight. Results that show significant deterrency compared to control at the 95% level are marked as S; those not significant in comparison to controls are marked as NS.

limited literature on the effects of ingested ecdysteroids on insect reproduction, development, and survivorship (summarized in Table 5) (reviewed in Matsuoka et al., 1969; Ellis et al., 1970) shows that species differ greatly in their sensitivity to ecdysteroids in the diet, but that levels greater than 500 mg/kg fresh weight diet may be hazardous. There are, however, examples of insects failing to respond at very high concentrations (Robbins et al., 1968; Kaplanis et al., 1968).

We conclude that the levels of ecdysterone measured in Skipwith bracken are 2×10^6 to 9×10^3 times lower than the lowest levels which would be required to affect development, reproduction, or survivorship of insects feeding on the plant. Similarly, the levels reported by Kaplanis et al. (1967) are 5×10^2 times lower than the lowest levels required. Furthermore, the higher levels of ecdysterone found in autumnal bracken occur at a time when the plant has no young growing aerial parts to protect and at a time when it is effectively at the "end of the season" for phytophagous insects (Lawton, 1976).

The fact that ecdysterone is the most common phytoecdysteroid is surprising if phytoecdysteroids were elaborated by the plant to influence insect development, reproduction or survival. Insects possess enzymes capable of degrading endogenous and exogenous ecdysteroids (Hikino and Hikino, 1970; King and Siddall, 1974; Nigg et al., 1974; Hikino et al., 1975), and this may explain the tolerance of insects to high ecdysteroids in their diet. (It may also be pertinent to note that predatory insects have presumably had to cope with ecdysteroids in their diets throughout their evolutionary history.) Thus if phytoecdysteroids were evolved as defense compounds, one might expect selective pressures on plants to elaborate physiologically active analogs less susceptible to degradation by the insect. Compounds such as cyasterone, inokosterone and ponasterone A are more effective, at lower concentrations, than ecdysterone in disrupting insect development, reproduction, and survival (Robbins, *et al.*, 1970), and yet relatively few plants have evolved to produce these compounds. It should also be noted that most phytoecdysteroids have been found in plants by biological screening for compounds with ecdysteroid-like activity (Imai et al., 1969a; Kaplanis et al., 1966). Consequently studies have been biased towards finding ecdysteroids with biological activity. Some biologically screened plants might contain high levels of nonphysiologically active ecdysteroid-like molecules (which would not be detected biologically). If this is the case, it would then be necessary to reconsider the hypothesis that plants evolved phytoecdysteroids as developmental or reproductive defences.

We suggest that the production of phytoecdysteroids for the purpose of influencing insect development or reproduction would, at best, be somewhat ineffective. Even if the insect attacking the plant was a susceptible species and at a susceptible stage of its life cycle, the consequences of ingesting an

TABLE 4. ECDYSTEROID LEVELS DETERMINED FOR PLANTS AS REPORTED IN THE LITERATURE

Species	Part of plant	Season of sampling	Ecdysteroid	FW content[a] (mg/kg)	Total ecdysteroid[b] content (mg/kg FW)	Reference
Torreya nucifera	Lvs[c]		Ecdysterone[d]	0.25	0.25	Staal, 1967
Pteridum aquilinum	(old) Fr	Jan.	Ecdysone	0.113	0.36	Kaplanis et al., 1967
			Ecdysterone	0.25		
Diplazium donianum	Rh		Makisterone A,B	0.075		
			Makisterone D	0.27	0.496	Hikino et al., 1977
	Fr		2 unidentified	0.076		
Morus sp.	Lvs		Inokosterone	2.5	2.75	Takemoto et al., 1967[a]
			Ecdysterone	0.25		
Podocarpus sospigliosii and P. univalis	Lvs		Ecdysterone[d]	3.75	3.75	Staal, 1967
Ajuga chia	Lvs, St	May	Cyasterone	18.75	18.75	Ikan and Ravid, 1971[a]
Trillium smallii	Rh		Ecdysterone	20	20	Imai et al., 1969[b]
T. schonoskii	Rh		Ecdysterone	25	25	Imai et al., 1969[b]
Blechum minus	Fr		Ecdysterone	25	32.5	Chong et al., 1970
			Deoxycrustecdysone	7.5		
Bosea yervamora	Rt		Ecdysterone	50	50	Takemoto et al., 1967[b]
Ajuga incisa	Wh		Ecdysterone	30	50	Imai et al., 1969[b]
			Cyasterone	20		
Achyranthes fauriei	Rt		Ecdysterone	25	50	Takemoto et al., 1967[c]
			Inokosterone			
Ajuga decumbens	Wh		Ecdysterone	30		Imai et al., 1969[b]
			Cyasterone	20	53.75	Imai et al., 1969[b]
	Lvs		Ajugasterone C	3.75		Imai et al., 1969[c]
Achyranthes obtusifolia	Rt		Ecdysterone	62.5	62.5	Takemoto et al., 1967[d]
Taxus canadensis	Lvs		Ecdysterone[d]	87.5	87.5	Staal, 1967

Taxus wallichiana	Lvs		Ecdysterone[d]	93.75	93.75	Staal, 1967
Ajuga iva	Wh	May	Ecdysterone	120	120	Ikan and Ravid, 1971[b]
Achyranthes rubrofusca	Rt		Ecdysterone	122.5	122.5	Takemoto et al., 1967[e]
Podocarpus elatus	Wd		Ecdysterone	2.5		Galbraith and Horn, 1969
	Bk		Ecdysterone	125	130.25	Galbraith and Horn, 1969
			Podecydsone A	0.25		Galbraith and Horn, 1969
	Lvs		Ecdysterone[d]	2.5		Staal, 1967
Stachyrus praecox	Bk		Ecdysterone	150	150	Imai et al., 1969[b]
Saxegothaea conspicua	Lvs		Ecdysterone[d]	175	175	Staal, 1967
Podocarpus andinus	Lvs		Ecdysterone[d]	200	200	Staal, 1967
Ajuga nipponensis	Wh		Ecdysterone	200	230	Imai et al., 1969[b]
			Cyasterone	30		
Taxus baccatta	Lvs		Ecdysterone	20		Hoffmeister et al., 1967
			Ecdysterone[d]	200		Staal, 1967
	Wd		Ecdysterone[d]	6.25	275	Staal, 1967
	Young buds		Ecdysterone[d]	6.25		Staal, 1967
			Ecdysterone[d]	187.5		Staal, 1967
Podocarpus chinensis	Lvs		Ponasterone A	300	300	Imai et al., 1967
Taxus baccatta cv "Amersfoort"	Lvs		Ecdysterone[d]	375	375	Staal, 1967
Podocarpus macrophyllus	Lvs		Ponasterone A	372.1		Imai et al., 1967
			Ecdysterone	6.97		Imai et al., 1967
	Lvs		Ecdysterone[d]	37.5	412.85	Staal, 1967
	Lvs	Summer	Makisterone A	2.51		Imai et al., 1967
			Makisterone B,D, Podecdysone A	0.25		Imai et al., 1967
			Ponasterone A	125		
			Ecdysterone	25		Imai et al., 1968
			Ecdysterone	1.75		Imai et al., 1967

Species	Part of plant	Season of sampling	Ecdysteroid	FW content[a] (mg/kg)	Total ecdysteroid[b] content (mg/kg FW)	Reference
Podocarpus nakaii	Lvs		Ponasterone A	395.75		Nakanishi et al., 1968
			Ponasterone B	16.75		Nakanishi et al., 1968
			Ponasterone C	4		Nakanishi et al., 1968
	Lvs		Ponasterone A	104	417.5	Nakanishi et al., 1966
			Ponasterone B	2.5		Nakanishi et al., 1966
			Ponasterone C	26		Nakanishi et al., 1966
			Ponasterone D	1		Nakanishi et al., 1966
Podocarpus neriifolius	Wd		Ecdysterone[d]	81.25		Staal, 1967
	Lvs		Ecdysterone	187.5	443.75	Horn, 1967
			Ponasterone A	175		Horn, 1967
Cyathula capitata	Rt		Cyasterone	52.5		Hikino et al., 1970
			Posterone	0.075		Hikino et al., 1970
	Lvs, Rt		Cyasterone	481.25	481.325	Miyazaki and Kuwafuji, 1970
	St		Cyasterone	50		Takemoto et al., 1967[f]
Taxus cuspidata	Lvs		Ponasterone A	454.5	542	Imai et al., 1967
			Ecdysterone[d]	87.5		Staal, 1967
Helleborus abchasicus	Lvs, Fl, St	Summer	Ecdysterone	600	600	Hardman and Benjamin, 1976
Serratula inermis	Lvs, Fl, St	Flowering	Ecdysterone	625	650	Yatsyuk and Segel, 1970
		Season	Ecdysterone	25		
Ajuga japonica	Lvs		Ecdysterone	500		
			Cyasterone	145	670	Imai et al., 1969[e]
			Ajugasterone C	25		
Helleborus viridis	Lvs, Fl, St	Summer	Ecdysterone	525	675	Hardman and Benjamin, 1976
			Polypodine B	150		
H. dumentorum ssp atrorubens	Lvs, Fl, St	Summer	Ecdysterone	525	800	Hardman and Benjamin, 1976
			Polypodine B	275		

Vitex megapotamica	Lvs		Ecdysterone	220	845	Rimpler and Shultz, 1967
			Viticosterone E	625		
Helleborus guttatus	Lvs, Fl, St	Summer	Ecdysterone	695	850	Hardman and Benjamin, 1976
			Polypodine B	175		
H. atrorubens	Lvs, Fl, St	Summer	Ecdysterone	625	1025	Hardman and Benjamin, 1976
			Polypodine B	400		
H. bocconei	Lvs, Fl, St	Summer	Ecdysterone	650	1025	Hardman and Benjamin, 1976
			Polypodine B	375		
H. cyclophyllus	Lvs, Fl, St	Summer	Ecdysterone	675	1075	Hardman and Benjamin, 1976
			Polypodine B	400		
H. dumentorum	Lvs, Fl, St	Summer	Ecdysterone	725	1075	Hardman and Benjamin, 1976
			Polypodine B	350		
H. multifidus ssp serbicus	Lvs, Fl, St	Summer	Ecdysterone	900	1125	Hardman and Benjamin, 1976
			Polypodine B	225		
H. orientalis Hybrid	Lvs, Fl, St	Summer	Ecdysterone	1125	1425	Hardman and Benjamin, 1976
			Polypodine B	300		
Cyanotis vaga	Lvs		Ecdysterone	1750	1750	Santos et al., 1970
						Santos et al., 1972
Polypodium vulgare	Rh		Ecdysone	5		Heinrich and Hoffmeister, 1967
			Ecdysterone	175		Heinrich and Hoffmeister, 1967
	Rh		Ecdysterone	>2500	>2600	Jizba et al., 1967
	Rh	April	Ecdysterone	200		Jizba and Herout, 1967
			Polypodine B	92.5		Jizba and Herout, 1967

[a] Ecdysteroid content is expressed in mg/kg fresh weight. Those levels recorded as dry weight have been converted to fresh weight on the assumption that plants contain approximately 75% water when freshly harvested. This conversion is to aid comparison with Table 5. Where conflicting levels of ecdysteroids have been found, the highest level is recorded in the table.

[b] Total ecdysteroids have been calculated from the sum of highest recorded levels for each part of the plant and only express total content as so far determined by other workers.

[c] Lvs = leaves; St = stem; B = bark; Fr = frond; Rh = rhizome; Rt = root; Wh = whole plant; Wd = wood; Fl = flowers.

[d] These results are expressed as ecdysterone moulting hormone equivalents and may not actually represent true ecdysterone content since the bioassay used is non-ecdysteroid-specific.

TABLE 5. MINIMUM ECDYSTEROID CONTENT IN mg/kg FRESH WEIGHT DIET REQUIRED TO INFLUENCE DEVELOPMENT, REPRODUCTION, OR SURVIVORSHIP OF VARIOUS INSECT SPECIES, AS REPORTED IN THE LITERATURE

Species	Ecdysteroid	Conc. in diet (min, dose mg/kg/FW)	Stage and diet	Effects	Reference
Musca domestica	Ponasterone A	150	Larval diet	Reduced pupation and adult emergence	Robbins et al., 1970
	Cyasterone	150	Larval diet	Reduced pupation and adult emergence	Kaplanis et al., 1968
	Ecdysone, Ponasterone A	1000	Adult ♀ diet	Ovarian inhibition	Robbins et al., 1968
	Inokosterone	2500	Adult ♀ diet	Ovarian inhibition	Robbins et al., 1968
	Cyasterone	500	Adult ♀ diet	Ovarian inhibition	Robbins et al., 1968
Tribolium confusum	Ecdysone, Cyasterone	5000	Larval diet	Inhibition of development	Robbins et al., 1968
	Ponasterone A	500	Larval diet	Inhibition of development	Robbins et al., 1968
	Ecdysterone	10,000	Adult ♀ diet	Inhibition of reproduction	Robbins et al., 1968
Aedes aegypti	Ponasterone A	10,000	Larval water	Inhibition of development	Robbins et al., 1968
Stomoxys calcitrans	Ecdysterone, Ecdysone	1000	Adult ♀ blood meal	Inhibition of ovarian development and hatching, and delayed hatching	Wright and Kaplanis, 1970 Wright et al., 1971
Ornithodorus monbata	Ecdysterone	5	Adult blood meal	Supermoulting, increased egg production and occasional mortality	Mango et al., 1976
	Ponasterone A	5	Adult blood meal		
Bombyx mori	Ponasterone A	100–200	Larval diet (4th instar)	Supermoult and mortality	Nakanishi, 1971
	Ponasterone A Inokosterone, Cyasterone, Ecdysterone	>1.5	Prepupae	Acceleration of onset of spinning and maturation	Matsuoka et al., 1969

Ephestia cautella	Ponasterone A	>>10,000	Larval diet	LD_{50}	Matsuoka et al., 1969
Prodenia litura	Ponasterone A	>>1000	Larval diet	LD_{50}	Matsuoka et al., 1969
Leucania separata	Ponasterone A	>10,000	Larval diet	LD_{50}	Matsuoka et al., 1969
	Ecdysterone	>1000	Larval diet	LD_{50}	Matsuoka et al., 1969
Pieris rapae	Ponasterone A	>>250	Larval diet	LD_{50}	Matsuoka et al., 1969
	Ecdysterone	>>500	Larval diet	LD_{50}	Matsuoka et al., 1969
Blatella germanica	Ponasterone A Ecdysterone	>250	Adult water	LD_{50}	Matsuoka et al., 1969
Drosophila melanogaster	Ecdysone	≃20	Larval diet	Premature moult and pupariation	Fourche, 1967
Kalotermes flavicollis	Ecdysone	0.025μg	Nymphal diet, fed directly	Premature moulting	Lüscher and Karlson, 1958
	Ecdysterone	12μg			
Glossina morsitans	Ecdysterone, Inokosterone	>80	Adult ♀ bloodmeal	Abortion, sterility and abnormal offspring	Whitehead, 1976
	Ponasterone A	>40	Adult ♀ bloodmeal		Whitehead, 1976

efficacious dose of phytoecdysteroids would not be manifest until after the plant had suffered the attack.

An alternative defensive role for phytoecdysteroids is as a nontoxic feeding deterrent. Experiments to test this hypothesis are reported in this paper. Ecdysterone has been shown to inhibit the feeding of larvae of *Pieris brassicae* and *Mamestra brassicae* at 200 mg/kg FW in sucrose solution (Ma, 1972) and to inhibit drinking in adult *Dysdercus koenigii*, *D. fulvoniger*, and *Spilostethus pandrus* at 100 mg/kg FW in the drinking water (Schoonhoven and Derksen-Koppers, 1973). Ability to recognize and avoid ecdysteroids could confer a selective advantage to an insect incapable of catabolizing large amounts of ecdysteroids. Deterrents are advantageous to the plant because it remains uneaten while competitors in the same environment may suffer attack instead. The present work has confirmed that ecdysterone and ecdysone can deter *Pieris brassicae* from feeding (Ma, 1972) and shows that other insects can also be deterred. However, concentrations in excess of 5 mg/kg FW diet are required for *Pieris brassicae*, and other species tested responded only at higher concentrations. For efficient (rather than detectable) inhibition of feeding in *Pieris brassicae*, concentrations in excess of 50 mg/kg fresh weight diet seem to be required. Obviously more work is needed on the deterrent activity of phytoecdysteroids against a wide range of phytophagous insects before one can accurately assess the efficacy of these compounds as natural feeding deterrents. It is quite clear, however, that concentrations of phytoecdysteroids in Skipwith bracken are 2×10^4 to 0.9×10^1 times less than the lowest levels required to deter feeding. These low levels make it extremely unlikely that phytoecdysteroids in bracken fern can act as a feeding deterrent to insect herbivores. In contrast to the inadequacy of ecdysteroids as feeding deterrents, methanolic extracts and fractions of bracken do contain other compounds more effective as feeding deterrents to insects at lower concentrations (Jones, 1977); the presence of these compounds and other potential defenses (Cooper-Driver, 1976; Lawton, 1976) do seem to provide an adequate defense against prospective predators.

In conclusion, we would suggest that the phytoecdysteroids of green bracken fronds occur in insufficient amounts to contribute significantly to defense against insect herbivores. Although plants containing much higher levels of phytoecdysteroids theoretically contain sufficient amounts to disrupt development or inhibit feeding, further careful study is required to determine whether these compounds are able to function efficiently as defenses in the natural habitat. In particular, evidence is required to demonstrate the occurrence of adequate amounts of phytoecdysteroids in the appropriate parts of the plant at the appropriate times throughout the plant's life cycle. It is not sufficient to monitor the plant once at an unspecified season.

We would suggest that the difference in ecdysteroid content between

Skipwith bracken and the bracken used by Kaplanis et al. (1967) may be due to random genetic variation between the two populations, on the grounds that the phytoecdysteroids of bracken are a redundant defense mechanism no longer under intense selection pressure. This variation could be further exaggerated by the reliance of the plant on vegetative rhizomes for most of its reproduction, resulting in the formation of cloned islands.

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BAROMETRIC FLUX

Effects on the Responsiveness of Bark Beetles[1] to Aggregation Attractants[2]

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Abstract—Exposure to artificial changes in atmospheric pressure depressed the responsiveness of bark beetles to their aggregation pheromones. *Scolytus multistriatus* and *Ips pini* held for 30 min in desiccator jars in which the pressure was modified ±25 mm experienced an inverse change when they were removed for laboratory bioassay. Relative response to pheromones among treatment groups of both species was as follows: ambient > increased > decreased > increased and decreased. In one series of 20 bioassays, response of *S. multistriatus* was significantly higher for 9 tests during which no change in atmospheric pressure was detected, as compared to 11 tests during which there was a change in barometric reading. However, in another series of tests with *S. multistriatus* and a series with *I. pini*, no depression in response was associated with natural barometric shifts. Large air bubbles in the foregut (ventriculus) confirmed that both beetle species swallow air in preparation for flight. The hypothesis advanced by other workers, that bark beetles may detect changes in atmospheric pressure by shrinking and swelling of the ventricular air bubbles, is consistent with our observations. Sensitivity to atmospheric pressure fluctuations may be the mechanism by which response in laboratory bioassays is depressed during stormy weather and flight in nature is concentrated in periods of calm air.

Key Words—Coleoptera, Scolytidae, bark beetle, *Scolytus multistriatus*, *Ips pini*, pheromone response, atmospheric pressure, dispersal, air swallowing.

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INTRODUCTION

In our laboratory bioassays of attractants, the European elm bark beetle, *Scolytus multistriatus* (Marsh.), and the pine engraver, *Ips pini* (Say), occasionally appeared subnormally responsive. The weather during days on which poor responsiveness occurred could usually be characterized as gusty or unstable.

Stormy weather was also associated with a low propensity of the Douglas fir beetle, *Dendroctonus pseudotsugae* Hopkins, and the striped ambrosia beetle, *Trypodendron lineatum* (Olivier), to fly in the flight-mill study of Bennett and Borden (1971).

During our studies and those of Bennett and Borden (1971) the temperature, light, humidity, and conditioning of the beetles were held constant. One uncontrolled factor was atmospheric pressure; Bennett and Borden hypothesized that pressure fluctuations associated with unstable weather might inhibit dispersal activities of the beetles and they suggested that these fluctuations are perceived through receptors that monitor variations in the size of an air bubble in the ventriculus of the beetle. Air swallowing that will produce this bubble was shown to be a part of the normal flight or preflight warm-up of *D. pseudotsugae* and *T. lineatum* (Chapman, 1955; Graham, 1961; Atkins, 1966; Bennett and Borden, 1971).

In bioassays with *S. multistriatus*, Elliott (1973) noted an apparent positive correlation between responsiveness and atmospheric pressure in 7 consecutive days during which barometric readings (in mm of mercury) varied from 74.6 to 71.6. We subsequently tested this apparent relationship over a period of 20 bioassays with *S. multistriatus*. In addition to these results, we report the effects of artificial pressure fluctuations on the responsiveness of *S. multistriatus* and *I. pini* as well as preliminary observations on the interaction of air swallowing and flight.

METHODS AND MATERIALS

The male *S. multistriatus* used in the bioassays were reared from naturally infested American elms (*Ulmus americana* L.) cut in Syracuse, New York. The female *I. pini* tested were taken from a laboratory colony originally collected from infested ponderosa pine (*Pinus ponderosa* Laws.) at Worley, Idaho, and reared on red pine (*Pinus resinosa* Ait.) in Syracuse. For each trial about 125 beetles were sexed and placed on damp filter paper in glass petri dishes. The beetles were illuminated under a fluorescent light at 60 foot-candles for 24 hr at 23°C and the ambient barometric pressure.

The olfactometer design by Moeck (1970) was used as a bioassay device, except the arena was open rather than covered with glass. The bioassay methods were described by Lanier et al. (1977). Briefly, beetles released at one side of the area were induced by their photopositive response to cross an attractant-bearing air stream. Beetles which turned 90° to follow the air stream for 2 cm were considered to have responded to the attractant.

The reaction of *S. multistriatus* to natural fluctuations in atmospheric pressure was first tested using as an attractant source logs infested with 50 virgin females 60 hr before the bioassay. The tests involving artificially fluctuated atmospheric pressure used standard solutions of the odorants collected by Porapak® extraction of volatiles released by beetles boring in logs (Peacock et al., 1975). The extract used for *S. multistriatus* was taken from virgin females boring in elm, while the *I. pini* extract was taken from males boring in red pine. In both cases the amount of odorant used was equivalent to that produced by 10 beetles.

Measurement of atmospheric pressure was accomplished with an anaeroid barometer calibrated by a mercury barometer. Ambient atmospheric pressure was measured before and after each trial.

Five bioassays were run for each trial. The first bioassay was to test the beetles' response to air containing no odorant. The second tested the response to an air flow containing the attractant without subjecting the beetles to barometric flux.

For the third test, the beetles were placed in a sealed desiccation chamber and subjected to decreased pressure equal to 25 mm (1 inch) of mercury less than the ambient barometric pressure (measured by a barometer inside the desiccator). This decrease was accomplished by pumping air out of the chamber with the laboratory vacuum line. After 30 min at this pressure, the beetles were removed, thus affecting a net increase in pressure experienced by the beetles immediately prior to their testing.

The fourth assay was similar to the third except the pressure inside the chamber was increased by 25 mm (1 inch), giving the net effect of a decrease in pressure at bioassay. The fifth assay varied the pressure inside the chamber by 12.5 mm (0.5 inch) of mercury above and below the barometric norm for the trial date. The pressure was reversed every 5 min for a 30-min period prior to bioassay.

Beetles were monitored for air-swallowing by removing their pronota under physiological saline. To stabilize the dissection, the insects were fixed in paraffin in the bottom of the dissecting dish.

Flight was induced by tethering beetles above the substrate on insect pins glued to the anterior half of their pronota. The dissection of "flown beetles" was usually completed within 90 sec of their removal from tethers.

RESULTS

Response under Natural Atmospheric Pressure

During the period of these tests (May 14 to June 8, 1973), response of *S. multistriatus* to the female-infested bolt ranged from 68 to 92%, but we could detect no relationship between response level and barometric reading. This is in contrast to the apparent positive correlation between these parameters observed by Elliott (1973). However, the variations in atmospheric pressure which we observed were much less than those which occurred during Elliott's tests. The difference between his maximum and minimum readings for the period was 29.7 mm and between March 16 and 17 the barometer dropped 22.6 mm, from 742.2 to 719.6 (at the date of the latter reading the atmospheric pressure at Hancock Field, 10 miles north of Syracuse, was the second lowest ever recorded at that locality). During our 20 tests for this experiment the largest between-day change was 4.1 mm and the highest and lowest readings were 75.0 (May 14) and 73.7 (May 28).

Upon regrouping our data we found a mean response of 85.6% for 9 tests during which we detected no change in atmospheric pressure compared to 73.2% for 11 tests during which readings varied (Table 1). The significance of these differences ($P < 0.001$, based on t test of raw data) indicates that shifts in atmospheric pressure, rather than pressure per se, affect the responsiveness of *S. multistriatus* in bioassays and that Elliott's (1973) data were misinterpreted owing to the unusual variability in pressure that occurred during his tests. Since only two of our tests were conducted during a period

TABLE 1. EFFECT OF CHANGE IN AMBIENT ATMOSPHERIC PRESSURE ON RESPONSE OF *S. multistriatus* AND *I. pini* TO THEIR AGGREGATION ATTRACTANTS

Barometer change (mm)	*S. multi.* series 1[a]		*S. multi.* series 2		*Ips pini*	
	No. tests	Mean response	No. tests	Mean response	No. tests	Mean response
0.00	9	85.6±2.15α[b]	4	65.5±1.50α	*[c]	
0.25 to 0.99	11	73.2±1.31β	8	63.5±2.56α	6	59.2±2.71α
1.00 to 1.49	0		8	67.0±3.25α	6	52.7±3.49α
1.50 to 3.30	0		0		6	58.0±2.68α

[a] *S. multistriatus* series 1 data are from test of attraction of beetles to females in logs; series 2 and *I. pini* data are from ambient tests summarized in Table 2.

[b] Differences among data in vertical columns followed by different letters (α and β) are significantly different ($P < 0.01$, t test for independent variables).

[c] Two observations at 0.00 category are combined with those in the 0.25 to 0.99 category. The anomalous 100% response (see text) is not included in these data.

when atmospheric pressure was falling and because it was impractical to depend upon natural fluctuations, we tested the response of beetles following exposure to artificially induced pressure changes.

Response following Induced Pressure Fluctuations

S. multistriatus. The response of the groups of beetles held at ambient pressure ranged from 56 to 82% in the 20 tests conducted for this experiment. These levels of response are low relative to those of the preceding experiment, but the stimuli are different; in the first experiment 50 virgin females boring in an elm log was the attractant, while in this test we used Porapak extract equivalent to 10 females in an elm log. The reduced stimulus was chosen in the hope of accentuating differences among the treatments.

Figure 1 shows that all artificial pressure fluctuations depressed beetle responsiveness. Moreover, the rates of response were consistently in the same order: ambient condition > increased pressure > decreased pressure > fluctuated pressure > ambient control (no odorant). Differences among means for all

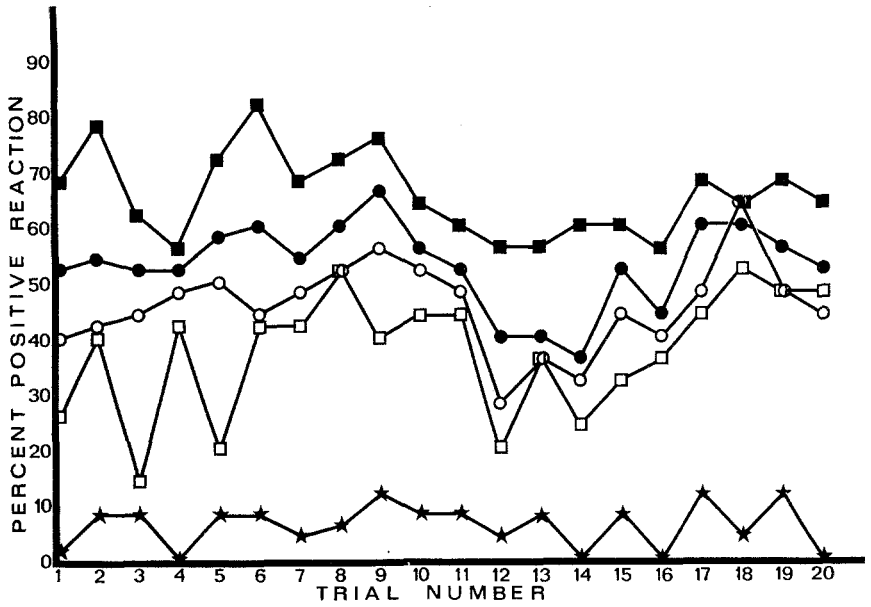

FIG. 1. Results of 20 tests of the response to attractants by *Scolytus multistriatus* exposed to natural and artificially fluctuating atmospheric pressures; solid square = ambient; solid circle = increased pressure; open circle = decreased pressure; open square = fluctuated pressure; stars = ambient, airstream without attractant.

TABLE 2. EFFECT OF INDUCED CHANGES IN ATMOSPHERIC PRESSURE ON RESPONSE OF *S. multistriatus* AND *I. pini* TO THEIR AGGREGATION ATTRACTANTS

	Percent response[a]	
	Scolytus multistriatus	*Ips pini*
Pheromone at:		
Ambient	$65.3 \pm 1.64\alpha$	$57.9 \pm 2.72\alpha$
Increase	$52.8 \pm 1.72\beta$	$51.9 \pm 3.76\alpha,\beta$
Decrease	$45.4 \pm 1.08\gamma$	$49.0 \pm 4.24\beta$
Fluctuating	$37.3 \pm 2.48\delta$	$47.8 \pm 4.04\beta$
Blank Control at:		
Ambient	$6.1 \pm 0.88\epsilon$	$6.7 \pm 1.64\gamma$

[a] Data in vertical columns followed by different letters are significantly different ($P < 0.05$, t test for independent variables).

of these treatments are significant ($P < 0.01$, t test for independent variables) (Table 2).

Our variations of 25 mm pressure are similar to the extraordinary drop and recovery of barometric readings observed by Elliott (1973). By using relatively high pressure differentials we were able to overcome natural variations, of which the maximum was 1.5 mm during the course of any of these tests. However, we did not find ambient pressure changes to be significantly correlated with response, as was the case for the previous test (Table 1).

Ips pini. During this experiment we observed maximum within-test fluctuations in the ambient atmospheric pressure of −4.6 (May 21, 1975) and +2.5 (May 23, 1975). Even though fluctuations were greater than those observed during the tests with *S. multistriatus*, there was no indication that response of the untreated groups of beetles was affected by natural pressure changes. Nevertheless, artificially induced changes in pressure reduced response of beetles, compared to the beetles held at ambient conditions ($P = 0.10$ for increasing pressure vs. ambient and < 0.05 for decreasing and fluctuating pressures vs. ambient). However, differences among the various induced pressure changes were not significant ($P > 0.30$).

On February 11, 1976, 100% of the *I. pini* in all of the treatments (except the air control) responded to the odorant stimulus. This extreme responsiveness was unparalleled during our experiments and has seldom been observed by the senior author during 12 years of bioassays with *Ips* species. The deletion of these anomolous data would slightly increase the levels of significance among differences for the various treatments.

Air Swallowing

S. multistriatus and *I. pini* taken from rearing cages as emergent adults usually had air in their ventriculi, whereas the guts of preemergent adults removed from the bark invariably did not contain bubbles. However, preemergent beetles which had been induced to fly by tethering them in the manner of Bennett and Borden (1971) generally had their ventriculi grossly distended by a large air bubble. As is the case for *D. pseudotsugae* and *T. lineatum*, *S. multistriatus* and *I. pini* appear to swallow air as a normal part of their flight or preflight activities. The data we present indicate that variations in atmospheric pressure affect the response of *S. multistriatus* and *I. pini* to their aggregation attractants and that these insects swallow air, but we can offer no direct evidence to support the hypothesis of Bennett and Borden (1971) that the ventricular air bubble acts as a barometer.

DISCUSSION

In reviewing the effects of atmospheric pressure changes on insects, Wellington (1946) generalized that insects were capable of reacting to slight pressure changes and that falling atmospheric pressure was often associated with increased insect activity. Falling atmospheric pressure may signal an advancing storm front (Sutton, 1960) and an opportunity for wind-aided dispersal. While these conditions may be advantageous for long-range mass movements of spruce budworm moths (Henson, 1951) and migratory locusts (Wellington, 1946), they would not be conducive to the concentration of bark beetles that must follow an odor trail in order to congregate on host material under attack. It seems reasonable that these insects would not move in response to an odorant during atmospheric conditions which are not conducive to their reaching the source.

It has been known for several decades that *S. multistriatus* withholds flight in windy weather (Wallace, 1940; Meyer and Norris, 1964). During several seasons of capturing these beetles on sticky traps baited with aggregation attractant, the senior author has noted that peak catches invariably occur during calm periods in mid-morning and just before sunset on clear days and immediately preceding rains on overcast days. Launching by *S. multistriatus* is consistent with periods for arrival of beetles at the source of the attractant; newly emergent beetles that had been secreted in crevices on the bark of brood logs while the wind was gusting abruptly scurried to exposed positions to take flight when the air became calm (Lanier et al., 1976).

It seems unlikely that air movements per se cue mass take-offs by beetles quite sheltered from this influence. We believe that microfluctuations in

atmospheric pressure associated with eddying of wind (Sutton, 1960) around the log pile suppressed pedestrian movement and dispersal flight by beetles, just as atmospheric pressure fluctuations apparently decreased the responsiveness of beetles during our tests.

Unlike *S. multistriatus*, we have observed that *I. pini* will arrive at odorant sources during periods of moderate wind (up to 20 km/hr, GNL estimate). This observation appears to corroborate the weaker influence of induced pressure changes on responsiveness of *I. pini*.

S. multistriatus is small relative to *I. pini* (2.3 vs. 3.6 mg/beetle, respectively) and would be more influenced by wind than the latter species. Wind buffeting potential could be cited as rationale for the specialization of mechanisms sensitive to atmospheric pressure fluctuations. Thus, beetle size may be correlated with flight patterns. In the southeastern United States, the tiny *Ips avulsus* (Eichh.) swarms in a manner similar to that we have related for *S. multistriatus* and flies mostly during the mid-morning and late afternoon while the large *Ips calligraphus* (Germar) flies throughout the day (Mason, 1970; Vité et al., 1964). However, the intermediate-sized *Ips grandicollis* (Eichh.) was trapped only during the late afternoon and the flight of the largest of the southern scolytid species, *Dendroctonus terebrans* (Olivier), was strongly restricted to a few hours in the early morning and late evening (Vité et al., 1964). Light intensity, temperature, and rhythms undoubtedly govern the activities of bark beetles as much as, if not more than, atmospheric pressure fluctuations. The physical and physiological factors that mediate the pheromone biology of insects appear to be fertile areas for research.

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EFFECTS OF SEX PHEROMONE ON LOCOMOTION IN THE MALE AMERICAN COCKROACH, *Periplaneta americana*

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Abstract—The locomotory activities of individual males of *Periplaneta americana* in a circular arena 2.5 m in diameter were investigated during the portion of the photocycle in which the cockroaches are most active. Control animals ran at an average speed of 10.86 cm/sec but remained immobile 68% of the time. Pauses in locomotion occurred frequently and at fairly regular intervals ($\bar{x} = 0.88$ sec). Males showed a strong tendency to remain near the sides of the arena. Sex pheromone presented in the center of the arena produced a reduction in immobility time and a slight increase in running speed. The frequency of pauses decreased, and the time between pauses became less regular. The proportion of time spent near the sides of the arena was greatly reduced also.

Key Words—Locomotion, sex pheromone, cockroach, *Periplaneta americana*, behavior.

INTRODUCTION

Periplaneta americana adults are not strong fliers. Although they may occasionally glide from a vertical surface to the ground, the vast majority of their movement from place to place is accomplished by walking or running. Many studies have been published which relate to locomotion in cockroaches: Hughes (1952) described locomotion in *Periplaneta americana* and *Blatta orientalis*. Studies by Delcomyn (1969, 1971a,b) on the coordination of leg movements have shown that *P. americana* uses an alternating tripod or alter-

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nating triangle gait at all speeds above 5 cm/sec. McConnell and Richards (1955) utilized photocells to measure running speeds over distances of 25 and 50 cm and found that *P. americana* was capable of speeds up to 130 cm/sec.

While the studies by Hughes and Delcomyn dealt primarily with the coordination of limb movements during locomotion, those of Darchen (1952, 1955, 1957) and Darchen and Richard (1960) described the search behavior of *Blattella germanica.*

To date, no information has been available concerning the search behavior of cockroaches in large open areas. Nothing is known about the paths that they follow, the distances they cover, their speed of movement, or even what proportion of their time is spent moving about. This study was conducted to provide information about the normal locomotory activities of cockroaches and the ways in which these activities are affected by the presence of sex pheromone.

METHODS AND MATERIALS

A group of 10 *P. americana* males was selected from laboratory colonies. The adult males selected were predominantly young because it was necessary that they have their antennae and all three pairs of legs intact. Some of these appendages are frequently lost during aggressive encounters rather early in life. Paper disks 9 mm in diameter coated with yellow luminescent paint (Dura tempera colors) were glued to the males' pronota. Clear plastic containers ($29 \times 18.5 \times 12.5$ cm) furnished with food and water served as housing cages for groups of five males. The animals were maintained on a 12:12-hr black light–white light cycle at $25 \pm 2°C$ in a room devoid of other cockroaches. They were kept under these conditions for two weeks before testing was initiated.

The locomotor activities of male cockroaches were examined in an 8-ft (2.5 m) circular arena. The floor of the arena consisted of two 4×8-ft sheets of $\frac{1}{4}$ in Masonite ($1.22 \text{ m} \times 2.44 \text{ m} \times 0.6$ cm) painted with flat black paint to reduce reflectance. The ends of a strip of galvanized sheet metal 20 cm high and 7.66 m long were joined together to form the sides. Petroleum jelly was applied to the metal to keep the test animals on the floor.

A motor-driven 35-mm single-lens-reflex camera was suspended from a wooden beam above the center of the arena. A 60 rpm synchronous motor was mounted on an adjacent beam in such a way that a slotted disk could be rotated in front of the camera lens. When photographing a cockroach in the arena, the disk rotating in front of the lens produced a stroboscopic effect, resulting in six exposures of 0.083-sec duration for each second the shutter remained open.

An Olympus OM-1 camera with a Zuiko 28-mm F3.5 lens was used for filming the cockroaches' movements. Kodak Tri-X film was rated at ASA 1600 and developed in Acufine for 6.5 min at 20°C and then fixed and washed normally. This technique produced dark sharp images in most cases and never gave unsatisfactory results.

During photographing, the arena was illuminated by low intensity black light (~365 nm). As a result, only the luminescent disks on the backs of the cockroaches reproduced on film. Using this technique, the light reflected from the disk on a moving cockroach produces a series of short streaks on the film, each of which represents the distance travelled in 0.083 sec. Although the cockroach can presumably see ultraviolet light, preliminary experiments with a 12:12-hr white light and black light photocycle revealed no significant differences in activity rhythms or mating behavior between the experimental animals and a group of controls maintained on a synchronized 12:12-hr light–total darkness cycle. It is likely that low-intensity ultraviolet illumination closely approximates the twilight conditions cockroaches often encounter at night.

Individual males were introduced into the arena, and after a 30-min adjustment period, their movements were photographed. One 20-sec exposure was taken each minute for 20 min. At the end of the 20-min test period, an inverted glass jar was placed over the male to restrict its movement (the inside of the jar was coated with petroleum jelly to prevent the male from climbing the sides). Thus confined, the male was moved to the perimeter of the arena, and a 5.5-cm disk of Whatman No. 1 filter paper treated with 10 μl of sex pheromone extract was introduced. After 5 min to allow for diffusion of the pheromone, the male was released and his activity sampled each minute for another 20 min. The extract was prepared from feces and filter papers taken from cages containing several hundred virgin females. The extraction technique used was that of Rust (1976) and used petroleum ether (Skelly F) as the solvent. Each of the 10 males was tested twice in this manner, with a minimum recovery time of 72 hr between tests. All observations were made during hours 3 and 4 of the black light (dark) cycle.

Laboratory-prepared sex pheromone extract was used in these experiments in order to ensure equal stimulus intensity for each of the males tested. Hawkins and Rust (1977) found that considerable variation in sex pheromone emission occurred between females and from day to day for individual females of *P. americana*. Bioassays similar to those of Block and Bell (1974) were conducted to determine approximately one female equivalent. It was determined that the level of male activity elicited by 10 μl of sex pheromone extract closely approximated the activity elicited by 24-hr filter papers from 250-ml beakers housing individual virgin females, i.e., one female equivalent.

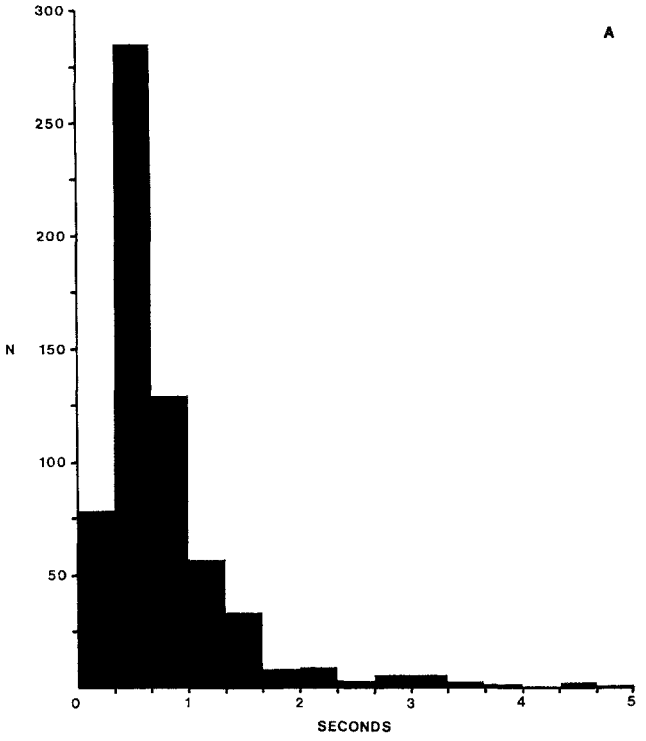
RESULTS AND DISCUSSION

When a cockroach is initially introduced into the arena, an escape response is elicited. The animal runs very rapidly in a nearly straight line until it reaches the wall. It then turns left or right and continues running rapidly along the perimeter of the arena for several seconds. It is not unusual for a cockroach to make several complete circuits around the arena before its running speed is noticeably reduced; it then begins to explore its new surroundings. After 30 min in the arena, the level of activity is considerably reduced. The male continues to walk about, but at a slower pace, and it may stop and remain stationary for minutes at a time. Locomotor activity during this period is presumably related to the circadian rhythm of activity rather than a continuation of the escape response.

P. americana males rarely maintain a steady pace for very long in this state. Instead, they walk a few centimeters, pause, walk a little farther, and pause again. A similar locomotor rhythm has been described for *Tenebrio* by Erber (1975). During test periods in which no prolonged inactivity occurred, the males paused an average of 8.6 times in each 20-sec sample or 25.8 times per min in the controls. In the presence of sex pheromone, the frequency of pauses declined to 6.2 per sample or 18.7 times per minute ($P < 0.001$; Wilcoxon's signed ranks test). These pauses show up on film as dark dots irregularly spaced, along the path. Because the dots are distinctly round and much darker than the streaks produced by a moving animal, they clearly denote where the cockroach has stopped. The distance traveled between pauses varies with the individual and is largely dependent on running speed, but the duration of locomotor activity is much more consistent, at least in the controls. In five randomly selected pairs of 20-min tests, the pauses in the controls were separated by 0.2–8.8 sec ($\bar{x} = 0.877$ sec) with 46% separated by only 0.5–0.67 sec of running time (Figure 1A). When pheromone was present, the duration of locomotor activity between pauses was less regular. Commonly, two or three pauses occurred in rapid succession followed by several widely separated ones. The time between pauses ranged from 0.2–7.7 sec with a mean of 1.27 sec (Figure 1B).

Although it is not possible to determine the duration of the individual pauses using this photographic technique, one can calculate the amount of time that the cockroach was immobile during a given 20-sec sample. Since the system provides six exposures per second during each sample, 120 streaks

FIG. 1. Frequency distribution of the duration of running activity between pauses in locomotion of *Periplaneta americana* males. Data from five randomly selected pairs of 20-min tests. (A) Control ($N = 699$; $\bar{x} = 0.88$ sec); (B) Sex pheromone present ($N = 407$; $\bar{x} = 1.27$ sec).


A
300
250
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N 150
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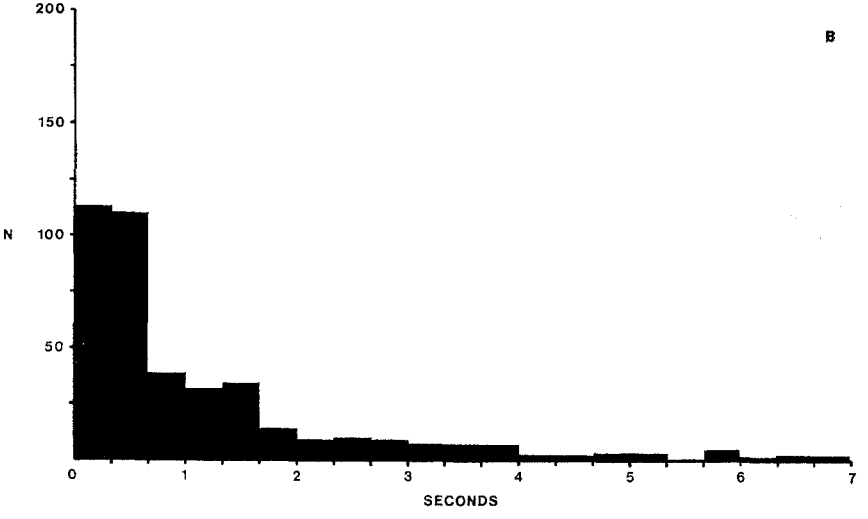
0 1 2 3 4 5
SECONDS


B
200
150
N 100
50
0 1 2 3 4 5 6 7
SECONDS

would result if the animal remained in constant motion. By subtracting the total number of streaks and dark dots from 120 and dividing the remainder by 6 (the number of exposures per second), the total immobility time is obtained. Immobility time per minute is calculated by multiplying this number by three.

Using this technique, immobility time was calculated for each trial. Figure 2 shows the mean immobility times for each minute of the 20-min test period. The mean level of activity was 14% greater in the presence of pheromone. The peak of sex-pheromone-induced locomotor activity occurred 10 min after initial exposure. This peak was followed by a gradual and irregular decline in activity, i.e., increasing immobility time. In the controls, no marked trends were evident except for a decline in immobility time toward the end of the 20 min. This insignificant increase in activity may have been due to the cockroaches' increased familiarity with the arena or simply due to sampling error.

The response of males to sex pheromone at the relatively low concentrations utilized in these experiments is thus greatly different from their response to very high concentrations. At high concentrations, the greatest activity occurs in the first minute after exposure and then declines steadily (Hawkins and Rust, 1977). While there is no way to correlate the data presented here

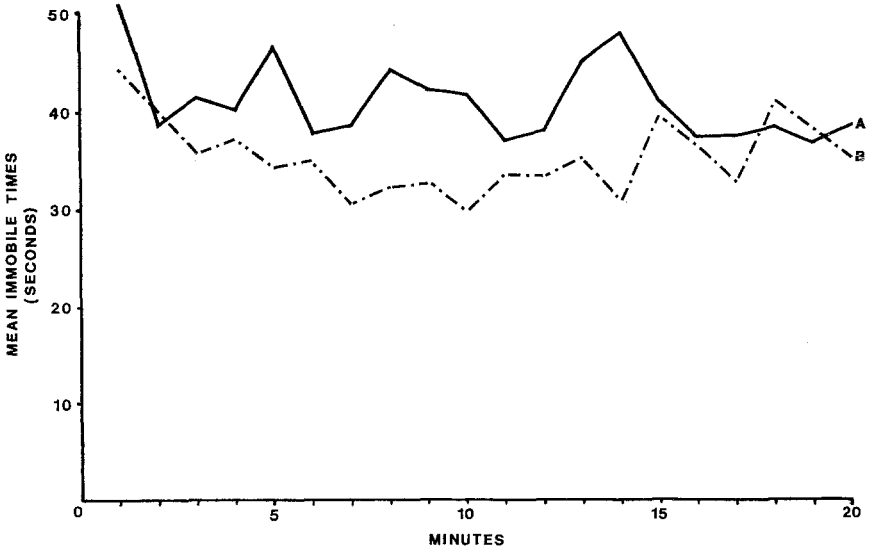


FIG. 2. Mean immobile times for males of *Periplaneta americana* during each minute of the 20-min testing period. (A = control; B = sex pheromone present.)

with the bioassays of Hawkins and Rust, it is evident that the maximum activity response does not necessarily occur at the moment of initial stimulation.

The controls remained immobile 68% of the time, i.e., 40.8 sec out of each minute, on the average. The variances were quite high from minute to minute for specific individuals as well as between individuals. Mean immobility time for individual animals ranged from 27.78 to 54.45 sec/min. Immobility times for the 20-sec samples ranged from 1.83 to 20 sec. As previously noted, it was not uncommon for a male to remain immobile throughout one or more of the 20-sec sampling periods. In fact, periods of prolonged immobility which produce just one very dark dot on the film occurred 1–10 times ($\bar{x} = 3.53$) per 20 exposures in the controls.

When sex pheromone was present, males still remained immobile for an average of 35 sec/min (58.4% of the time). Mean immobility times for individual animals ranged from 17.76 to 52.38 sec/min. As in the controls, immobility time ranged from 0 to 20 sec/20 sec sampling period in the pre-

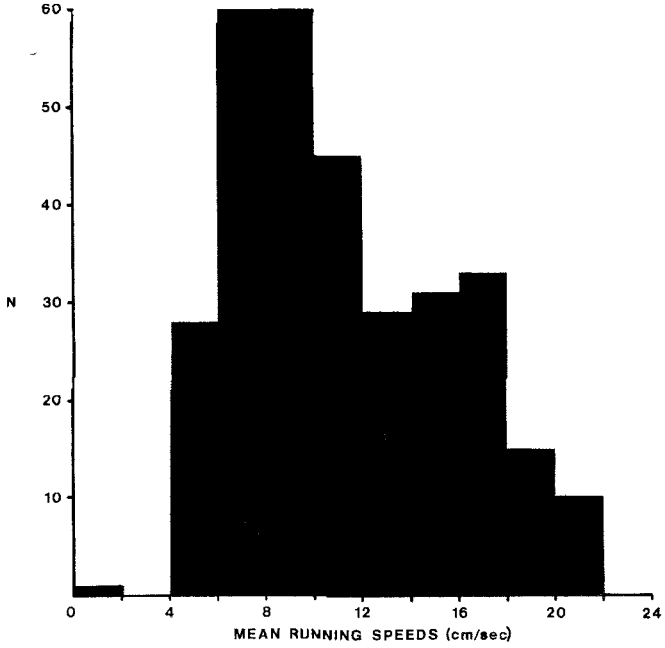


FIG. 3. Frequency distribution of the mean running speeds of the 312 control samples in which the cockroach did not remain stationary for the entire 20 sec. The distribution was not changed significantly by the addition of sex pheromone.

sence of pheromone. The decrease in immobility time due to pheromone is highly significant ($P < 0.01$; Wilcoxon's signed ranks test).

When they are not standing still, *P. americana* males move about at widely varying speeds. The over-all mean running speed in the controls was 10.86 cm/sec with the means of the 20-sec samples ranging from 5.22 to 17.32 cm/sec (Figure 3). Speeds in excess of 20 cm/sec were not uncommon, and the highest speed recorded was 38.8 cm/sec. Sex pheromone increases the speed of male locomotion but not to the extent that might be expected. The average running speed for the 20 trials was 11.42 cm/sec (range: 5.89–19.88). Maximum running speed within 20-sec sampling periods was generally higher than in controls, and the highest value recorded was 52.4 cm/sec, only 13.6 cm/sec faster than the peak without pheromone. Even this value is considerably below the highest speed measured by McConnell and Richards (1955), but they were measuring speeds generated by the cockroach's escape response.

Running speed may be influenced to some degree by whether the cockroach is turning or going straight, and whether it is following the side of the arena or crossing the center. However, the majority of drastic changes in direction are accomplished by positional changes during pauses, as in *Tenebrio* (Erber, 1975). Thus, many broad loops consist of a series of short, relatively straight runs with frequent pauses for directional changes. Lengthy pauses also occur frequently during long straight runs. Running speeds vary greatly in all portions of the arena, but the highest speeds recorded in the controls occurred during straight runs across the center. Males are able to change speed rapidly, and increases or reductions in speed of 10–15 cm/sec within a 0.167-sec interval frequently occur.

An interesting phenomenon occurs in the presence of sex pheromone. As the male orients toward the pheromone source in the center of the arena, and enters areas of progressively higher pheromone concentration, mean running speeds first increase and then decline close to the source (Table 1). The highest speeds occur in a broad band 30–90 cm away from the source. Speeds outside this band, either less than 30 cm or more than 90 cm from the source, were significantly slower with the slowest speeds occurring close to the source. No such trend was evident in the controls. The increase in locomotor speed that occurs at intermediate distances might be expected as a result of higher pheromone concentrations, but the slower speeds in the area of highest concentration are somewhat surprising. Presumably, the slower running speeds facilitate chemotactic orientation which occurs close to the source (Hawkins, 1978).

Darchen (1957) found that *Blattella* spent 94% of a 75-min test period in the outer 3 cm of a circular arena 28 cm in diameter. Although it was not possible to determine the exact amount of time spent by *Periplaneta* males

TABLE 1. MEAN RUNNING SPEEDS (CM/SEC) OF 14 *P. americana* MALES STIMULATED BY SEX PHEROMONE

Sample	Distance from source (cm)			
	0–30	31–60	61–90	91–120
1	16.53	19.70	20.79	19.72
2	4.93	5.83	6.95	6.51
3	13.18	17.93	18.48	12.91
4	13.68	16.94	15.12	9.28
5	13.04	22.12	21.44	19.34
6	10.81	16.99	16.10	14.73
7	13.61	17.60	17.18	19.67
8	14.41	18.39	13.43	13.31
9	11.71	18.34	19.88	19.00
10	9.42	15.77	14.68	12.20
11	12.62	12.89	14.89	15.40
12	5.19	5.57	7.64	5.43
13	11.56	17.26	20.12	18.24
14	11.66	14.00	15.71	14.98

	Wilcoxon's Signed Ranks Test		
	Groups		
	1–2	2–3	3–4
T_s	0[a]	37.5	15[b]
P	0.01	N.S.	0.05
N	14	14	14

[a] $P<0.01$.
[b] $P<0.05$.

in the outer portion of the arena, fairly accurate estimates were possible. By estimating to the nearest 25% what portion of an animal's path was restricted to the outer 30 cm of the 2.5-m arena, it was determined that control animals spent more than 70% of the time in that region. Of the 400 individual paths examined, 62% (248) lay entirely within the outer 30 cm while less than 10% (38) were completely outside it. Thirty-four percent of the time was spent within 2.5 cm of the sides. These data suggest a strong wall-seeking tendency which is presumably related to predator avoidance. Cracks and crevices in which cockroaches can hide from predators are likely to be more abundant along walls and the edges of other vertical structures such as rocks and tree

trunks. When pheromone was present, the wall-seeking tendency was considerably reduced. Males spent 63% of the time in the outer 30 cm. Forty-five percent of the paths were entirely within the outer 30 cm, and only 10% of the time was spent within 2.5 cm of the sides.

From an ecological point of view, perhaps the most interesting parameter is the distance that cockroaches travel. If an animal moves forward at a given speed at a more or less constant rate, it must necessarily traverse a certain amount of territory, i.e., speed and distance are interrelated. In attempting to control cockroach populations, it is important to have some knowledge of how far they are likely to wander during periods of activity. Dispersal studies of sewer populations of *P. americana* in Arizona by Jackson

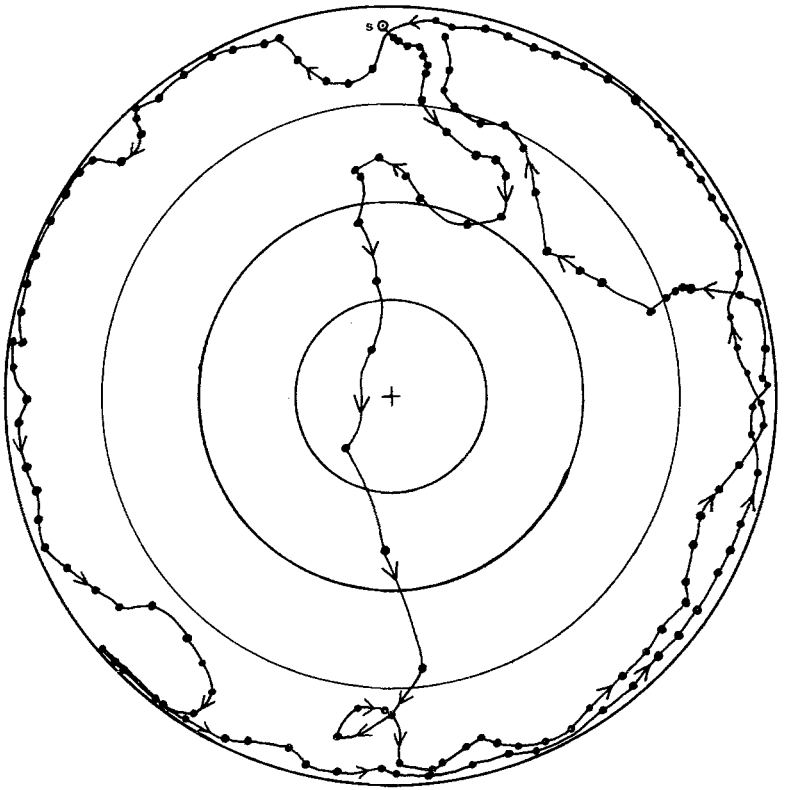


FIG. 4. A typical path traveled by an adult male of *Periplaneta americana* during 7 min of continuous filming with no sex pheromone present. Dots mark the locations of pauses of varying duration; arrows indicate the direction of movement (s = starting point).

and Maier (1955) and studies of the home-range behavior of populations in a deserted building by Wetherell and Breed (unpublished data) have suggested that individuals rarely travel very far during a given 24-hr period. This may be the case, but the results of these studies were far from conclusive.

In the 2.5-m circular arena, control animals moved about with an over-all average speed of 3.65 cm/sec for 20 min of testing during the portion of the dark cycle when Hawkins and Rust (1977) found them to be most active. (This figure includes numerous periods of prolonged inactivity as well as active periods.) This translates to a distance of 131.4 m/hr and suggests that males of *P. americana* are easily capable of traveling several hundred meters during a single night. However, since their paths are usually quite convoluted, total distance may not be correlated with linear distance. In fact, cockroaches rarely maintained the same directional heading for more than 1–2 sec at a time when they were not following the sides of the arena. A typical path across the open central portion of the arena can best be described as meandering—not a zigzag or a series of loops—but a path with frequent irregular changes of direction (Figure 4).

CONCLUSIONS

The locomotion of *P. americana* males in the 2.5-m circular arena is characterized by a generally low level of activity. They remain immobile approximately 68% of the time, and when they move, it is only at speeds averaging 5–14 cm/sec over 20 sec ($\bar{x}$ = 10.86 cm/sec). Pauses occur frequently, often being associated with marked changes of direction. Males exhibit a marked tendency to remain close to the sides of the arena and might be expected to follow walls and stay near corners under natural conditions. Even at such low levels of activity, cockroaches are easily capable of traveling several hundred meters in a single night, but their paths are apt to be so greatly convoluted that the linear distance from start to finish might be 30 m or less.

When a source of sex pheromone is placed in the center of an arena, immobility time decreases, running speed and distance traveled increase, the frequency of pauses decreases, and the time between pauses becomes less consistent. The proportion of time spent near the sides of the arena also decreases considerably. Thus the principal changes are a general increase in the over-all level of activity and reduction of the wall-following tendency.

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PENTATOMID NATURAL PRODUCTS

Chemistry and Morphology of the III–IV Dorsal Abdominal Glands of Adults

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Abstract—In addition to the primarily defensive metathoracic glands, adult Pentatomoidea possess a pair of active exocrine glands that open between the III and IV abdominal tergites. In the southern green stink bug, *Nezara viridula*, and other phytophagous species examined, the glands are small ($< 10\,\mu$g secretion/individual) and of approximately equal size in both sexes. In some, but not all, of the predaceous pentatomids (Asopinae), the III–IV dorsal abdominal glands are small in females ($< 10\,\mu$g secretion/individual) and extremely large in males ($> 500\,\mu$g secretion/individual). Using a GC-MS system, the secretion from both males and females of *N. viridula* (Pentatominae) was found to contain (*E*)-2-hexenal, hexanal, 1-hexanol, and *n*-tridecane. Females contained about three times more *n*-tridecane than males. The capacious glands of *Podisus maculiventris* (Asopinae) males produce (*E*)-2-hexenal, benzyl alcohol, α-terpineol, linalool, terpinen-4-ol, and *cis*-piperitol. The composition of the previously unanalyzed secretions from the adult III–IV dorsal abdominal glands is compared and contrasted to that of secretions from the metathoricic gland, and the role of coexisting exocrine glands in adult Heteroptera is discussed.

Key Words—Pentatomidae, Heteroptera, Asopinae, sex attractant, pheromone, (*E*)-2-hexenal, α-terpineol, benzyl alcohol, exocrine glands.

INTRODUCTION

The pungent odors emitted by true bugs when disturbed have captured the attention of numerous students of the Heteroptera. The capacious

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metathoracic gland of adults and the dorsal abdominal glands of nymphs seem certain to have evolved as agents of chemical warfare against the onslaught of microbial, vertebrate, and invertebrate attackers (Remold, 1963). Careful observation by several authors, however, has revealed isolated instances of a sexual dimorphism in the metathoracic gland of Heteroptera. For examples, adult male Belostomatidae in the genus *Lethocerus* produce much more secretion in their metathoracic gland than do the females (Butenandt and Tam, 1957; Pattenden and Staddon, 1970), and the milkweed bug, *Oncopeltus fasciatus* (Lygaeidae), exhibits a sexual dimorphism in the metathoracic gland (Johansson, 1957; Carayon, 1971) which is correlated with a dimorphism in the composition of the secretion (Games and Staddon, 1972). Apparently in the course of evolution, a sexual function has been superimposed upon the original defensive role of the metathoracic gland in some groups of Heteroptera.

While functional parsimony of exocrine glands is a widespread phenomenon, particularly among the Hymenoptera (Blum and Brand, 1972), its occurrence in the Heteroptera may be the exception rather than the rule. Compared to the large defensive glands, other heteropteran exocrine glands

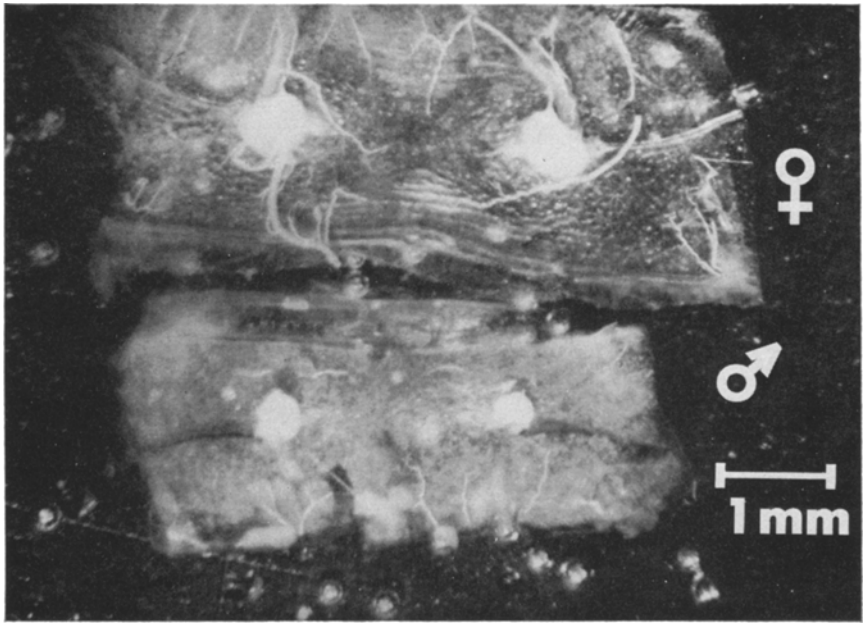


FIG. 1. The III–IV dorsal abdominal glands dissected from an adult female (top) and an adult male (bottom) of *Nezara viridula* (Pentatomidae: Pentatominae).

often appear minute, and in some cases appear to be inactive (Roth, 1961; Everton et al., 1974). But since pheromones are active at much lower concentrations than allomones, one would generally expect their associated glands to be smaller. Furthermore, individuals calling a mate may continually release volatiles from a gland, whereas in defensive glands the secretion is stored until needed to deter an aggressor.

In fact, a bewildering array of active exocrine glands are known to coexist with the ubiquitous metathoracic glands in adult Heteroptera. Adult males of coreoid bugs possess a VII–VIII ventral abdominal gland (Thouvenin, 1965; Merle, 1969) which produces a unique blend of aromatic volatiles (Aldrich et al., 1976). Some anthocorid males have ventral abdominal glands opening in the middle of the third visible abdominal sternite (Carayon, 1954). At least some Rhopalidae retain an active III–IV unpaired dorsal abdominal gland in adults of both sexes (Aldrich, unpublished data). Adult males of a cotton stainer bug, *Dysdercus fasciatus* (Pyrrhocoridae), possess dermal glands in abundance scattered over the IV abdominal sternite (Lawrence and Staddon, 1975). In the Pentatomoidea, adult males and females have a pair of III–IV dorsal abdominal glands under the wings. In all the phytophagous

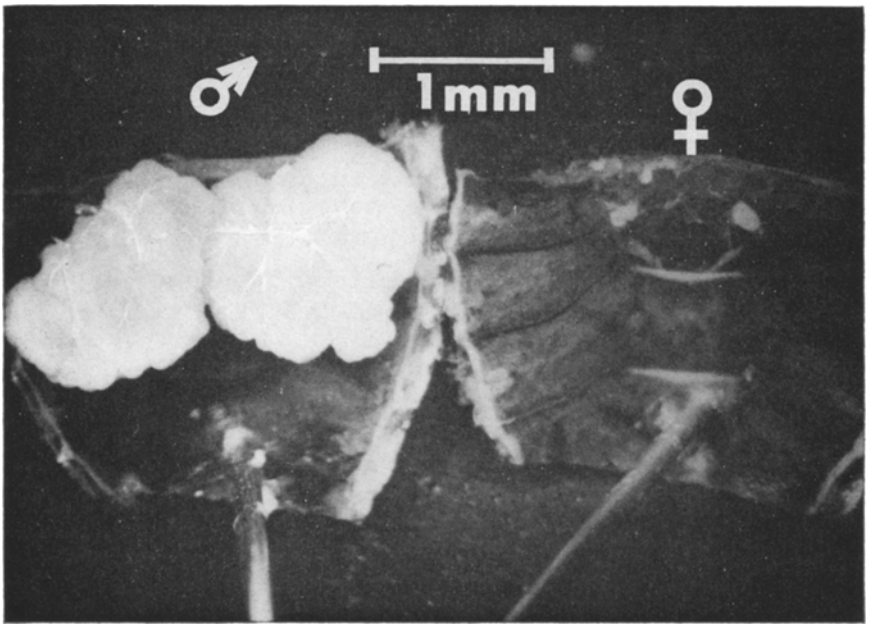


FIG. 2. The III–IV dorsal abdominal glands dissected from an adult male (left) and an adult female (right) of *Podisus maculiventris* (Pentatomidae: Asopinae).

species observed and in some of the predaceous species (Asopinae), these glands are small relative to the metathoracic gland and about the same size in the sexes (Figure 1). However, in some asopine species, the glands appear the same in the females as in the phytophagous species, but in the adult males the glands are greatly enlarged (Figure 2) (Dupuis, 1949, 1952). In addition, sexually mature adult males of some phytophagous pentatomids (e.g., *Nezara viridula*) have differentiated glandular patches of epidermis under the II and III abdominal sternites which secrete volatiles attractive to adult females (Dr. B. Brennan, University of Hawaii, Department of Entomology, personal communication).

The present paper reports on the chemistry of the adult male and female III–IV dorsal abdominal gland secretion in the southern green stink bug, *Nezara viridula*, and the III–IV dorsal abdominal gland secretion from adult males of the predaceous spined soldier bug, *Podisus maculiventris*. Morphological comparisons with related species are also included.

METHODS AND MATERIALS

Insects. A laboratory culture of *P. maculiventris* was developed from specimens obtained from the Biological Control Laboratory in Columbia, Missouri. The bugs were easily reared on mealworm (*Tenebrio molitor*) larvae and pupae and were provided with water from cotton wicks. A few specimens from the original culture in Missouri, reared on cabbage looper larvae (*Trichoplusia ni*), were analyzed in order to observe the effect of diet, if any, on the composition of the III–IV dorsal abdominal gland secretion. Adult males and females of three additional species of Asopinae, *Euthyrhynchus floridanus*, *Alcaeorrhynchus grandis*, and a species in the genus *Stiretrus*, were collected near Athens, Georgia, and the morphology of the III–IV dorsal abdominal glands was observed, but the secretions have not been chemically analyzed.

Field-collected specimens of the southern green stink bug, *N. viridula*, were obtained from the Georgia Coastal Plain Experiment Station in Tifton for chemical analysis. Field-collected specimens of a *Euschistus* sp. (Pentatomidae), *Acrosternum hilare* (Pentatomidae), *Stethaulax marmoratus* (Scutelleridae), and *Tetyra bipunctata* (Scutelleridae) were collected near Athens or Tifton and their glandular morphology was observed, but none of the secretions were analyzed.

Insects were maintained under a 16:8-hr day–night regime.

Preparation of Extracts. For all species investigated, extracts of the III–IV dorsal abdominal glands were prepared after microdissection of the glands from the bugs. For the phytophagous pentatomids, in which the glands are

much smaller than the metathoracic glands, the glands from 10–15 bugs were pooled in a single sample. In adult males of predaceous species having enlarged III–IV dorsal abdominal glands, the secretion from a single individual was ample for analysis. After removal of the glands from bugs dissected under tap water, the excess water was drawn off the gland and forceps with tissue paper, and then the gland was gently macerated in 2 drops of carbon disulfide in a spot plate. Finally, the extract was drawn into a 100-μl capillary tube, flame sealed, and frozen until analysis.

Chemical Analysis. Extracts were examined gas chromatographically on two different columns and instruments. Most of the chromatographic work was carried out with a Micro Tek 160 GC equipped with a flame ionization detector on a 10% SP-1000 AW Chromosorb W (60/120) column programed from 60 to 195°C at 5°C/min using nitrogen as the carrier gas. Samples were also examined on a Tracor 222 GC with a flame ionization detector coupled to a Varian CDS 111 automatic peak area integrator, using helium as the carrier gas, and a 3% OV-1 on Chromosorb W (80/100) column programed from 80 to 205°C at 5°C/min. The relative proportions of the constituents in a secretion were ascertained from peak-area integration on the Tracor 222-Varian CDS 111 system. Absolute weights of the major constituents in the secretions were determined on the Micro Tek 160 GC by preparing standard curves calculated from peak weights of a dilution series for each compound.

All gas chromatographic–mass spectrometric analyses were conducted with an LKB-9000 mass spectrometer at 70 eV, with a source temperature of 270°C, separator at 260°C, and 60 μA ionizing current. Separations were achieved using either a 1% SP-1000 column or a 1% OV-17 column, programed from 50 to 225°C at 10°C/min. All compounds identified from *N. viridula* and *P. maculiventris* by their mass spectra were cross-checked by coinjection of extracts from the bugs with standard compounds.

RESULTS

Gland Morphology. The dorsal abdominal glands of *N. viridula* and *P. maculiventris* are homologous in position, opening between the third and fourth abdominal tergites. Figure 1 shows the III–IV dorsal abdominal glands dissected from an adult female (top) and an adult male (bottom) of *N. viridula*. The glands consistently appeared slightly larger in females than males. In the two scutellerid species examined (*S. marmoratus* and *T. bipunctata*), as well as in the other Pentatominae examined (*Euschistus* sp. and *Acrosternum hilare*), the glands were always small as in *N. viridula* and approximately of equal size in both sexes. Figure 2 shows the III–IV dorsal abdominal glands of an adult male (left) and adult female (right) of *P. maculi-*

ventris. The female glands were always approximately the same size as the glands in *N. viridula*, but the glands in males were always greatly enlarged. Even freshly molted adult males had enlarged glands, although in the course of a few days the glands would become increasingly distended with secretion. The adult female of *A. grandis* had III–IV dorsal abdominal glands very similar to those observed in *P. maculiventris*, and the adult male had large glands comparable to those of adult male *P. maculiventris*. On the other hand, the other two species of Asopinae examined did not exhibit the striking sexual dimorphism of these glands that occurs in *A. grandis* and *P. maculiventris*. No indication of a sexual dimorphism of the III–IV dorsal abdominal glands was seen in over 30 specimens of *E. floridanus* dissected. Likewise, none of the *Stiretrus* sp. males dissected (5 individuals) had capacious glands; the male and female glands appeared identical to each other and similar to those of *N. viridula*.

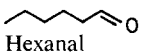
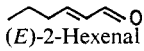
In nymphs of *N. viridula* and *P. maculiventris* the III–IV dorsal abdominal glands were always small and paired. Posteriorly there are two large unpaired exocrine glands opening between the IV–V and V–VI tergites. Although the III–IV abdominal glands in nymphs appear extremely small by comparison to the IV–V and V–VI dorsal abdominal glands, they may still be active. The IV–V and V–VI dorsal abdominal glands are colorless and empty in the adults.

The glands in all the species dissected, except those of the adult male *A. grandis*, consisted of a glandular cell wall surrounding a reservoir, as has been described for the IV–V and V–VI dorsal abdominal glands of immature coreid bugs (Aldrich et al., 1972). The III–IV dorsal abdominal glands of the adult male *A. grandis* were unique in that the gland cells did not form the wall of the reservoir. Rather, there were numerous glandular tubules, analogous to short Malphighian tubules, each of which apparently emptied its secretion independently into the large, translucent cuticular reservoir. Also, the gland cells in the *A. grandis* male were white, whereas in all the other species examined the gland cells were bright orange.

Sexually mature male *P. maculiventris* did not appear to have any modification of the epidermis under the second and third sternites as could be seen in sexually mature males of *N. viridula*. This is particularly interesting since *Nezara* lacks the sexual dimorphism of the III–IV dorsal abdominal glands that is so pronounced in *Podisus* and *Alcaeorrhynchus*.

Chemistry. Four compounds were detected in both male and female III–IV dorsal abdominal glands of *N. viridula*: (*E*)-2-hexenal, hexanal, 1-hexanol, and *n*-tridecane (Table 1). Each compound matched spectra on file in the computerized mass spectral search system (Heller, 1972) and was verified by coinjection of extract with authentic standards. Based on peak-area integration, (*E*)-2-hexenal, hexanal, and 1-hexanol were present in a ratio of

TABLE 1. COMPOUNDS PRESENT IN THE III–IV DORSAL ABDOMINAL GLANDS OF ADULT MALE AND FEMALE *Nezara viridula* (0.1–1.0 μg SECRETION/ADULT)

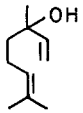
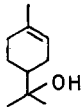
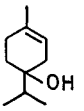
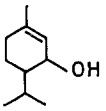
Compound	Percentage of total[a]	
	Male	Female
 Hexanal	8	1
 (*E*)-2-Hexenal	43.5	22
 1-Hexanol	3.5	3
 *n*-Tridecane	18	68

[a] Percentages represent the GC peak area of each compound relative to the total secretion determined with a Varian CDS 111 automatic peak area integrator.

about 1:9:1, respectively, in both male and female gland secretions. However, there was about 3.5 times more *n*-tridecane in female secretion than male secretion, which in some way may be correlated with the presence of slightly larger glands in the female adults of *N. viridula*. In females, *n*-tridecane comprised nearly 68% of the total secretion, whereas in males *n*-tridecane comprised only about 18% of the total secretion. The total weight of secretion per one male or female is in the range of 1–10 μg.

The III–IV dorsal abdominal gland secretion from *P. maculiventris* adult males proved to be one of the most chemically diverse exocrine blends ever analyzed from a heteropteran (Table 2). The mass spectra of the first four peaks were identical to published spectra of (*E*)-2-hexenal, linalool, benzyl alcohol, and terpinen-4-ol, respectively (Stenhagen et al., 1969). The fifth, and major peak, matched the spectrum of authentic α-terpineol. Standards of (*E*)-2-hexenal, linalool, α-terpineol, and benzyl alcohol were available and each gave a single peak when coinjected with gland extract. A sixth peak, eluting at the tail end of the α-terpineol peak, is probably *cis*-piperitol, based on *m/e* fragments at 86, 139, and 154 superimposed on the α-terpineol spectrum. The three major compounds in the blend are (*E*)-2-hexenal, α-terpineol, and benzyl alcohol, implying the coexistence of aliphatic, terpenoid, and

TABLE 2. COMPOUNDS PRESENT IN THE III–IV DORSAL ABDOMINAL GLANDS OF ADULT MALE *Podisus maculiventris* (~500 μg SECRETION/MALE)

Compound	Percentage of total[a]
 (*E*)-2-Hexenal	43
 Benzyl alcohol	9
 Linalool	< 1
 Terpinen-4-ol	< 1
 α-Terpineol	47
 *cis*-Piperitol	< 1

[a] Percentages represent the GC peak area of each compound relative to the total secretion determined with a Varian CDS 111 automatic peak-area integrator.

aromatic biosynthetic pathways in the gland. These three compounds are each present in very large amounts (Table 2), totaling over 500 μg/male which is 2–3 orders of magnitude more secretion by weight than is produced by females of the species and by the homologous glands in *N. viridula* males and females. It seems unlikely that such large amounts of these compounds could be concentrated from food. Indeed, specimens of *P. maculiventris* reared on

cabbage looper larvae produced a secretion indistinguishable from that of bugs reared on mealworm larvae and pupae. Thus, the III–IV dorsal abdominal gland secretion appears to be synthesized *de novo*, although the possible involvement of symbiotic microorganisms cannot be excluded.

DISCUSSION

Our chemical analyses of the heretofore little-studied abdominal exocrine glands of adult Heteroptera show considerable divergences from those of the metathoracic gland and reveal a biosynthetic versatility previously unsuspected for the Heteroptera. Prior to our identification of 1-hexanol in the III–IV dorsal abdominal glands of *N. viridula*, this compound had never been reported from Pentatomidae, not even in the particularly thorough analysis of the metathoracic gland secretion of *N. viridula* (Gilby and Waterhouse, 1965). In the Coreidae, on the other hand, 1-hexanol has been repeatedly found in the metathoracic gland exudates (Waterhouse and Gilby, 1964; Baker et al., 1972; Aldrich and Yonke, 1975). Benzyl alcohol is a major constituent of the male-specific VII–VIII ventral abdominal gland blend of *Leptoglossus phyllopus* and is a major constituent of the capacious III–IV dorsal abdominal glands of male *P. maculiventris*. Indeed, only two species, both aquatic, have been demonstrated to produce aromatic compounds in their metathoracic gland, *Notonecta glauca* (Notonectidae) and *Ilyocoris cimicoides* (Naucoridae) (Pattenden and Staddon, 1968; Staddon and Weatherston, 1967).

Of over 80 species of Heteroptera whose metathoracic gland secretions have been analyzed, only one species, *Dysdercus intermedius*, is reported to produce a monoterpene (Calam and Scott, 1969), although we have recently discovered a species of rhopalid which secretes only monoterpenes from the metathoracic gland. Even with the paucity of chemical data on the secretions from adult glands other than the metathoracic glands, it is obvious that coexisting exocrine glands have often independently evolved their own chemical theme.

Not only do the different exocrine glands in an individual secrete highly distinctive volatile blends, but the composition of a gland's secretion may change with time. Compositional shifts in the metathoracic gland scent with age have been observed in other heteropterans (MacLeod et al., 1975; Aldrich, unpublished data). In fact, nondiapausing adult males of *N. viridula* have been found to be significantly more attractive to females than diapausing males (Brennan et al., 1977).

The sexual dimorphism exemplified by the various additional exocrine glands, but rarely by the metathoracic gland itself, argues in favor of a reproductive role. This view is substantiated chemically; for example, the metathor-

acic scents of coreid bugs in the genus *Leptoglossus* are not species specific (Aldrich and Yonke, 1975), whereas the male-specific blends from the VII–VIII ventral abdominal glands are species specific, both qualitatively and quantitatively (Aldrich et al., 1976; Aldrich, unpublished data).

While exocrine glands other than the metathoracic gland appear to be the most likely source of sex pheromones in bugs from a morphological and biochemical perspective, at the present time we do not know what the behavioral response(s) is to the volatiles in question or at what distance(s) the presumed response(s) occurs. Removal of the VII–VIII ventral abdominal gland from male *L. phyllopus* did not impair mating of caged adults, suggesting a long-range effect for the secretion; however, males of *Podisus modestus* reportedly emit a strong odor during courtship (Tostowaryk, 1971). It is conceivable that a series of behavioral responses are elicited with increasing concentration of the volatiles. Nymphs of *N. viridula* employ the same compound, (*E*)-2-hexenal, as both an alarm pheromone (Ishiwatari, 1974) and an aggregation pheromone (Ishiwatari, 1976), the response elicited being a function of concentration. Unfortunately, it was not determined if the low concentration of (*E*)-2-hexenal, which causes aggregation of nymphs, emanates continually from the small pair of III–IV dorsal abdominal glands or is leaked from the large IV–V and V–IV dorsal abdominal glands. The much greater relative volume of III–IV dorsal abdominal gland secretion produced by some Asopinae compared to other Pentatomidae could thus be interpreted either as an adaptation needed to aggregate predatory individuals for reproduction (predatory species are by nature more dispersed than phytophagous species) or as an adaptation of males to overcome the predatory instincts of females.

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Addendum—Since the writing of this paper some samples of adult male III–IV dorsal abdominal gland secretion from *P. maculiventris* have been found to contain (*E*)-2-hexene-1-ol.

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CHEMOSENSORY ORIENTATION TO FOOD BY A HAWAIIAN GOATFISH (*Parupeneus porphyreus*, MULLIDAE)

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Abstract—The roles of olfaction and gustation in feeding arousal and food locating behavior are described for the first time for a marine teleost. The external taste sense located on the chin barbels of the goatfish mediates both arousal and searching in response to diluted prey homogenate and rinse of intact live prey. Electrophysiological monitoring of the barbel nerve (VII) reveals a sensitivity to amino acids dominant in prey tissues, but not to those present in trace amounts.

Key Words: Fish feeding arousal, food search, gustatory tracking, barbel sensitivity, aquatic chemoreception.

INTRODUCTION

Food acquisition in fishes is generally accepted as proceeding through three major phases: arousal, searching/locating, and control (Atema, 1969; Bardach and Villars, 1974). Arousal and food search behavior initiated solely by dissolved food extracts has been observed in several species of fish (Van Weel, 1952; Tester, 1963; Hobson, 1963, Bardach and Case, 1965; Atema, 1969, 1971; Kleerekoper, 1969; Sutterlin, 1975; Carr et al., 1976; Carr and Chaney, 1976; Pawson, 1977), but for most species the precise roles of the different chemosensory systems have not been established. Tuna show behavioral responses to the presence of prey odors and dissolved food subtances (Atema et al., 1977) as do sharks, some species of which

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perform chemotropotaxes, and others of which embark on rheotactic responses, swimming upstream in the swiftest currents they can find (Hobson, personal communication). Indeed, the distinction between rheotactic, phobotactic, and tropotactic responses by fishes is a source of continued lively debate in the field of aquatic chemosensory physiology (Kleerekoper, 1969; Bardach and Villars, 1974).

It is only in the freshwater catfishes of the genus *Ictalurus* that both the behavioral and neural substrates of chemosensory orientation to food have been firmly demonstrated. Bardach et al. (1967) and Timms and Kleerekoper (1972) demonstrated the ability of the catfish to perform true tropotactic responses, and Atema (1969) revealed a distinct division of labor between the olfactory and gustatory senses. Arousal, food searching behavior, and food pick up are mediated largely, if not exclusively, by facially (VII) innervated external taste organs (such as barbels), whereas social interactions are predominantly mediated by olfaction. Such data are not available for any marine teleost.

Electrophysiological investigations of the sensitivity and specificity of external taste organs have revealed the nature of some of the key substances involved food searching. Prey tissue homogenates, certain amino acids and polypeptides, sugars, and some electrolytes have been shown to evoke discharges in sensory nerves. There is variation between species as to which compounds are effective in eliciting responses (Bardach and Case, 1965; Konishi and Zotterman, 1961, 1963; Tateda, 1961, 1964; Konishi et al., 1966; Fujiya and Bardach, 1966; Kiyohara and Hidaka, 1975; Caprio, 1975). Nerves responsive to the traditional four taste modalities—sweet, sour, salt, and bitter—have been observed. However, neurons with an exceptional specificity to a limited range of amino acids and nucleotides, yet which are not responsive to substances representing the traditional taste modalities, have been found innervating the lip receptors of a marine puffer fish (Kiyohara and Hidaka, 1975). Moreover, these units are excited only when the amino acids most abundant in homogenates of the tissues of the puffer's natural prey organisms are applied to the lip receptors.

Stereotyped arousal and food search behavior can be elicited in the Hawaiian goatfish (*Parupeneus porphyreus*) (Mullidae, Hawaiian name: kumu) by the introduction of dissolved food extracts made from crabs and other small crustaceans which are the dominant food organisms of this animal (Mahi, 1968). This stereotyped food acquisition behavior in an animal with its sense of external taste limited to one set of organs (chin barbels) makes it an ideal subject for an investigation of how taste and olfaction interact to mediate arousal and searching behavior in a marine teleost.

If complexity of ventilation systems and the number of lamellae are indicators of olfactory sophistication (Kleerekoper, 1969; Bardach and

Villars, 1974), then this fish is well equipped for olfactory sensing. The nasal capsule is an elongated tube with two anterior accessory sacs with the quite large olfactory rosette (20–24 lamellae) located where the accessory sacs anastomose immediately below the anterior naris.

The barbels are covered throughout their length with taste-bud-like structures similar to those described for several other species of fish (Bardach and Villars, 1974). The facial (VII) nerve innervating the taste buds is closely applied to a skeletal element of the first branchiostegial ray (Mahi, 1968) as it proceeds through the barbel. From the barbel base the nerve runs caudad into the facial lobe of the brain.

Behavioral experiments were undertaken to describe and quantify arousal and food search patterns and to then ascertain the relative importance of the different chemosensory organs in these behaviors. Electrophysiological monitoring of the sensory system(s) primarily responsible for food finding was then performed to determine active components of food extracts. In light of the puffer fish lip receptor data (Kiyohara and Hidaka, 1975), the effects of glycine, proline, and arginine were of particular interest since these amino acids are present in large amounts in the tissues of several marine crustaceans (Schoffeniels, 1967; Florkin and Schoffeniels, 1969). In fact, in Hawaiian crabs, arginine and glycine are the most prevalent amino acids, comprising 30% of protein tissues (Balatz, personal communication).

METHODS AND MATERIALS

Behavior

Test Tank. All of the behavioral experiments were conducted in a 320-liter test tank 2.5 m long, 60 cm wide, with a sand bottom and filled with running seawater to a depth of 25 cm (Figure 1). Water entered through a row of openings at one end of the table and exited through a standpipe at the other. At the standpipe (downstream) end a small shaded area was used by the fish as a shelter. Test subtances inconspicuously introduced via an inlet pipe were carried down the tank by the flow of water. Fluorescein dye traveled between the inflow and outflow pipes in 3.5 min, the leading edge of the cloud forming a more or less straight line across the tank.

Test Substances. The standard test subtance was prepared by blending 100 g of whole Hawaiian crab (*Podophthalamus vigil*) in 100 ml of seawater. The homogenate was allowed to stand for several hours and then the clear supernatant was removed. Various amounts of supernatant were diluted with 200 ml of seawater and introduced into the tank, in which form it was invisible to the human eye. Introduction of pure seawater through the inlet pipe never elicited any response, thus precluding stimuli other than of olfactory

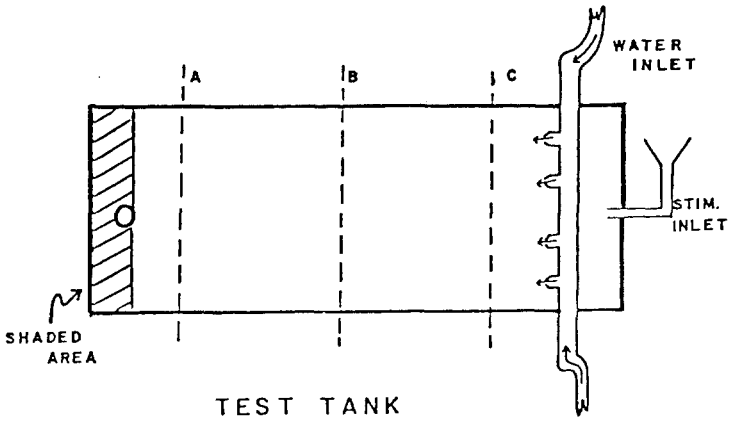

FIG. 1. Test tank. Length: 2.5 m; width: 60 cm; water depth: 25 cm. In the absence of stimuli animals spend most time under shaded (downstream) area, making occasional forays into remainder of tank. Transections of imaginary lines A, B, and C are used to quantify swimming activity.

or gustatory cues (e.g., sound). Test substances were used both fresh and after being frozen and thawed. No loss of efficacy resulted from freezing. The absolute concentration of test substances in the tank could not be calculated but the relative concentrations were known.

Tests were also conducted using a clear supernatant made from homogenized crab carapaces which had been thoroughly cleaned of tissue. Normal and anosmic fish were also exposed to crab rinse, i.e., water piped from an adjacent aquarium containing a colony of five Hawaiian crabs.

Animals 15–25 cm long were trapped in Kaneohe Bay and kept in open pens in the bay until used. Captive fishes were fed crushed Hawaiian crabs which are sympatric with the kumu in Kaneohe Bay and which are probably a normal food item. Tests were only conducted when the fish in the test tank were feeding eagerly, had normal coloration, and swam in a normal fashion. Animals were not fed for one day before an experiment, and were not fed immediately after a test to avoid conditioning phenomena.

Animals were made anosmic by anesthetizing them with MS222 and then cauterizing the sensory olfactory epithelia which had been exposed by small incisions in the anterior nares. All animals recovered. Testing began 1–3 weeks after the operation, at which time the fishes were in good health and feeding normally. Control animals were anesthetized and had small slits cut in their nares.

The "chin" barbels were removed by cutting them off as close as possible to the lower jaw. The wounds were soaked in Furacin anitibiotic. Again, all animals recovered.

Removal of the barbels has advantages over the facial lobe ablation technique of Atema (1969, 1971) in that more animals survive for testing because of the simpler surgery and, more important, the sensory loss is total (central ablations can leave some connections intact) and is restricted to the organs amputated.

Test Procedures and Data Analysis. In all test series only one fish was used per trial. Swimming activity was quantified by counting the number of times the fish transected three equidistant line markings on the tank during 15-min periods immediately preceding and following the introduction of test substances. Only transections that occurred as the fish was traveling towards the source were counted. The cumulative total difference between activity levels in the 15-min periods before and after substance introduction were statistically analyzed with a t test (Snedecor and Cochran, 1967).

Fin movements, barbel manipulations, body posture, and swimming activity were observed for the entire 30-min test period. Thus, for any given test, background swimming activity, the occurrence of arousal characteristics, response latency, elicited swimming activity, and stimulus type and relative concentration were noted.

The following tests were conducted: (1) exposure of normal, anosmic, and barbel-less fishes to various amounts of clear crab homogenate diluted in seawater; (2) exposure of normal fishes to a clear homogenate made from crab carapaces only; and to pursue the concept of the barbels being "long-range" food perceptors, (3) exposure of normal and anosmic fishes to seawater piped from an aquarium containing live crabs.

Electrophysiology

An *in vivo* preparation was divised whereby a small incision in a flap of loose skin allowed electrodes to be applied to the facial nerve where it leaves the base of the barbel (Figure 2). The fishes were able to survive the operation and subsequent 3-hr recording sessions. Prior to surgery the animal was lightly anesthetized with MS222, and a neuromuscular blocking agent (Flaxedil, 2 mg/kg) injected into the dorsal musculature. When placed in the restraining chamber the fish was held in a head-up posture and irrigation water entering the mouth flowed out of the gills and thus did not interfere with the stimulus solutions being applied to the barbels. A few fibers were teased out of the main nerve trunk and placed over a pair of stainless-steel hook electrodes. The animal was grounded by inserting a silver wire into adjacent tissues. Differential recordings were made of background activity,

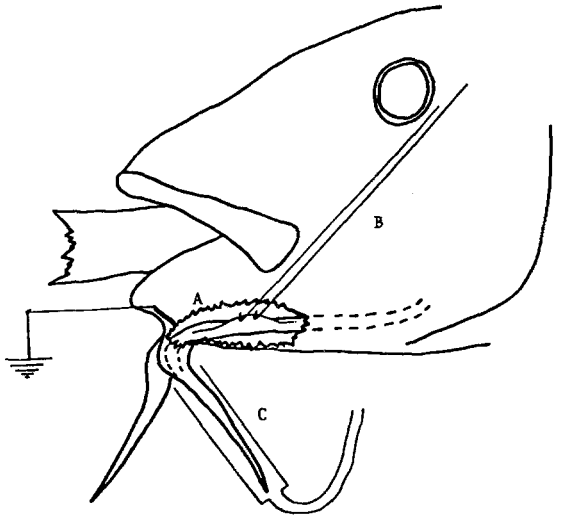

FIG. 2. Barbel nerve preparation. A small incision (A) reveals the barbel nerve. Fibers are teased out of the main bundle and looped over stainless-steel electrodes (B). Taste stimuli are applied to the barbel through tube (C).

activity during stimulus application, and activity occurring up to 2 min after stimulus application.

The extent of barbel immersion was controlled by inserting the barbel into a liquid-filled pipette. The pipette was attached to a Tygon tube through which test substances could be introduced and removed (Figure 2). The barbel was immersed in a constant level of liquid prior to, and during, stimulus application thereby avoiding pressure and cooling artifacts.

Seawater solutions of various amino acids and weak solutions of crab extract were tested.

RESULTS

Behavior

Typically, a fish acclimated to the test tank would spend most of its time in the shaded areas of the tank, making occasional brief forays into the other regions. Exposure of normal, unconditioned fishes to 10 ml of clear crab extract resulted in a consistant pattern of response. The initial phase, lasting only a few seconds, consisted of a pronounced, repeated flicking

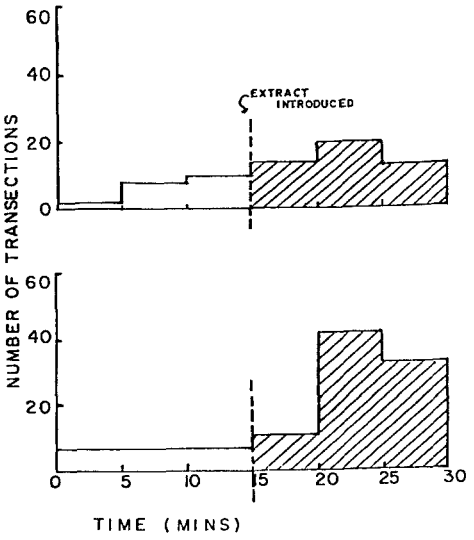

FIG. 3. Swimming activity of normal fish following arousal. In normal (and anosmic) fish swimming activity increased significantly following stimulus (10 ml crab homogenate). Eleven fish were tested singly in a total of 20 trials. Shown from this series are two trials displaying median (upper) and maximum (lower) differences in "before" and "after" activity.

of the pectoral and pelvic fins, and increased opercular pumping. Frequently the barbels were flicked forward two or three times and the first dorsal fin was partially erected several times in quick succession. The previously quiescent fish then launched into swimming activity. I have characterized this initial pattern of activity as the "arousal" phase of food detection behavior.

Immediately following arousal the fish began a period of greatly increased swimming activity (Figure 3). A high swimming speed was maintained and was accompanied by circling and extensive exploration with the barbels of irregularities in the bottom and sides of the test tank. Additionally, the fish often stopped to excavate small areas of the sand substrate. This very active response following arousal I have termed the "food search" phase of food locating behavior, and typically reached a maximum between 2 and 6 min after the onset of arousal. These and subsequent behavioral experiments were analyzed in terms of the occurrence of these "arousal" and "food search" characteristics.

The cumulative difference in swimming activity before and after introduction of the extract in 20 trials using a total of 11 animals was highly significant (t test, $P < 0.001$).

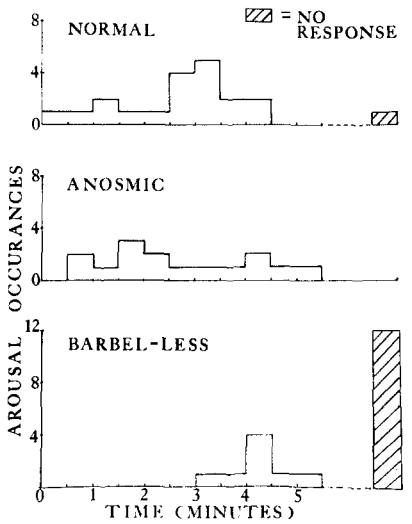

FIG. 4. Arousal occurrence and latency in three test groups. Occurrence of arousal characteristics in response to 10 ml crab homogenate shows no differences in detection acuity or latency between normal and anosmic animals. Barbel-less fish seldom become aroused and, when they do, show increased latencies. The shaded columns represent the number of times test animals failed to respond.

TABLE 1

Fish type	Arousal characteristics			Food search characteristics in response to 10 ml standard homogenate	Responses to	
	Frequency	Acuity	Latency		Crab carapace	Crab rinse
Intact	95%	Normal	Normal	Normal	Normal	Normal
Anosmic	100%	Normal	Normal	Normal	Not tested	Normal
Barbel-less	40%	Reduced	Increased	Absent	Not tested	Not tested

The background activities and responses to stimuli of fish with cauterized nasal rosettes were indistinguishable from those of intact fish, and show no statistical differences. Fifteen tests were run using a total of seven animals, which, postmortem examination showed, were without olfactory rosettes. No differences in acuity were observed between normal and anosmic animals since both groups gave consistent responses to 3.2 ml of dissolved clear homogenate (this approximates to 10 ppm, assuming maximum uniform dilution in the tank). There was no difference in the response latency exhibited by the two groups (Figure 4), and all components of arousal were normal, as were all aspects of the food searching behavior which accompanied the increased swimming activity.

Although background activity was not significantly different, the barbel-less fish (20 trials using 11 fishes) showed marked differences from the intact and anosmic fish in their responses to chemical stimuli (Table 1). Overall, the incidence of arousal was sharply reduced and food-searching behavior was entirely eliminated. In only a few trials were arousal characteristics observed and, although increased swimming activity was occasionally noted, the pooled differences of "before" and "after" activity were not significant, and in no trials was swimming activity accompanied by other characteristics of food search behavior such as substrate investigation or tight circling. In the few trials where arousal characteristics occurred, they were observed later than in the other two classes (Figure 4).

The control fish (anesthetized and sham-operated) responded normally to stimulant introduction.

Barbel-less animals only occasionally showed the initial arousal responses even when exposed to stimulus doses eight times as large as those evoking responses in the other animals. In the barbel-less animals a low frequency of arousal persisted for the three highest concentrations tested (3.2, 10.0, 250 ml supernatant). In none of these cases was food search forthcoming.

Normal fishes tested with extracts produced only from blended carapaces showed arousal in eight of nine trials. Food search behavior followed and the activity increase was significant at $P < 0.005$. In the tests using the 'rinse' of live crabs, the dissolved compounds are doubtless much more dilute than when the standard homogenate was used and yet arousal and food search behavior were elicted in both normal and anosmic fish with very pronounced activity changes ($P < 0.005$). Thus, the gustatory system alone is capable of perceiving the presence of "hidden" prey organisms and initiating and mediating food search behavior.

Electrophysiology

The results reported here are from 62 few-fiber nerve preparations

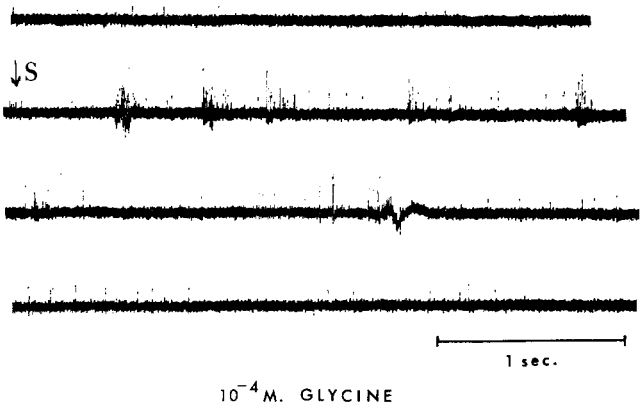

FIG. 5. Responses to 10^{-4} M glycine (retouched). Top line: background responses to seawater; arrow: stimulus arrival; bottom four lines: response to 10^{-4} M glycine. This few fiber preparation was not responsive to mild tactile stimuli.

which (1) showed no apparent injury; (2) had consistent and/or low background frequencies which returned after cessation of stimulus presentation; and (3) were responsive to dilute solutions of crab homogenate.

Nearly all chemosensitive preparations included touch-sensitive fibers, but some preparations exhibited purely chemosensitive responses (Figure 5). Touch-sensitive fibers showed greater spike frequencies and amplitudes than chemosensitive fibers. Tactile responses closely followed the temporal characteristics of the stimulus, responding in short bursts to stimuli such as water dripping onto an exposed barbel or showing continuous tonic firing when the barbel was laying against an object.

When the barbel was bathed in seawater, individual chemosensitive fibers were quiescent, showing background frequencies of between zero and 30 spikes/min (Figure 5, top trace). Responses to amino acid solutions exhibited an initial phasic response (Figure 5, second trace) followed by a tonic phase which sometimes persisted as long as the stimulus was applied (up to several minutes). This facilitated the demonstration of chemosensitive responses by oscillograph recordings taken a few seconds after stimulus application, at which time stimulus presentation artifacts had ceased and yet the tonic chemical response was continuing.

Chemosensitive fibers (as defined above) showed marked responses to crab extract and a limited number of amino acids: 10^{-4} M proline, glycine, arginine, glutamic acid, and alanine. These fibers were not responsive to similar concentrations of serine, histidine, tyrosine, or cystine.

Invariably the response to crab extract was greater than for single amino acids.

Although no threshold concentration data were obtained, all stimulatory amino acids elicited responses at 10^{-5} M concentrations.

In summary, dilute crab extracts which elicited marked behavioral responses also elicited greatly increased spike activity in fibers of the barbel nerve. These same preparations were also sensitive to solutions of glycine, proline, arginine, alanine, and glutamic acid (all of which occur in large amounts in crustacean tissues), but not to solutions of cystine, serine, histidine, or tyrosine, which combined, total only about 8% of Hawaiian crab tissue protein.

DISCUSSION

The kumu is a crepuscular carnivore feeding primarily on small crustaceans which live buried in the sandy surrounds of coral reefs. In adapting to this situation, this animal emphasizes chemosensory techniques for localizing food.

Since anosmic animals showed no differences in feeding arousal or food search from normal animals, whereas barbel-less animals were much more refractory and did not display food search characteristics, it can be concluded that the barbels are the primary organs of food orientation. These appendages are capable of mediating all phases of food localization including the initial arousal to the presence of food organisms somewhere in the general vicinity. That is, they are distance perceptors as well as the organs responsible for the last stages of prey localization. Unlike some sharks (Tester, 1963), olfaction is not the dominant food orientation sense, and, unlike catfish (Atema, 1969), loss of the barbel taste sense severely reduces the frequency of arousal and eliminates the swimming activity associated with food search. In catfish arousal and food search persisted in animals which were both anosmic and which had also had facial lobe ablations. Atema (1969) suggests this persistent arousal might have been due to gustatory connections that survived the lobectomies.

In both species tactile sense is doubtless extremely important in augmenting chemosensory input. However, touch is involved in close range behaviors that occur subsequent to arousal and initial exploratory swimming. The positive responses to carapace-only homogenate suggest that, at close range, the shell itself may provide chemosensory clues to the predator.

The occasional occurrence of arousal characteristics in barbel-less animals suggests that olfaction (or possibly internal taste) may mediate some aspects of feeding arousal. This may take the form of a generalized increase in

activity upon which the barbel taste-mediated characteristics of arousal and food search are superimposed. In this respect the "right context" (e.g., appropriate interaction of olfactory and gustatory stimuli, mixture profile of dissolved substances, correct light intensities, hunger state) is doubtless important for normal feeding behavior to occur in the field.

The electrophysiological data also attest to the feeding (as opposed to social) role of the barbels. Only those substances seemingly strongly indicative of the presence of food stimulate the population of barbel taste receptors which were selectively studied in this experiment. Furthermore, the greatest neural activity was noted when these amino acids were applied in the form of a crustacean homogenate (however, interpretation of this phenomenon is not possible since the concentration and precise composition of the homogenate was not known). These findings are in agreement with the selective sensitivity data for the puffer fish lip receptors (Kiyohara and Hidaka, 1975) and lobster data of Mackie and Shelton (1972) and pinfish data of Carr et al. (1976), who found natural prey mixtures more behaviorally evocative than either pure amino acids or synthetic mixtures. If there are receptors specifically tuned to food constituents then stronger neural (and presumably behavioral) responses will result when many stimulus types are simultaneously encountered.

The food acquisition system of this marine species fits (with the above noted differences) the arousal, search, control/ingest sequence proposed by Atema for the catfishes. This is to be expected since, in many respects, both species acquire their foods in the same way. Preliminary experiments suggest that the well-developed kumu olfactory system may subserve social interactions as they do in catfish (Bardach, personal communication). Further research is required in this direction and into the finer aspects of barbel nerve threshold sensitivities and specificities. Differences in barbel sensitivity may assist in resource partitioning by the several sympatric species of Hawaiian goatfish.

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SUSTAINED-FLIGHT TUNNEL FOR MEASURING INSECT RESPONSES TO WIND-BORNE SEX PHEROMONES

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Abstract—The design, construction, and uses of a relatively simple and inexpensive wind tunnel for studying responses of flying insects to sex pheromones are presented with special reference to research on moths. A variable-speed continuous belt installed on the tunnel floor proved to be a valuable feature, allowing the observer to manipulate the speed of the responding insect and thus to measure the duration and persistence of sustained anemotactic flights in the pheromone plume. It is concluded that flight tunnels of this kind will prove to be extremely valuable multipurpose tools in pheromone research.

Key Words—Flight tunnel, wind tunnel, sex pheromones, insect sexual behavior, anemotaxis, *Argyrotaenia velutinana*.

INTRODUCTION

In nature pterygote insects stimulated by airborne sex pheromones ("attractants") released from calling individuals normally exhibit a sequence of complex behavioral responses which may include: (1) activation—antennal erection, undirected or directed ambulation, and wing-fanning; (2) flight initiation; (3) positively anemotactic flight up the pheromone plume; (4) landing near the releaser; (5) finding the releaser; (6) precopulatory courtship, and (7) copulation. Many of these responses can be elicited by the release of man-made pheromones; however, the resultant stimulus–response sequence may differ significantly from the natural situation in those steps following source finding. With copulation precluded by the absence of a

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mate and under continuous stimulation by pheromone, the responding insect may approach, explore, and leave the source a number of times. Its behavior when finally leaving the vicinity of the chemical source and plume may be extremely complex and drastically altered from that before exposure to pheromone. A better understanding of each aspect of mating behavior will increase the chances that pheromones can be used to manipulate pest populations in man's favor.

In the laboratory the great majority of bioassays for insect responses to sex pheromones have employed olfactometers that allow only a partial enactment of the normal stimulus–response sequence (see Kennedy, 1977a, for a critical review of bioassay procedures). As pointed out by several of the pioneers of insect olfactory responses to distant odor sources (Wright, 1958; Kellogg and Wright, 1962; Kennedy 1977a, b), the behavioral steps most neglected in laboratory bioassays of insect attractants are those involving free flight in moving air which for their study require the use of wind tunnels.

Despite their proven utility in studies of insect responses to host volatiles (Visser, 1976; Kennedy, 1977b, and references therein), such flight tunnels have been used only sparingly in pheromone studies. Notably, the mechanisms by which moths orient to and follow a plume of pheromone have been investigated in flight tunnels (Farkas and Shorey, 1972; Farkas et al., 1974; Kennedy and Marsh, 1974). Pheromone bioassays conducted in other flight tunnels of varying degrees of sophistication (Traynier, 1968; Dahm et al., 1971; Mayer, 1973; Hendry et al., 1973) have successfully incorporated anemotactic flight as one of the required responses. In spite of such encouraging results, flight tunnels have yet to become popular tools in pheromone research. Possibly the tunnels hitherto reported may have appeared either too complicated or too crude to serve as broadly applicable tools in pheromone behavioral studies.

We now describe the design, construction, and uses of a relatively simple and inexpensive flight tunnel that has proven eminently useful in studying all of the aforementioned aspects of insect behavioral responses to pheromones.

METHODS AND MATERIALS

Apparatus

The flight tunnel (Figure 1), constructed largely of plywood and plexiglass, is housed in an environmental chamber where temperature and humidity are closely controlled. Various features in the design of the present apparatus were influenced strongly by the work of Kennedy and Marsh (1974). The 2.44-m-long tunnel is a flattened cylinder formed by bending and butting together two $1.22 \times 2.44 \times 0.002$-m clear plexiglass sheets. In cross

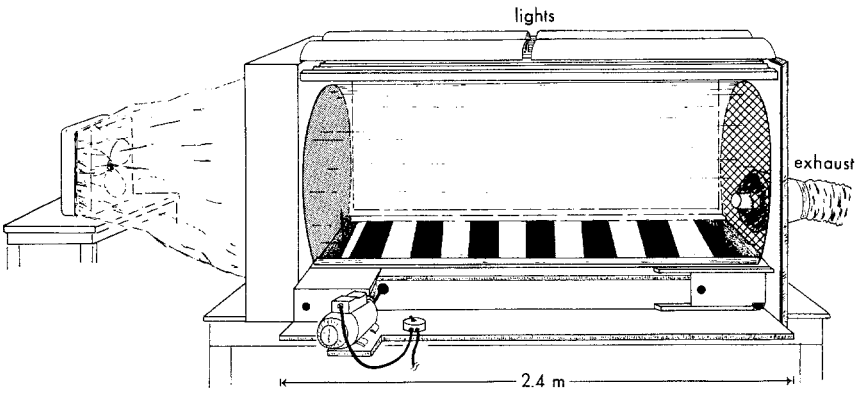

FIG. 1. Over-all view of the sustained-flight tunnel. See text for details.

section the tunnel measures 0.89 m wide at floor level and 0.96 m at its widest and highest points. Mounted immediately above the apex of the tunnel is a white corrugated fiberglass light diffuser. For behavioral studies of day-flying insects, light is provided by twelve 1.2-m fluorescent bulbs affixed 0.14 m above the diffuser. Light intensity on an upward-facing photocell at the top, midpoint, and floor of the tunnel is 2400, 1400, and 1000 lux, respectively, and the lateral distribution of light from above is very even. Light readings when a photocell is directed in any horizontal position or downward toward the tunnel floor fall within 500–700 lux. For night-flying insects light provided by incandescent lamps covered by red cellophane is reflected 1.5 m from the white walls of the environmental chamber. By varying the lamp arrangement and voltage, a range of illuminations down to 0.05 lux can be achieved within the tunnel.

Kennedy and Marsh (1974) demonstrated that certain male moths flying up a plume of pheromone "appear to be guided anemotactically by optomotor reactions to the ground pattern". The aspect of anemotactic flight that was clearly shown to be influenced by the ground pattern is airspeed. By moving the ground pattern either backward or forward, moths flying up a pheromone plume could be caused to decrease or increase their airspeeds accordingly and thus their positions within a flight tunnel could be manipulated.

To exploit the optomotor responses to ground pattern in pheromone behavioral studies a 0.80-m-wide continuous canvas belt painted with alternate orange and black 0.15-m-wide transverse stripes (Kennedy and Marsh, 1974) was installed on the floor of our tunnel. The belt is driven in the same direction as the wind by an electric motor and speeds are regulated by a

sliding-gear variable speed transmission controlled by a joy stick. A selection of highly uniform belt speeds from approx. 2 to 60 m/min has been found desirable. An odometer for recording belt displacement is attached to the belt drive.

The air supply for the flight tunnel is generated by an ordinary 0.51-m variable-speed window fan that delivers room air into a 0.9-m-long mixing chamber bounded by 0.2-mm polyethylene sheeting (Figure 1). Air then passes through a turbulence damper that consists of a $1.2 \times 1.1 \times 0.22$-m plywood box into which openings having the same shape as the tunnel have been cut on each side. A lining was positioned across the two cut edges and around the perimeter of the opening so that it became an extension of the tunnel. Two thicknesses of 9 mesh/cm cheese cloth, functioning as damping screens, were stretched tightly over each side of the opening. As measured by a hot wire anemometer, emerging air flow is extremely uniform at all points in the tunnel except those areas immediately adjacent to the plexiglass and canvas floor of the tunnel where frictional drag reduces the air flow rate by at least 60%. Air velocities of 65 and 47 cm/sec were chosen arbitrarily.

Sources of pheromone are introduced at the upwind end of the tunnel on 2.4-cm filter paper disks or other suitable dispensers held on wire clips and positioned 18–19 cm above the canvas belt. Higher positioning of pheromone sources resulted in reduced optomotor responses to the moving floor by insects engaged in anemotactic flights. Access to the tunnel interior is available through doors at each bottom corner and through the downwind end. At all times care was taken that pheromone vapors did not accumulate in the room housing the wind tunnel.

Moths to be tested are placed in 7-cm-diam $\times$ 22-cm-long 7-mesh/cm wire gauze cylinders which are inserted through a 7-cm-diam hole in the 7-mesh/cm wire screening enclosing the end of the tunnel. Such release cages are carefully aligned directly in the pheromone plume. A 30-cm-diam exhaust port, connected by 20-cm-diam flexible tubing to a 5 m^3/min exhaust fan vented to the outside of the building, is positioned approx 30 cm directly behind the release cage so that all of the pheromone-bearing air is removed from the room.

Total expenditure for all materials for the apparatus was less than $400.

Plume Characterization

The physical characteristics of plumes of airborne chemicals generated in the flight tunnel at an air speed of 65 cm/sec were characterized by various methods. Firstly, plumes of titanium dioxide smoke generated from 2.4-cm filter paper disks soaked with titanium tetrachloride and placed edgewise to

the wind were measured and photographed. However, it was recognized that smoke particles and pheromone molecules might not flow through the tunnel identically.

Secondly, human subjects determined the dimensions of a plume generated from a 2.4-cm filter paper disk saturated with "apple oliffac" (International Flavors and Fragrances, Inc., New York). While keeping their eyes closed, subjects familiar with the odor of apple oliffac were positioned with their noses within several cm of the screened downwind end of the tunnel. From a position outside the plume they were instructed to move toward the center of the plume at approx. 1 cm/sec while sniffing continuously and shallowly and to stop immediately and mark the spot where they first detected the fragrance. Finally, subjects were instructed to move in a line transecting the center of the plume while describing verbally the relative chemical concentrations.

Thirdly, a filter paper grid (Figure 4) was impregnated with an aqueous 0.02% crystal violet solution, allowed to dry, and then mounted on the screen at the downwind end of the tunnel. A plume of vapor from a 2.4-cm filter paper disk saturated with concentrated hydrochloric acid passed over the blue grid causing exposed areas to turn yellow.

Fourthly, electroantennogram (EAG) recordings from the antennae of newly emerged male gypsy moths, *Lymantria dispar* (L.), were used to characterize a plume of (±)-disparlure generated from a 2.4-cm filter paper disk bearing 10 mg of the pheromone. The general EAG procedures of Roelofs (1977) were followed; however, the excised ends of an antenna were inserted into the drawn ends of two opposing disposable Pasteur pipettes containing saline and either the indifferent or the recording electrode. The preparation was mounted on a mobile stand that was drawn horizontally through the plume at the downwind end of the flight tunnel at a rate of approx 2.5 cm/sec.

General Operating Procedures

Testing procedures vary with the nature of the experiment. In tests such as bioassaying fractions during pheromone isolations or in evaluating the attractancy of synthetic compounds, up to 10 moths were introduced in one release cage. When first placed at the end of the tunnel, moths often became more active than they had been when held in still air and hence were given several minutes to settle before the upwind end of the release cage was opened and the pheromone source was introduced. Replications, using fresh cages each time, followed in succession. The metal release cages and the screening at the tunnel end were cleansed frequently with acetone. Moths placed in cages that had previously had an exposure to a pheromone plume often

exhibited sexual excitement, indicating that chemicals can be adsorbed onto the metal.

In trapping experiments many insects can be released simultaneously in the flight tunnel and allowed to respond to the attractant baits spontaneously. In detailed behavioral studies one moth at a time was released into the flight tunnel. Some behavioral observations were recorded directly, while those happening rapidly were spoken into a tape recorder and later transcribed.

Detailed Observations of Male Argyrotaenia velutinana *Behavior*

Preliminary data on detailed behavioral responses of male redbanded leafroller moths to their pheromone are provided to illustrate the kinds of data that can be recorded in a flight tunnel. The moths were reared on a pinto-bean-based medium (Miller and Roelofs, 1977a) and were held at 24 ± 1°C on a 16-hr photophase until testing at 3–5 days posteclosion. Behavioral tests were conducted 1–4 hr prior to the onset of scotophase. A 15-min exposure to temperatures of 20 ± 0.5°C in the flight tunnel room advanced sexual receptivity (Cardé et al., 1975) allowing the tests to be conducted in bright light. The pheromone, a 12:1:19 neat mixture of (*Z*)-and (*E*)-11-tetradecenyl and dodecyl acetates, was dispensed from sixty 0.22-mm (ID) hollow fibers (Conrel Corp., Norwood, Massachusetts) stuck to two $1.5\times$ 2.5-cm strips of red adhesive tape. Wind velocity was 65 cm/sec.

RESULTS AND DISCUSSION

Plume Characteristics

Studies using titanium dioxide smoke indicated that turbulence in the flight tunnel was very low and that resultant plumes had very discrete boundaries. A closeup view (Figure 2) shows that generated smoke plumes were not uniformly dense within, but were rather filamentous. Within 1 m of a smoke source the width of the plume increased from an initial 2–3 cm to 10 cm and spread to approx. 14 cm by the time it reached the end of the tunnel (Table 1). When sighted in cross section, smoke plumes appeared most dense at the center.

Plume boundaries established by the other methods agreed well with the smoke tests (Table 1). Also, all results indicated that plumes were most dense in the center and that in moving away from center the density decreased and suddenly became undetectable. This was testified to by persons characterizing plumes of "apple oliffac" and was best documented in EAG tracings of gypsy moth antennae drawn through a disparlure plume (Figure 3). Time averaging representations of HCl vapor plumes (Figure 4) also indicated that

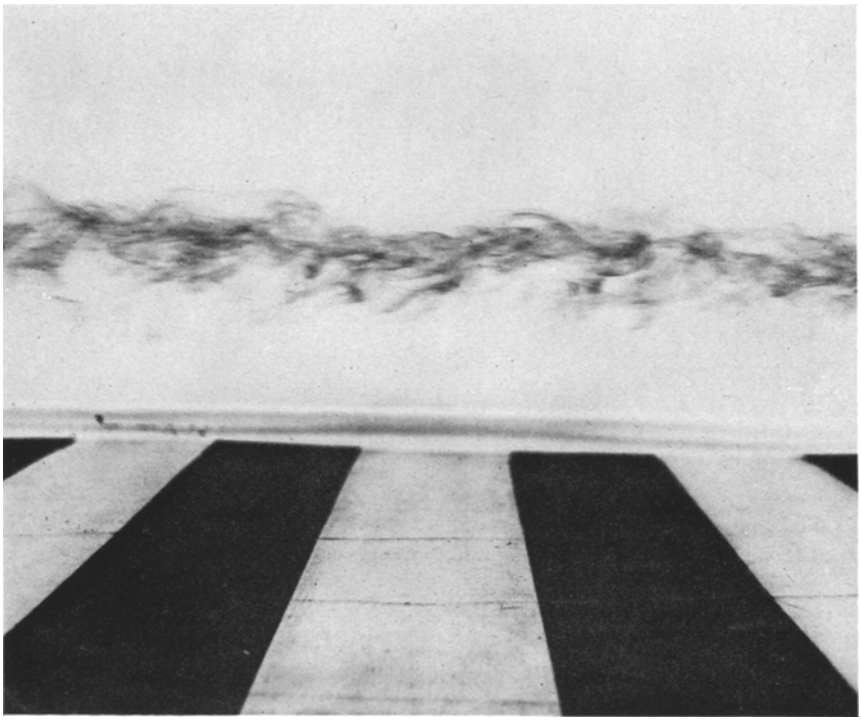

FIG. 2. Negative image closeup view of titanium dioxide smoke plume. The stripes are 15 cm wide.

TABLE 1. COMPARATIVE MEASUREMENTS OF PLUMES[a] GENERATED IN A SUSTAINED-FLIGHT TUNNEL

Method	Width (cm)	Height (cm)
Titanium dioxide smoke	14.1±1.1 (SD)	18.3±1.0
Detection of "apple oliffac" by human nose	14.6±1.4	17.7±2.0
Detection of HCl vapors by pH-sensitive paper	15.5±2.0	17.8±1.8
Detection of disparlure plume by gypsy moth electroantennogram	13.5±0.9	—

[a] All compounds were applied to 2.4-cm-diam filter paper disks. Measurements were taken at the downwind end of the tunnel and wind velocity was 65 cm/sec.

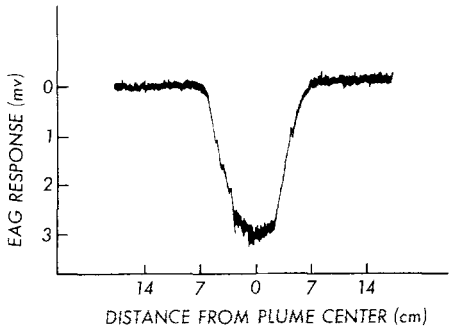

FIG. 3. Electroantennogram tracing of a gypsy moth antenna drawn horizontally through a plume of (±)-disparlure.

boundaries were sharp. During the time course of color development an area approx. 6 cm in diameter always developed uniformly and more rapidly than other areas of the pH sensitive paper, indicating a dense plume center.

Uses of the Flight Tunnel

Behavioral tests on over a dozen different species of moths from 7 different families have been conducted in the flight tunnel in widely differing types of experiments. One very important use is for discriminative bioassays during pheromone isolation and identification. Before beginning isolation procedures, it is wise to ascertain that an extract contains compounds that elicit positive anemotaxis and not just sexual excitation. It is our experience that *sustained* positive anemotactic flights in the tunnel are elicited only by treatments approximating very closely the natural pheromone and that such treatments have a good chance of yielding trap catches in the field. During fractionations the loss or absence of a requisite pheromone component is readily detected by flight tunnel bioassay. The present flight tunnel provided key information allowing the identification of a requisite second pheromone component from the black cutworm, *Agrotis ipsilon* (Hufnagel) (Hill et al., 1977) and is presently being used in a number of other pheromone identifications.

Likewise, flight tunnel measurements of the attractancy of synthetic compounds can be made directly relevant to field performance. For example, it was known from trap catches in the field (Miller and Roelofs, 1977b) that the (+) enantiomer of disparlure was highly attractive to male gypsy moths and that addition of small amounts of the antipode drastically reduced trap catch. An identical relationship was found (Miller and Roelofs,

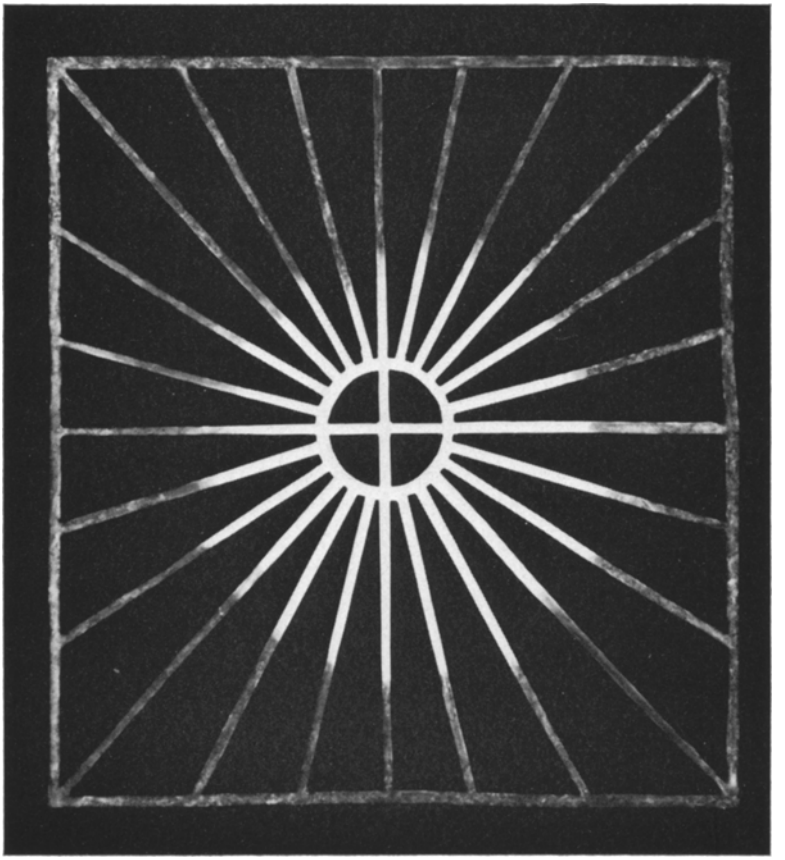

FIG. 4. HC1 plume tracing on a 33 × 35-cm filter paper grid impregnated with crystal violet dye.

1978) between the optical purity of (+)–disparlure and the duration of anemotactic flights measured in the flight tunnel. In these experiments flying moths were held in place at the center of the tunnel by means of the moving belt.

In the same study traps were hung in the tunnel for determining both improved trap design and relative trap catches by various ratios of disparlure enantiomers. Also, several plumes were generated simultaneously from separate and chemically different sources for choice tests. It should be noted that tests in which flights were not prolonged by the belt were informative but showed little difference among disparlure treatments. The shortcoming of such tests was that most responding insects reached the source very quickly

so that duration and persistence of response could not be measured. The persistency of response is likely to be a very important factor affecting trap catch in the field, and this emphasizes the necessity of a moving floor for sustaining anemotactic flights in the tunnel. Without this provision it would be difficult to relate behavioral observations in the flight tunnel to trap catch results in the field.

Movement of the tunnel floor has directly influenced the airspeeds of all moth species so far tested, including those such as the black cutworm flown at 0.05 lux. Under optimal conditioning the flights of moths responding to their natural pheromones in the tunnel were surprisingly long. For example, the mean flight duration for male gypsy moths responding to (+)-disparlure

TABLE 2. KINDS OF BEHAVIORAL RESPONSES RECORDED FOR *Argyrotaenia velutinana* EXPOSED TO ITS SEX PHEROMONE IN A FLIGHT TUNNEL WITH FLOOR PATTERN STATIONARY[a]

Type of behavior	$\bar{x}\pm$SD	Range
Time[b] from source introduction until first visible response	6.9± 2.1	3 – 9
Time walking or running before flight	8.3± 4.4	3 –15
Time wing-fanning before flight	4.2± 3.3	0 – 9
Time from source introduction until flight initiation	27.3±26.8	12 –85
Time hovering in plume before making upwind progress	5.1± 3.3	1 –11
Flight speed (ground) over a 70-cm sector in the downwind half of the tunnel (cm/sec)	17.1± 7.1	10.8–31.8
Flight speed (ground) over a 70-cm sector in the upwind half of the tunnel (cm/sec)	14.4± 3.8	9.0–21.3
Number of approaches to within 2–3 cm of the source before landing	1.9± 0.9	1 – 3
Number of times dropping back more than 30 cm and reorienting before landing	0.3± 0.8	0 – 2
Spot where lands:		
Directly on chemical source	71%	
On structures supporting source	29%	
Time landing after first coming within 45 cm of source	10.9± 5.3	4 –19
Elapsed time from flight initiation until landing on source	24.3± 5.7	15 –32
Time walking or running on source	8.0± 9.3	3 –29
Time wing-fanning on source	8.2± 9.7	3 –30
Time genital claspers extended	8.2± 9.7	3 –30
Number of copulatory attempts	0.1± 0.4	0 – 1
Time quiescent on source	0.1± 0.2	0 – 0.5
Total time on source	9.9±14.2	3 –42

[a] The data are from a preliminary experiment ($N = 7$) and characterize the first in what is usually a series of orientations. Wind velocity was 65 cm/sec.
[b] In all cases time is expressed in seconds.

was 36.7 min (range 3–83) (Miller and Roelofs, 1978) and flight durations of 60 min and longer have been recorded for male redbanded leafroller moths.

Detailed Observations of Male Argyrotaenia velutinana *Behavior*

The flight tunnel has proven to be very useful in detailed behavioral studies of the total sequence of moth response to pheromone. Presented in Table 2 is a listing of the kinds of data that are being recorded for male redbanded leafroller moths studied individually without movement of the floor pattern. Attention was paid to the behavior of moths leaving as well as approaching the pheromone source. After leaving the source a male usually flew downwind and began reorienting to the source immediately. The mean number of successive orientations was 3.4 ± 2.5 SD (range 1–10). Eventually moths left the vicinity of the source and plume and after a period of flying and walking, often accompanied by wing-fanning, they became sedentary. If captured and returned to the release cage 20–30 min after leaving the source, most males proceeded through multiple series ($\bar{x} = 1.7 \pm 0.8$, range 1–3) of orientations, and some began sequential series of orientations spontaneously. A comparison of detailed data for each successive orientation and interim rest period should provide a clearer understanding of the behavioral effects of previous exposures to pheromone. A detailed report on *Argyrotaenia velutinana* responses will be forthcoming.

CONCLUSIONS

The sustained-flight tunnel described herein has proved to be a very valuable multipurpose tool in moth pheromone research and similar apparatuses should prove useful in behavioral studies on other winged insects. Considerable variability can be envisioned in the design of the apparatus; however, one important feature that should be incorporated is a moving floor allowing measurement of response persistency. Just as flight tunnels can be very useful in elucidating the behavioral responses whereby insects locate a point source of pheromone, they should prove equally useful in elucidating how sexual communication is disrupted by the technique of atmospheric permeation with pheromone.

Acknowledgments—We thank Dr. J.S. Kennedy for inspiring discussions on the mechanisms by which insects follow an odor plume and for impressing upon us the utility of exploiting the optomotor response to control the flights of insects responding anemotactically. We also thank Mr. S.A. DeMaria for his competent assistance in the construction of the flight tunnel, the USDA Gypsy Moth Methods Laboratory, Otis AFB, Massachusetts, for supplying gypsy moths, Ms R. McMillen and G. Catlin for preparing the figures, and Dr. R.M. Silverstein for suggesting the use of perfumes for plume characterization.

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SOME ASPECTS OF OVIPOSITION SITE SELECTION IN *Monochamus notatus* AND *M. scutellatus* (COLEOPTERA: CERAMBYCIDAE)

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Abstract—The composition of monoterpene hydrocarbons and water content of freshly felled white pine logs (unattractive to *Monochamus* for oviposition) was compared to that of the same logs after aging long enough to become attractive to *Monochamus*. No significant differences were found. Variations between trees was the greatest source of variance in the data. If monoterpenes are used in host selection they are probably used to locate the host species and not to identify logs suitable for oviposition.

Key Words—Host selection, *Monochamus*, terpenes, oviposition.

INTRODUCTION

Wood borers of the genus *Monochamus* are the greatest single threat to softwood logs that must remain in the woods during the insect's breeding season in eastern Canada. They are also a threat to standing trees killed by fires or insect pests.

To date there has been little work published on the host attraction of *Monochamus*. Although *M. scutellatus* has been seen ovipositing on freshly felled logs (Wilson, 1961), several reports indicate that *Monochamus* will not oviposit on logs until they have been felled for at least a week (Raske, 1972), and the logs remain attractive for up to a year, although trees cut within a few months of insects' breeding season were most attractive. Clemens (1916) reported that the factor attracting *M. scutellatus* (Say) to injured or felled trees is its resinous odor.

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Several scolytid bark beetles were attracted to the same host trees as sawyer beetles. Chapman (1963) found that scolytids respond to log odors. There were varying degrees of specificity exhibited by different species.

Rudinsky (1966) concluded that scolytid dispersal flight is directed toward breeding material in the forest by volatile terpenes. This "primary" attraction precedes the "secondary attraction" that occurs in several species in response to pheromones (Renwick and Vité, 1970; Borden et al., 1975). It therefore seemed appropriate to look for chemical clues to the difference between logs attractive to *Monochamus* and those which are not.

There are extensive reports in the literature on the terpene content of various parts of many members of the family Pinaceae. Mirov (1946) states that each pine has its peculiar turpentine, the chemical composition of which is specific. *Pinus strobus* is a preferred host of *Monochamus*, but in New Brunswick *M. scutellatus* is also found on *Abies balsamea*, *Picea glauca*, and *Larix*. The turpentine of fresh wood chips of *Pinus strobus* contains 67% alpha-pinene, 2.9% camphene, 18% beta-pinene, 0.9% myrcene, and 0.9% limonene; varying concentrations of these same monoterpenes are found in *Abies balsamea*, *Picea glauca*, and other Pinaceae (Drew and Pylant, 1966).

Oudin (1958) reported large variations in individual *Pinus pinaster* from the "typical" turpentine composition. Mirov (1961) did an extensive study on the gum turpentines obtained by steam distillation of samples from a comprehensive collection of pines. He noted that there is often considerable variability in the turpentine composition within a species, which he speculated was caused by interbreeding, mutation, or individual variability.

A study on ponderosa pine (*Pinus ponderosa*) by Smith (1964a) on the effects of age of the tree on the wood oleoresins indicated that the proportions of each terpene remained remarkably constant in any one tree over many years. He previously ascertained that oleoresin, once produced, does not change over time in a living tree. Smith (1964b) concluded that the principal source of variation in terpenes in *P. ponderosa* appears to be intraspecific variation even in trees growing in the same plots.

In *Pinus pinaster*, Bernard-Dagan et al. (1971) report that the terpene content of the wood did not change with age of the sample. Seasons were shown to have no effect on the monoterpene content of either wood or cortical tissues, but injury by insects caused an increase in the beta-pinene content in both tissues.

In summary, in addition to marked species differences, it appears that the pines are characterized by a high level of individual variation in the monoterpene content of their wood, although in any individual the amounts of terpene produced are quite constant.

METHODS AND MATERIALS

Method of Terpene Analysis

Ten *Pinus strobus*, 20.3–30.5 cm dbh (diameter at breast height), from a small plantation in the University of New Brunswick woodlot were felled and cut into logs as needed for maintenance of cultures of *Monochamus scutellatus* (Say) and *M. notatus* (Drury). The logs were stored in the laboratory after the ends were coated with paraffin to reduce moisture loss.

On the day of felling, two samples of bark and phloem (approximately 20 g) were taken from each tree, one about 3 ft from ground level; the other, in the region where the diameter approximated 7.6 cm.

An additional set of samples were taken from these same regions 23–27 days later. To determine whether the logs were attractive to *Monochamus* at this time, the logs were placed in the breeding cages for one week, and checked for evidence of larval activity after a month.

Extracts were prepared as follows: Ten grams of each sample were coarsely chopped, then ground for three minutes in a Waring blender with 80 ml of redistilled petroleum ether. The remainder of each sample was weighed before and after oven drying to determine water content.

The ground material was transferred to an Erlenmeyer flask together with 30 ml of the solvent used to rinse the blender, stirred for 24 h, filtered into a volumetric flask, and dried with anhydrous magnesium sulfate.

The prepared extracts were analyzed by injecting 0.1 or 2.2 μl into a Perkin Elmer 990 gas chromatograph, equipped with a flame ionization detector. A 3 m $\times$ 6.35 mm OD stainless-steel column packed with 4% Carbowax 20 M on 60/80 mesh Chromosorb G NAW was used. The injection port temperature was 165°C; detector temperature, 180°C; and column temperature, 90°C for 24 min, after which it rose 12°C/min to a final temperature of 120°C. Helium carrier gas flow was approximately 30 ml/min. Recorder attenuation was adjusted to permit all peaks to be shown in their entirety.

Qualitative determinations of the terpenes in the sample were made by comparing retention times with those of pure commercial terpenes dissolved in redistilled petroleum ether, either alone or in combination. The solvent was analyzed periodically to ascertain its purity.

Quantitative determinations were made by measuring the relative area under the peaks. A Model 1 analysis of variance was used to test for significant difference between treatments.

Oviposition Observations

Insects of known age were obtained from a laboratory colony. Mature

female beetles were placed with a fertile male into individual cages supplied with a pine bolt, 7.6–15.2 cm in length and 3.8–7.6 cm in diameter cut from the top of a tree felled at least two weeks previously. The moisture content of the tree top was kept high by immersing one end of the log in water. At the end of each week the bark was removed and the eggs counted. The water content of the bark and of the wood was then measured by weighing it before and after oven drying. Each week, the cages were supplied with a new pine bolt.

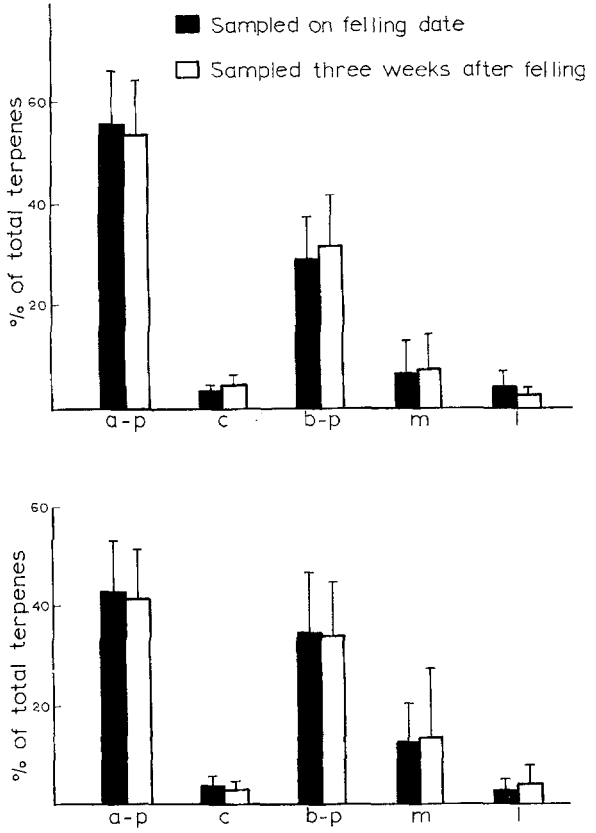

FIG. 1. A comparison of the mean terpene composition of freshly felled logs with the composition in the same logs 23–26 days later. Above: samples taken near the base of the trees. Below: samples taken near the top of the tree. Legend: a-p, alpha-pinene; b-p, beta pinene; c, camphene; m, myrcene; l, limonene; vertical lines, SD $\bar{X}$.

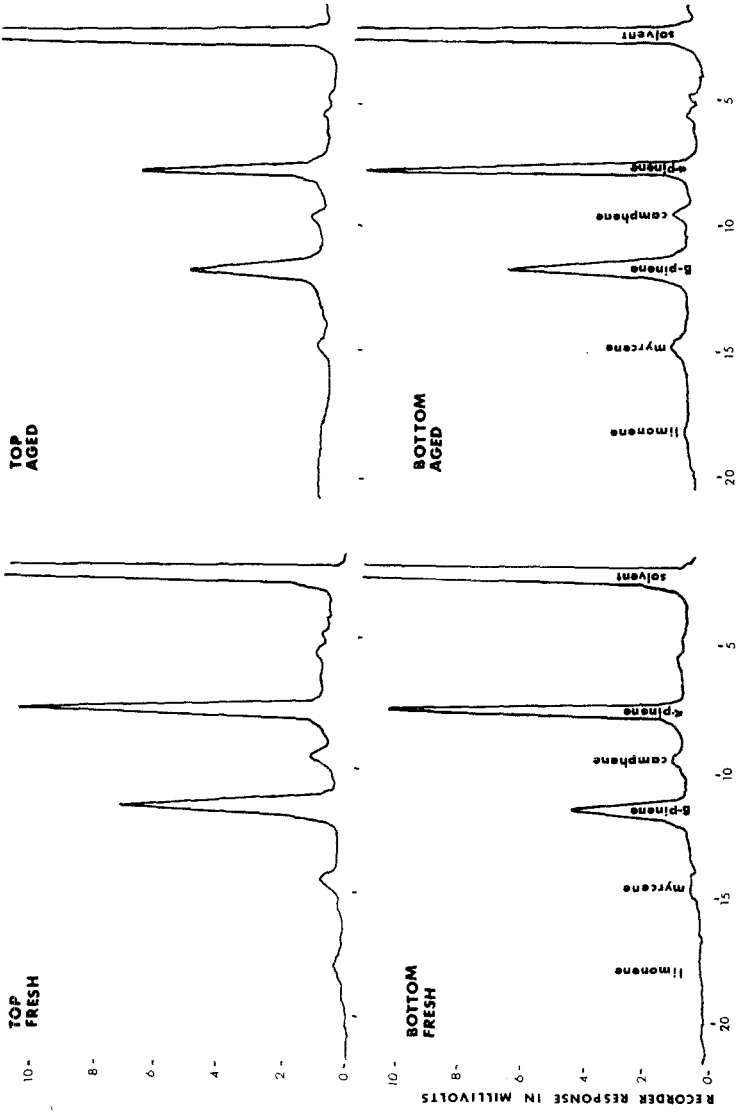

FIG. 2. Gas chromatograph outputs of extracts from samples taken near the bottom and at the 7.6-cm-diameter level of a white pine compared with data from samples taken from the same logs 26 days later.

RESULTS

Chemical Analysis of White Pine Logs

The analyses of the terpene composition of the bark and phloem of freshly felled pine trees and of the same trees several weeks later are summarized in Figure 1. The recorder outputs for representative analyses of two trees are presented in Figures 2 and 3.

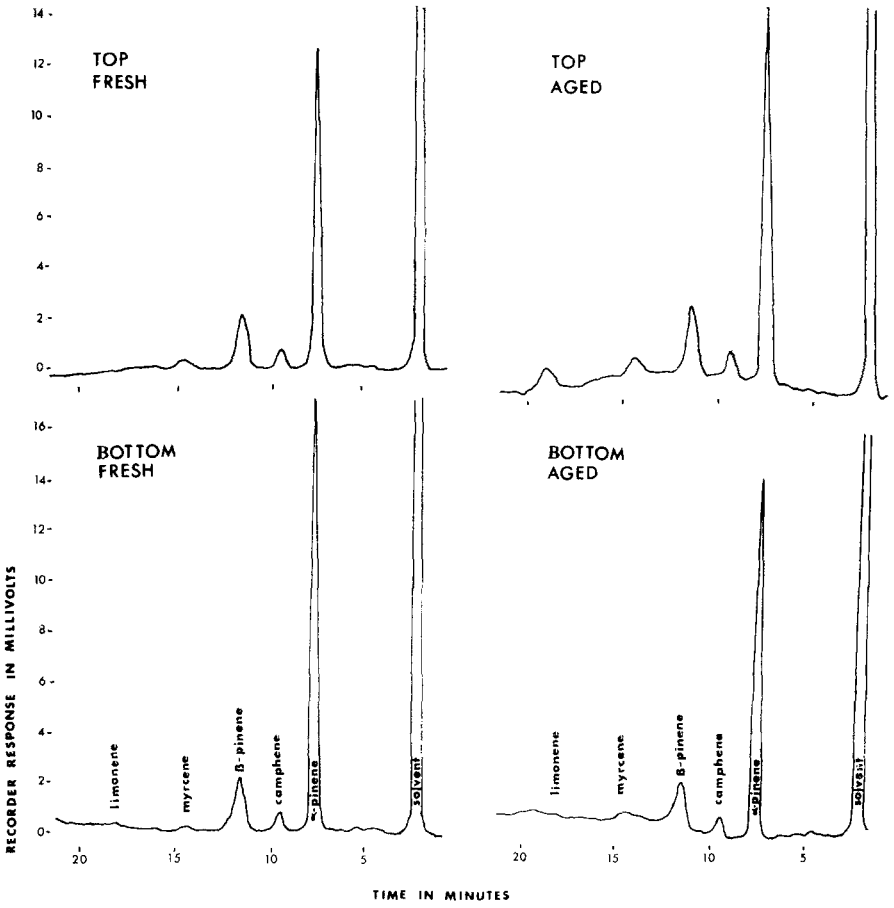

FIG. 3. Gas chromatograph output from samples taken near the bottom and at the 7.6-cm-diameter level of a white pine compared with data from samples taken from the same logs 23 days later. Note the difference in the terpene composition between this white pine and the tree analyzed in Figure 2.

An analysis of variance of these data showed no significant change in the percent of any of the five terpenes detected over the time tested and no significant interaction between the time of felling and the location on the tree from which the sample was taken.

However, for two terpenes there was a significant difference between the percent concentration of terpene found near the bottom of the tree and that found near the top. In freshly cut logs, there was an average of 56.3±11.5% alpha-pinene in the total terpenes at the bottom of the tree, but only 43.5± 11.8% at the top. Several weeks later, the percentage of alpha-pinene at the bottom of the tree was still larger, at 54.4±11.6%, than at the top, 42.2± 10.5%. This difference due to location on the tree was significant at the 1% level. The percentage of myrcene at the top of the tree, 13.5±8.5% in newly cut logs and 14.0±14.4% in older logs, was also significantly different at the 5% level from the percentage at the bottom, 6.3±7.4% and 7.6± 6.8%, respectively.

In general, the variation in terpene composition between trees was larger than the variation due to either location on the tree from which the sample was taken, or to age differences of several weeks.

An analysis of variance showed that the water content of the bark of

TABLE 1. WATER CONTENT OF PINE BARK IN FRESHLY FELLED LOGS AND IN THE SAME LOGS AFTER AGING 23–26 DAYS

	Water content (%)		Change (%)
	Freshly felled logs	Aged logs	
Near top of tree			
1	57.4	61.0	+3.6
2	52.9	57.0	+4.1
3	58	45.5	−12.5
4	56	52.5	−3.5
5	56.2	56.4	+0.2
Mean	56.1±2.0	54.5±5.8	
Near base of tree			
1		52	
2	39.4	41.0	+1.6
		55.4	
4	51	55.3	+4.3
5	53.8	53.4	−0.4
Mean	48.1±7.0	51.4±6.2	
Over-all mean	53.1±6.0	52.9±5.8	−0.2

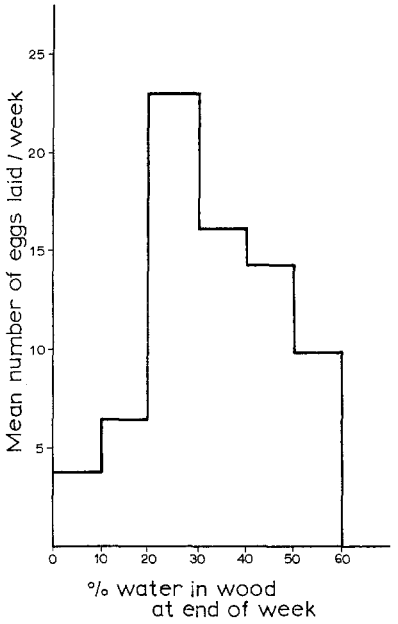

FIG. 4. Effect of the moisture content of white pine blocks on the oviposition of *Monochamus* spp. Moisture content of wood kept in a cage with mature females for one week was measured at the end of the week and compared with the number of eggs found in the bark at the end of the week.

freshly felled logs was not significantly different from that of the same logs aged for 23–26 days after felling (Table 1). However, it should be noted that the number of samples analyzed successfully in this case was quite low. In some cases the water content rose during the elapsed time, and in other cases it fell slightly.

Oviposition

Females laid few or no eggs if the water content of the pine blocks available to them was below 10% or above 60%. The relationship between the moisture content of the wood and oviposition is presented in Figure 4.

DISCUSSION

The analyses of white pine (Figure 1) presented in this paper failed to show a distinct change in the proportions of monoterpenes present over time

comparable to those found by Werner (1972) in loblolly pine. The fact that he used a more uniform, even-aged population of trees than those used for this study should not influence the results. Several reports indicate that the terpene content of a tree remains unchanged over most of its life (Smith, 1964a, b; Bernard-Dagan et al., 1971).

Another factor other than species difference which could contribute to the difference between the results of terpene analysis in white pine reported here and in loblolly pine is the difference in techniques of collection and analysis of samples. Werner sampled living trees in the field, sealed the samples in plastic bags and froze them immediately in a dry ice–isopentane solution. In this study on white pine, freshly felled logs were brought to the laboratory before samples were taken, making it unnecessary to freeze them for transport. It is quite possible that significant changes occur in the terpene content during the hour or two between felling and sampling times. However, freshly felled logs do not become attractive to *Monochamus* for oviposition until after a week or more. Therefore the use of felled logs rather than living trees is valid in a search for a chemical difference between attractive and unattractive host material.

No significant differences were detected in the percent concentration of monoterpenes in attractive and freshly felled, unattractive white pine logs (Figure 1). Also, the individual differences in terpene composition are so large (Figures 2 and 3) that changes after death would have to be extreme to be separable from them. The large individual variations are consistent with those found in other pines (Oudin, 1958; Tobolski, 1968; Werner, 1972; Smith 1964b). Extreme variation of a tree's terpene content from the usual range could explain the observation that some logs in many infested piles are not attacked (Graham, 1925; Raske, 1972).

Although the percent concentrations of monoterpenoid hydrocarbons present in attractive logs are indistinguishable from those of freshly felled logs (Figure 1), this does not necessarily mean that these or other terpenes are not host attractants. Behavioral tests showed that the mixtures of terpenes are indeed attractive to these insects (Dyer and Seabrook, unpublished results). Terpenes may be used to find specimens of the preferred host species, and some other signal could be used to indicate the physiological status of the host. This hypothesis would fit with the observed fact that the beetles do select both living *Pinus* for feeding activity and dead trees for oviposition.

There are several possible ways that *Monochamus* could assess host suitability for oviposition. They may be sensitive to decay products of wood or terpenes, or to changes in the concentration of attractant terpenes associated with felling. Other possibilities which are not olfactory in nature are a sensitivity to change in resin pressure or to changes in water relations associated with death of the tree.

Decay products such as ethanol or methanol are not specific to a particular host, and therefore could not alone explain the narrow host preference of *M. notatus*. Beetles such as *Trypodendron* which do use ethanol as a primary attractant (Moeck, 1970) have an extremely wide range of hosts. However, ethanol can synergize the attractiveness of host terpenes for scolytids (Nijholt and Shonherr, 1976). Thus ethanol may be the signal indicating the suitability of the host for oviposition when combined with terpenes. Other candidates are the decay products of terpenes themselves. Sumimoto et al. (1975) have implicated alcohols produced by the oxidation of terpenes as the host attractants in a Japanese scolytid, but it is not clear from their paper if these compounds are oxidized in the host tree. They did find some but not all of the attractants in host material. Perttunen et al. (1970) also found degradation products of terpenes which were attractive to bark beetles in host trees.

Any insect that has selected a conifer as a host must be able to tolerate or avoid the detrimental effects of the host oleoresin system. Trees vary greatly in all aspects of this system. The complexity and diversity of oleoresin systems, especially the close relationship with the water status of the tree, provided a logical basis for the observed selectivity of hosts by insects. Such selectivity can be influenced by environmental conditions; Vité (1961) found that *Pinus ponderosa* trees or portions thereof in a stressed or weakened condition due to prevailing environmental conditions have the resin pressure reduced to a level which makes it innocuous to invading bark beetles. The use of this clue by *Monochamus* cannot be ruled out on available evidence. It could not operate in long-distance attraction, but may well be a factor in the release of ovipositional behavior.

A determination of the time necessary for the oleoresin pressure to drop to zero after tree cutting would provide evidence of the probable importance of this clue in white pine. In ponderosa pine, Vité (1961) has found that the resin pressure drops to zero within 24 hr, if the branches are left on a felled tree, but the process takes several days if the branches are removed. This is less time than is usually needed for felled white pine to become attractive to *Monochamus*, although there are isolated reports of attack within one or two days of death (Morgan, 1948; Wilson, 1961).

The water content of white pine also did not change significantly between freshly felled trees and the same logs several weeks later (Table 1). Water content of the tissues would be comparable to the presence of ethanol in its lack of specificity to a particular species of tree. It can thus be eliminated as a possible primary attractant for *Monochamus*. On the other hand, it probably is used as one indicator of host suitability for oviposition (Figure 4). The inhibition of oviposition in dry wood is not surprising in view of the long period of time spent by the larval stages in wood prior to pupation (1–2

years). A supply of water would be essential during this period.

Ovipositional behavior is not necessarily mediated by the same signals as host attraction. Observations of prolonged palpation of newly cut ovipositional slits indicate that contact chemoreception plays a large part in oviposition site selection.

Both olfactory clues such as the presence of decay products, particularly of terpenes, and nonolfactory clues such as a reduced resin pressure combined with the presence of a favorable phloem moisture content could be effective signals of the physiological state of a tree over the necessary timespan. Any combination of these may well be used in addition to the presence of volatile host monoterpenes to discriminate trees suitable for oviposition. It is likely that a combination of factors are used for different aspects of host attraction. Host selection for oviposition is probably not a single reflex response, but rather requires considerable integration of a variety of sensory information.

CONCLUSIONS

The composition of monoterpenoid hydrocarbons in freshly felled white pine logs, which are unattractive to *Monochamus*, is not significantly different from the monoterpene composition of the same logs aged several weeks, which are attractive. The water content also did not change significantly. Therefore these host monoterpenes could not be used by the beetles to discriminate live trees from logs suitable for oviposition, although they may be used to identify host species. These insects will not oviposit unless the water content of the logs is between 10% and 50% of total wet weight.

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IDENTIFICATION OF THE CALIFORNIA RED SCALE[1] SEX PHEROMONE[2]

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Abstract—Pheromone components of female California red scale, *Aonidiella aurantii* (Maskell) were isolated from airborne collections and found to be 3-methyl-6-isopropenyl-9-decen-1-yl acetate and (*Z*)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate. Both enantiomers of the latter compound as well as the corresponding enantiomers of the *E* isomer were prepared from (*S*)- or (*R*)-carvone. Bioassays with each of the four isomers showed that only the *R,Z* isomer attracted male red scale.

Key Words—California red scale, *Aonidiella aurantii*, pheromone, attractant, enantiomer, isomers (*E,Z*)-3-methyl-6-isopropenyl-9-decen-1-yl acetate, (*Z*)-3-methyl-6-isopropenyl-3,9-decadien-1-yl acetate.

INTRODUCTION

The California red scale, *Aonidiella aurantii* (Maskell), is a serious pest of citrus in many parts of the world, particularly in California, Australia, and the Mediterranean countries. Tashiro and Chambers (1967) found that the female scales produce a sex pheromone that can be used to attract male red scales. Rice and Moreno (1969) demonstrated that a trap consisting of sexually mature virgin females on a lemon in a pint carton with an attached

[1] *Aonidiella aurantii* (Maskell) (Homoptera: Diaspididae).
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sticky card was highly effective in catching males in citrus groves, and Shaw et al. (1971) showed that such traps were an excellent substitute for the laborious visual inspection for detecting infestations.

Interest was generated in chemically defining the sex pheromone components because it would represent the first for a Homopteran species, and also because the synthetic pheromone could replace the cumbersome and expensive virgin female traps in the field. Furthermore, the risk of a trap bearing virgin females becoming damaged, allowing mating and subsequent infestation by these females, would be eliminated by a synthetic pheromone. Laboratory rearing procedures were developed (Tashiro, 1966), as well as a good turntable olfactometer for bioassays (Tashiro et al., 1969). Studies (Tashiro and Chambers, 1967) showed that an active extract was obtained with cold-trap condensate from air passed over female red scale-infested lemons and with whole-body extractions. Preliminary identification tests indicated that the pheromone could be an unsaturated acetate (Warthen et al., 1970).

METHODS AND MATERIALS

Pheromone Collection and Bioassay

Our initial studies were conducted with cold-trap condensate that was processed by extracting its steam distillate with ether–hexane (3:1). The resulting extract contained large quantities of contaminating terpenes and other volatiles from the lemons and made the pheromone purification steps more difficult. The host material was changed to potatoes (Rice and Moreno, 1969), and the cold trap was replaced with a Porapak-Q® solid absorption trap (Byrne et al., 1975). This improved system was used to collect an estimated 400 million female day-equivalents of pheromone for the identifiction of two sex pheromone components.

Airborne collections were made by passing air over scale-infested host material contained in a 45-liter glass jar and through a 2.5×30-cm column of Porapak-Q (Waters Associates, Inc.) (Figure 1). Initially male scales were selectively killed while female scales were in the second molt by dipping scale-infested lemons into a 0.5% suspension of dichlorvos (Shaw et al., 1973); the females were used for pheromone collection when sexually mature (approx. 27 days old) for a period of 2 weeks. The same isolation technique was used with various other host materials, such as rose cuttings, butternut squash, banana squash, summer squash, the wild gourd *Cucurbita foetidissima*, oranges, and grapefruit, but lemons were preferred because of their year-around availability, size, and long shelf-life. However, the large quantities of lemon volatiles obtained in airborne collections created problems in

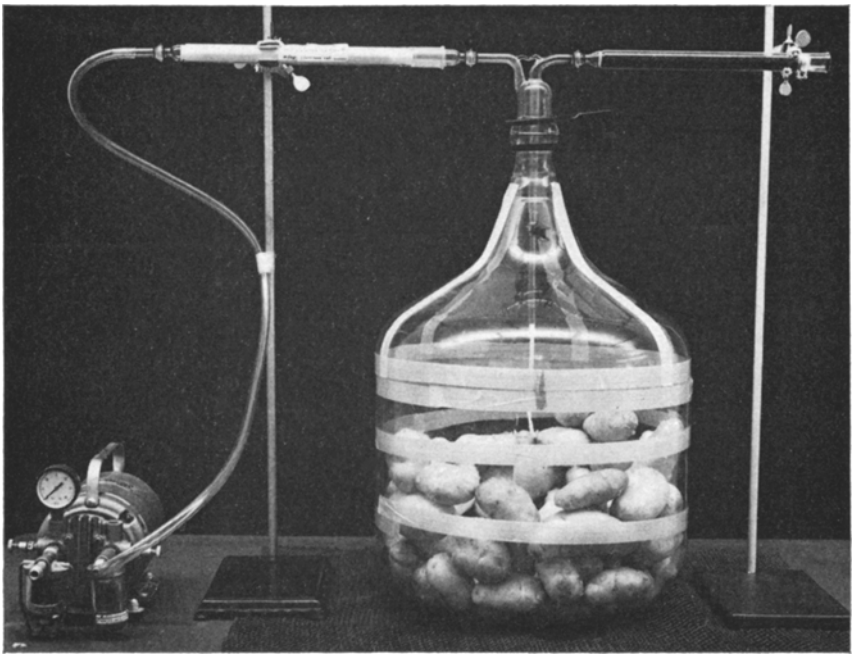


FIG. 1. Airborne collection with Porapak-Q of pheromone emitted by virgin female red scales on potatoes.

the isolation procedure. An alternative host with less volatiles was sought. Rice and Moreno (1969) found that white rose, russett, and sweet potatoes were suitable hosts, although the use of dichlorvos to isolate female scales was not applicable with potatoes because it killed all the scales. Juvenoids were found (Moreno et al., 1976) to selectively prevent male maturation, and so a formulation of 1 ppm of triprene was sprayed on first-molt scales to prevent male metamorphosis. Female scales matured at approx 35 days old, and their pheromone was collected for a period of 2 weeks. Thus, the predominant host in the last year of pheromone collection was potatoes. In a typical run, air was drawn over about 150 infested potatoes per jar for 7 days before changing the Porapak column. The used Porapak was extracted with 200 ml of pentane, the solvent evaporated, and the extracted residue used in further purification steps.

Bioassays were conducted in the greenhouse with a turntable olfactometer modified from Tashiro et al. (1969). The 1.8-m-diam table traveled at the rate of approx. 0.17 rpm. It accommodated 24 Munger cells (Munger and Gilmore, 1963), which contained either a section of lemon with virgin female

scales or test sample on a 2.4-cm-diam filter paper disk placed in a 3.5-cm steel planchet. Treatments were set out in the early afternoon, and male scales emerging the same afternoon from male and female-infested lemons were captured on 7.5×12.7-cm sticky cards (Bird Tanglefoot®) positioned above the Munger cells. Counts were made the next morning.

Pheromone Purification

The oily residue from the airborne collection first was chromatographed on a 1.5×50-cm column of florisil (60–80 PR mesh, Floridin Co.) eluted with 900 ml of a 0–50% gradient of diethyl ether in Skelly B. A 0.25-ml aliquot from every second fraction (15 ml each) was bioassayed.

Active fractions from the florisil column were combined and injected onto a 1×100-cm-high pressure column packed with 10% $AgNO_3$ on Bio-Sil A (20–44 μm, Bio-Rad Laboratories). The column was eluted with 500 ml of 10% diethyl ether in benzene and then with 500 ml of 20% ether in benzene. A 0.25-ml aliquot of every third fraction (15 ml each) was bioassayed, and the fractions containing each active component were combined.

Each component was purified further on a 2×500-mm LiChrosorb® (10 μm) high-pressure column by eluting (1 ml/min) with 20 ml of 1% ethyl acetate in heptane. Fractions (0.5 or 1.0 ml) were bioassayed or assayed by GLC.

GLC columns were 3% OV-1 or 3% OV-101 (methyl silicone) on 100-120 mesh Gas-Chrom Q, and 10% XF-1150 (50% cyanoethyl methyl silicone) or 3% Carbowax 20 M on 100-120 mesh Chromosorb W-AW-DMCS.

Identification

Microchemical reactions were carried out in 3.7-ml (1-dram) vials. The reduction of acetates to alcohols was accomplished by adding $LiAlH_4$ to an aliquot of active component in diethyl ether. A few drops of ethanol were added after 1 hr followed by a few drops of water. The ether was decanted, dried over $MgSO_4$, and assayed by GLC and greenhouse activity. The product was acetylated by adding a few drops of acetyl chloride and evaporating the excess acetyl chloride after 0.5 hr.

Microozonolyses (Beroza and Bierl, 1967) in CS_2 were analyzed by GLC with the Carbowax column. Hydrogenations were carried out at atmospheric pressure with either 5% palladium on calcium carbonate in methanol or with platinum oxide in methanol or hexane. Synthetic 3-oxobutan-1-yl acetate was prepared by acetylating 3-methyl-3-buten-1-ol and ozonizing the resulting acetate.

The pheromone hydrocarbon skeleton was prepared by saponifying the

hydrogenated product with 2% sodium hydroxide in aqueous methanol for 1 hr at 100°C. The product was extracted with ether and the ether removed under N_2 after drying over $MgSO_4$. The resulting alcohol was converted into the corresponding bromide by treating it with an excess amount of triphenylphosphine dibromide prepared according to Sonnet and Oliver (1976). After stirring for 4 hr at room temperature the mixture was filtered and treated with $LiAlH_4$ to produce the hydrocarbon skeleton.

Ultraviolet spectra were obtained with a Cary 15 spectrophotometer using 4×10-mm cells and pentane as the solvent. The mass spectrometer was a Hitachi RMU-6E interfaced with an OV-1 or Carbowax 20 M column. All mass spectra were obtained at 20 eV.

Nuclear magnetic resonance spectra were obtained with either a Varian XL 100 (Fourier transform) spectrometer (College of Environmental Science and Forestry, Syracuse, New York), a Varian HR 300 (Fourier transform) spectrometer (TNO, Delft, The Netherlands), or a Varian HA-100D spectrometer (Geneva, New York).

RESULTS AND DISCUSSION

Crude material from the airborne collections was fractionated on the florisil column (Table 1) and the active fractions (11–22) were combined. The active material was fractionated on the $AgNO_3$ column (Table 1), and activity was found in fractions 11–14 (AI) and fractions 29–38 (AII). The two active components were purified further on the LiChrosorb column (Table 1), and activity in each case was found in the same 0.5-ml fraction (fraction 10). The carbon numbers relative to saturated acetates for AI and AII were 12.25 and 12.06, respectively, on OV-1, and 13.08 and 13.00, respectively, on XF-1150.

The components were shown to be acetates by $LiAlH_4$ reduction to the corresponding alcohols and subsequent acetylation. The alcohol product was inactive, but acetylation restored the activity at the original retention time on OV-1. Hydrogenation of AI and AII produced the same compound, which had carbon numbers relative to saturated acetates of 12.39 on OV-1 and 11.90 on XF-1150. Mass spectra of both hydrogenated products were identical (Figure 2, AH_2) and indicated that the compound was a branched 14-carbon acetate. There was an immediate loss of acetate to give peaks at *m/e* 196 (M-60) and 61, and the next highest mass fragment (*m/e* 153) indicated a facile loss of three carbons. A mass spectrum of the hydrocarbon skeleton (Figure 2, A-Sk) of AH_2 also indicated a branched 14-carbon compound with a molecular ion peak at *m/e* 198 and an immediate three-carbon loss to *m/e* 155.

Mass spectra of AI and AII (Figure 3) showed that AII possesses three

TABLE 1. MALE RED SCALE CATCHES IN GREENHOUSE BIOASSAY OF PURIFICATION COLUMN FRACTIONS

Florisil[a]		$AgNO_3$[b]		LiChrosorb[c]	
Fraction	Males caught	Fraction	Males caught	Fraction	Males caught
2–	0	2–	9	1–	1
4–	3	5–	0	2–	2
6–	1	8–	2	3–	0
8–	1	11–	338	4–	4
10–	1	14–	17	5–	1
12–	92	17–	1	6–	0
14–	225	20–	5	7–	0
16–	212	23–	2	8–	0
18–	252	26–	2	9–	0
20–	135	29–	15	10–	128
22–	26	32–	422	11–	1
24–	3	35–	210	12–	1
26–	8	38–	10	13–	0
28–	2	41–	1	14–	0
30–	2	44–	1	15–	0
32–	3	47–	1	16–	0
				17–	0
48–	2	62–	0		

[a] Crude pheromone extract from airborne collection.
[b] Active fractions (11–22) from florisil.
[c] Active fractions (11–14) from $AgNO_3$.

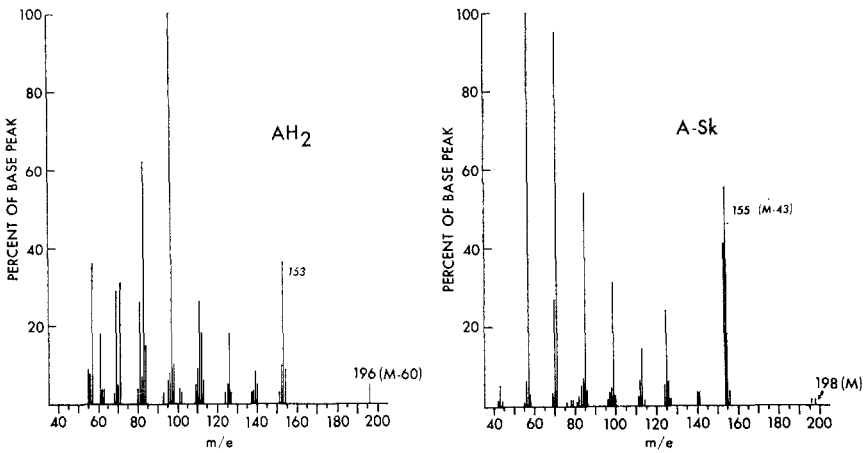


FIG. 2. Mass spectra (20 eV) of hydrogenated AII (AH_2) and of its hydrocarbon skeleton (A-Sk).

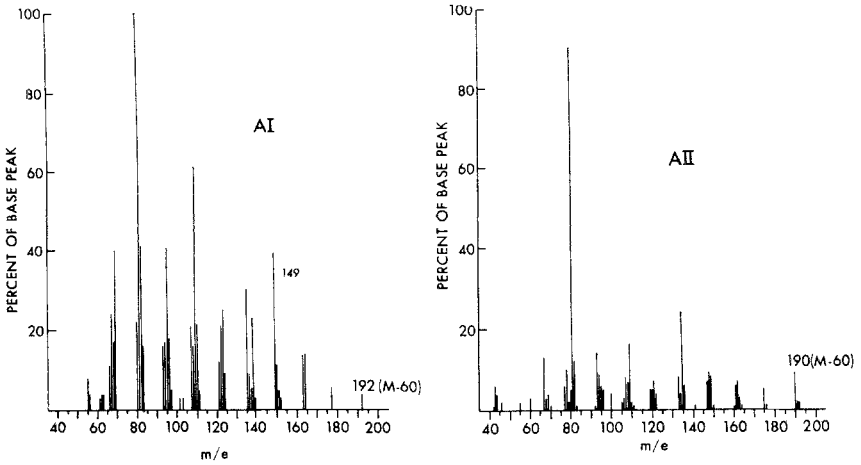


FIG. 3. Mass spectra (20 eV) of AI and AII.

units of unsaturation and AI only two, since the M-60 peaks were found at *m/e* 190 and 192, respectively, compared to 196 for the saturated AH_2.

Ozonolysis of AI did not yield any GLC visible product, but ozonolysis of AII produced one major product that was identified as 3-oxobutan-1-yl acetate by comparison of its retention time on Carbowax 20 M (6.5 min when programed from 80° at a rate of 10°/min) and its mass spectrum with

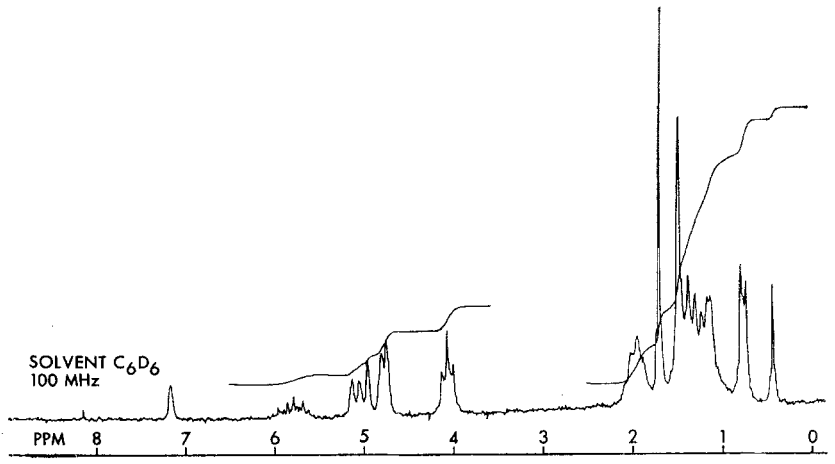
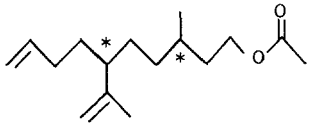


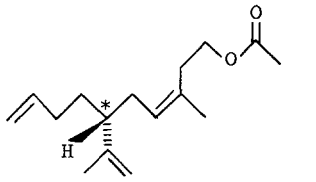
FIG. 4. NMR spectrum of AI (C_6D_6; 100 MHz).

those of a synthetic standard. This information defined a 3-methyl group on both pheromone components, and a double bond in the 3 position for AII. An ultraviolet spectrum did not exhibit any absorption attributable to a conjugated system.

Pheromone was accumulated until approximately 30 μg of each component was available for an NMR spectrum. The spectrum of AI (C_6D_6; 100 MHz) (Figure 4) was consistent with structure I with δ values as follows: 5.75 (1H, *m*), 5.07 (1H, *d*), and 5.02 (1H, *d*) ($\underline{H}_2C{=}C\underline{H}{-}CH_2{-}$); 4.82 (1H, *s*) and 4.76 (1H, *s*) ($R_2C{=}C\underline{H}_2$); 4.09 (2H, *t*, $J = 7$ Hz) and 1.7 (3H, *s*) ($-CH_2-CH_2-O-\overset{\overset{O}{\|}}{C}-C\underline{H}_3$); 1.95 (3H, *m*, allylic); 1.49 (3H, *s*) ($C\underline{H}_3-\overset{\overset{R}{|}}{C}{=}C-$); 1.1–1.6 (9H, *m*); and 0.77 ppm (3H, *d*, $J = 6$ Hz) ($R_2CH-C\underline{H}_3$).



I



II

The spectrum of AII (CS_2; 300 MHz) (Figure 5) was consistent with structure II with δ values as follows: 5.71 [1H, *m*, "*J*" = 7, 10, 16 Hz (*t,d,d*, respectively)], 4.92 (1H, *d*, "*J*" = 16 Hz), and 4.87 (1H, *d*, "*J*" = 10 Hz) ($H_2C{=}C\underline{H}{-}CH_2{-}$); 5.12 (1H, *t*, "*J*" = 6 Hz) ($R_2C{=}C\underline{H}{-}CH_2{-}$); 4.7 (1H, *s*) and 4.64 (1H, *s*) ($R_2C{=}C\underline{H}_2$); 3.95 (2H, *t*, $J = 7.5$ Hz) and 1.92 (3H, *s*) ($-CH_2-C\underline{H}_2-O-\overset{\overset{O}{\|}}{C}-C\underline{H}_3$); 2.27 (2H, *t*, $J = 7$ Hz) ($-\overset{\overset{R}{|}}{C}{=}C-C\underline{H}_2-CH_2-$); 1.9–2.1 (5H, *m*, allylic); 1.69 (3H, *s*) and 1.60 (3H, *s*) (two $C\underline{H}_3-\overset{\overset{R}{|}}{C}{=}C-$); and 1.39 ppm (2H, *m*).

An alternative structure possessing a vinyl group on the 6 position and a 9-methyl group also is generally consistent with the data, except for the

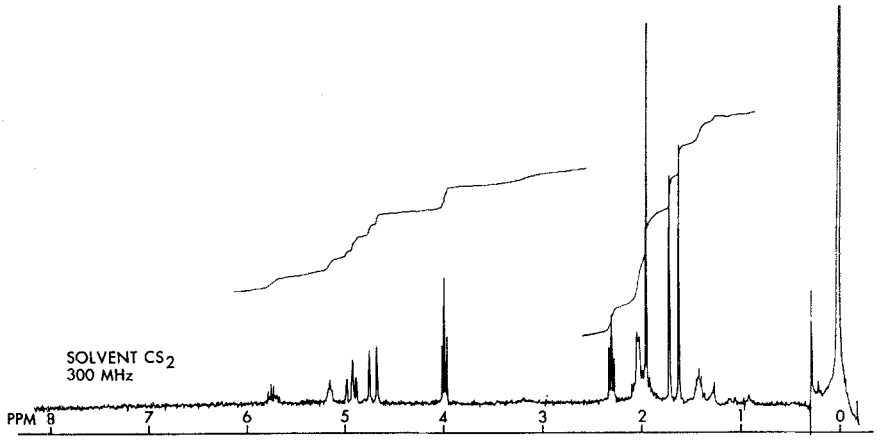


FIG. 5. NMR spectrum of AII (CS_2; 300 MHz).

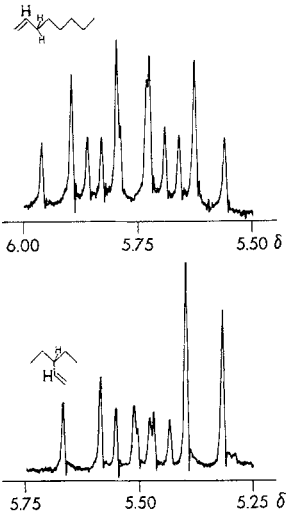


FIG. 6. The NMR splitting pattern of a vinyl proton (bold faced) with two adjacent allylic protons (top, 1-octene) and of a vinyl proton with one adjacent allylic proton (bottom, 3-ethyl-1-pentene).

characteristic splitting pattern of the 9-position vinyl proton of I and II. This proton has two adjacent allylic protons (Figure 6, top), whereas the corresponding vinyl proton of the alternative structure would be adjacent to only one allylic proton (Figure 6, bottom). The NMR spectra of standards in Figure 6 show the splitting patterns produced by the two different systems. The splitting patterns of AI and AII are consistent with that of the vinyl proton adjacent to two allylic protons.

The pheromone component AII was determined to possess a *Z* configuration by comparison of its NMR spectrum and its GLC retention times on OV-101 to those of the synthetic Z and *E* isomers of II. The 3-methyl group of the *E* isomer resonated at δ 1.60 (in CS_2), whereas the 3-methyl group of the *Z* isomer resonated at δ 1.69. The natural component had the same retention time on XF-1150 (2.9 min at 165°C) as the synthetic *Z* isomer, and there was no evidence for the *E* isomer at its retention time (3.8 min).

The NMR spectrum of the natural component AII (300 MHz) was similar to that of synthetic II, and the mass spectra of AII, AH_2, and A-Sk were identical to the corresponding synthetic compounds.

Synthesis of II

Since neither the stereochemistry of the trisubstituted double bond nor the absolute configuration of the pheromone II was known, a synthesis of all four of the possible isomers was designed. Thus the *R,Z* and *R,E* isomers of

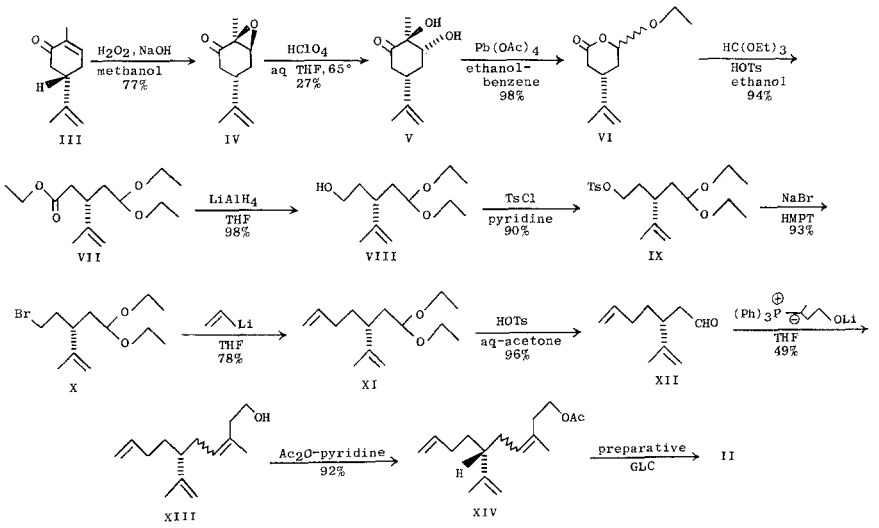


FIG. 7. Synthesis of the *R,Z* and *R,E* isomers of compound II.

compound II were prepared from (*S*)-(+)-carvone as shown in Figure 7, and the *S,Z* and *S,E* isomers were obtained from (*R*)-(−)-carvone in an analogous manner. In each case the *Z* and *E* isomers were readily separable by preparative GLC (OV-1 at 170°C, retention times of 16 and 14 min, respectively).

(*S*)-(+)-Carvone (III) was converted to the epoxide IV with alkaline hydrogen peroxide in methanol (Klein and Ohloff, 1963). Treatment of IV with dilute perchloric acid in tetrahydrofuran (THF) gave a mixture from which the keto diol V crystallized [mp 104–107°C; IR (CCl_4) 3590 and 3490 (OH), 1715 cm^{-1} (C=O); NMR ($CDCl_3$ and D_2O, δ) 4.67 and 4.87 (br *s*, 2H), 3.77 (*d* of *d*, 1H, "*J*" = 5, 11 Hz), 1.78 (*s*, 3H), and 1.37 ppm (*s*, 3H)]. Oxidation of V with 2 equivalents of lead tetraacetate in ethanol–benzene gave directly a mixture of the diastereomeric lactones VI [IR (CCl_4) 3080 ($C=CH_2$), 1760 cm^{-1} (C=0); NMR ($CDCl_3$, δ) 5.35 (*m* 1H), 4.83 (br *s*, 2H), 1.77 (br *s*, 3H), and 1.23 ppm (*t*, 3H, $J = 7$ Hz)], which were converted to the acetal ester VII with triethyl orthoformate in ethanol [VII; IR (CCl_4) 3075 ($H_2C=C$), 1740 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 4.80 (br *s*, 2H), 4.45 (*t*, 1H, $J = 6$ Hz), and 1.70 ppm (br *s*, 3H). Lithium aluminum hydride reduction of VII gave the alcohol acetal VIII [IR (CCl_4) 3630 and 3490 cm^{-1} (OH); NMR ($CDCl_3$, δ) 4.82 (br *s*, 2H), 4.47 (*t*, 1H, $J = 6$ Hz), 1.68 (*s*, 3H), and 1.20 ppm (*t*, 6H, J = 7 Hz)], which on reaction with *p*-toluenesulfonyl chloride in pyridine gave the acetal tosylate IX [NMR ($CDCl_3$, δ) 4.40 (*t*, 1H, $J = 5.5$ Hz), 3.98 (*t*, 2H, $J = 6.5$ Hz), 2.47 (*s*, 3H), and 1.18 ppm (*t*, 6H, $J = 7$ Hz)]. The tosylate was then converted to the bromo acetal X with NaBr in hexamethylphosphoric triamide (HMPT), and reaction of X with vinyllithium in THF gave the diene acetal XI [NMR ($CDCl_3$, δ) 4.43 (*t*, 1H, $J = 6$ Hz), 1.62 (*d*, 3H, $J = 1$ Hz), and 1.18 ppm (*t*, 6H, $J = 7$ Hz)]. Hydrolysis of the acetal produced the key intermediate XII [IR (CCl_4) 3080 ($H_2C=C$), 1730 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 9.75 (*t*, 1H, $J = 2$ Hz) and 1.67 ppm (br *s*, 3H)]. This diene aldehyde XII was then reacted with the ylide generated from the corresponding 3-hydroxy-1-methylpropyltriphenylphosphonium salt to give the triene alcohols XIII [IR (CCl_4) 3625 and 3540 (OH), 3075 cm^{-1} ($H_2C=C$); NMR ($CDCl_3$, δ) 3.67 (*t*, $J = 7$ Hz), 3.61 (*t*, $J = 6.5$ Hz), and 1.62 and 1.70 ppm (both br *s*, 6H)]. GLC analysis of XIII indicated that the *Z* and *E* isomers were formed in the Wittig reaction in a ratio of 52:48, respectively. Acetylation of XIII with acetic anhydride in pyridine gave the corresponding triene acetates XIV [IR (CCl_4) 3080 ($C=CH_2$), 1745 cm^{-1} (C=O); NMR ($CDCl_3$, δ) 4.10 and 4.13 (two *t*, 2H, $J = 7$ Hz), 2.03 (*s*, 3H), and 1.62 and 1.70 ppm (both br *s*, 6H)].

Pure samples of the *R,Z* and *R,E* isomers of XIV were obtained by preparative GLC separation of the mixture. The enantiomeric purity of the

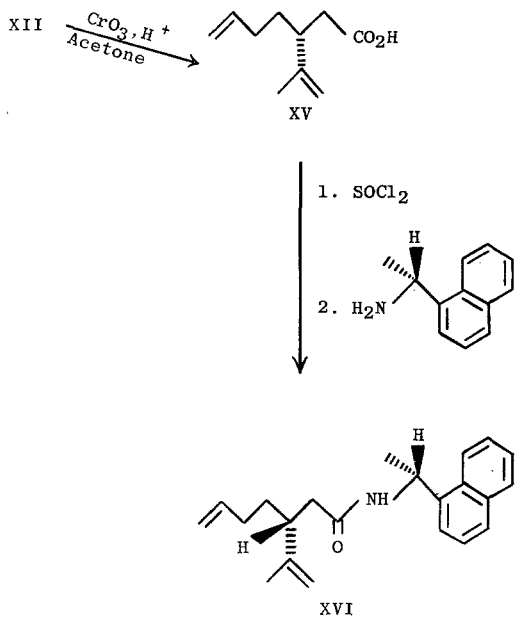


FIG. 8. Preparation of a derivative for determination of enantiomeric purity.

synthetic *R* and *S* isomers of II was determined by using a technique developed earlier in our laboratories (Zoecon) (Figure 8) (Bergot et al., 1978). Thus the *R* diene aldehyde XII was oxidized with excess Jones reagent to give the corresponding acid, XV. This acid was then converted to the acid chloride ($SOCl_2$, ether, catalytic dimethylformamide), which on treatment with (*R*)-(+)-1-(1-naphthyl)ethylamine gave the amide XVI. Similarly, the corresponding diastereomer was prepared from the *S* isomer of XV and (*R*)-(+)-1-(1-naphthyl)ethylamine. These diastereomeric amides were completely resolved by high-performance liquid chromatography (HPLC) (22×0.46 cm Zorbax-SIL, DuPont, eluted with water-saturated 10% ethyl acetate in pentane at 1.8 ml/min). From such a HPLC analysis, the percentage enantiomeric purity of XVI [and hence of (*R*)-XIV and also of the synthetic (*R,Z*)-isomer II)] was shown to be 98.4% (i.e., enantiomeric composition of 99.2% *R* and 0.8% *S*). In a like manner the *S* isomers of II were shown to be of 99.0% enantiomeric purity (i.e., enantiomeric composition of 99.5% *S* and 0.5% *R*).

Bioassays of II

Greenhouse flight tests (Table 2) with the four stereoisomers of II, *R,Z*,

TABLE 2. MALE RED SCALE CATCHES IN GREENHOUSE FLIGHT TESTS

Treatment	$\bar{x}$ males/treatment[a]
Test 1 (25 ng each isomer)	
R,Z	266 a
R,E	7.5 b
S,Z	8.5 b
S,E	9.0 b
Blank	7.5 b
Virgin female[b]	395 a
Test 2 (25 ng each isomer)	
R,Z	141 a
R,Z+*R,E*	123 a
R,Z+*S,Z*	135 a
R,Z+*S,E*	140 a
All 4 isomers	155 a
Virgin female[b]	150 a
Blank	4 b
Test 3 (125 ng each isomer)	
R,Z	219 a
R,Z+*R,E*	246 a
R,Z+*S,Z*	246 a
R,Z+*S,E*	227 a
All 4 isomers	240 a
Virgin female[b]	184 a
Blank	6.5 b

[a] Treatments were replicated twice and run two different days. Means followed by the same letter are not significantly different at the 5% level as determined by Duncan's new multiple-range test.

[b] The two female treatments averaged 33 females/trap.

S,Z, *R,E*, and *S,E*, showed that only the *R,Z* isomer was active by itself (Table 2, test 1). The *R,Z* isomer was competitive with virgin female red scales at load rates of 25 and 125 ng on filter paper. This activity was not diminished when equal amounts of *S,Z* (racemic mixture) or the other isomers were added to *R,Z* (Table 2, tests 2 and 3).

A preliminary field test in California using sticky 1-pint ice cream carton traps showed that 200 μg of II (*R,Z*) on a rubber septum was as attractive as standard traps containing 200 virgin females. In 2 days three replicates of II captured 150 red scale males, the standard female traps captured 9 males, and blank traps caught 1 male. In another test over 28 days with II (*R,Z*+*R,E*; 1:1), the mean number of male red scales caught in five replicates were

14.5, 21.4, and 14.6 for 300 μg, 1000 μg, and 200 virgin females, respectively, compared to 1.2 for the blank traps. Further field testing will be conducted with the various isomers of II and with I when they become available.

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SHORT-CHAIN FATTY ACIDS AS GROWTH INHIBITORS IN DECOMPOSING WHEAT STRAW[1]

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Abstract—The aqueous extract of decomposing wheat straw in water was inhibitory to the seedling growth of wheat (*Triticum aestivum* L.). The major compounds isolated from the toxic fractions were salts of acetic, propionic, and butyric acids. Amounts of these acids increased gradually up to 12 days and the toxicity of the straw extract increased accordingly. Traces of isobutyric, pentanoic, and isopentanoic acids were also identified by gas chromatography–mass spectrometry.

Key Words—Allelopathy, crop residue, growth inhibitor, wheat straw, acetic acid, propionic acid, butyric acid.

INTRODUCTION

Stubble mulch farming of wheat (*Triticum aestivum* L.) by leaving wheat straw on the surface of soil occasionally causes yield reduction of the successive wheat crop (McCalla and Army, 1961). Evidence has accumulated indicating that phytotoxic compounds are produced from wheat straw residues under certain field conditions. Similar phytotoxic effects have also been observed from other crop or noncrop plant residues (cf. McCalla and Haskins, 1964; Patrick et al., 1964).

Phytotoxic compounds such as ferulic, *p*-coumaric, vanillic, and *p*-hydroxybenzoic acids have been identified by Borner (1960). These phenolic acids were found both in cold-water extracts of the straw of barley, rye,

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wheat, and in alcoholic extracts of their roots. Recently, Chou and Patrick (1976) identified, in addition to the above phenolic acids, 13 other substituted phenyl compounds and butyric acid from decomposing corn residues. Most of these compounds exhibited some phytotoxicity. On the other hand, Norstadt and McCalla (1971) reported that a number of common soil fungi of *Aspergillus* and *Penicillum* family on wheat straw residue produced patulin, an antibiotic that inhibited the growth of wheat. Kimber (1967) in Australia believes that due to rather different weather conditions, the toxin problem in the wheat field there may be quite different from that in the northern United States. He reported (Kimber, 1973) that the most toxic material separated from rotted rye straw had molecular weights ranging from 10,000 to 50,000.

While the different results obtained in the aforementioned reports may have been caused by the variations of experimental conditions and materials, we have decided to investigate the toxic substances produced from a simple system, namely, a wheat straw–water mixture. Fractions toxic to wheat seedling growth were isolated by column chromatography. Further separation and identification were carried out by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). A quantitative GC method is described which may permit the evaluation of large numbers of straw or soil samples.

METHODS AND MATERIALS

Bioassay

A 2% agar solution was autoclaved and dispensed in 6 ml portions into 16 × 150-mm culture tubes. Sample solutions for bioassay were added into the agar immediately with a micropipette and agitated vigorously on a test tube mixer. The tubes were then cooled at a slanted position (45 degrees). Two wheat seeds were placed on the surface of the agar slot. The tube was then loosely capped and kept in a wooden rack which provided a dark surrounding to the bottom 40-mm section of the culture tube.

The length of the longest primary root penetrating into the agar medium was measured after 7 days of incubation at ambient temperature. An arbitrary rating system (footnote, Table 1) was used to estimate the toxicity of the testing material.

Sample Preparation and Clean-Up

Air-dried wheat straw was obtained from the Palouse Conservation Field Station, Pullman, Washington. Sixty grams of the straw were minced to

approx. 1 cm sections and mixed with 1.2 liters of distilled water in a 2.8-liter Fernbach culture flask. The flask was covered with cheese cloth and kept in the dark at ambient temperature for 12 days. The extract was filtered through Whatman No. 1 filter paper and freeze-dried. The residue was redissolved in a small quantity of distilled water and mixed thoroughly with powdered α-cellulose (ICN Pharm. Inc., 1 g equivalent of straw to 1 g of α-cellulose), freeze-dried, and packed into a column as a hexane slurry. The column was washed with two volumes of hexane, two volumes of ethyl acetate, ten volumes of methanol, and one volume of water, consecutively.

Column Chromatography

The methanol eluate from the α-cellulose adsorbent was dried under vacuum and the residue dissolved in distilled water to make a solution of 2 g equivalent of straw per milliliter. Ten milliliters of the samples was applied onto a Sephadex LH 20 column (Pharmacia Fine Chemicals, 5×100 cm) and eluted with distilled water at a flow rate of 300 ml/hr. The combined inhibitory fractions were freeze-dried. The residue was redissolved in methanol and precipitated by adding acetone into the methanol solution. After repeating the methanol–acetone procedure, a white amorphous precipitate was obtained.

Gas Chromatography (GC)

A Hewlett-Packard F and M Model 810 dual flame-ionization gas chromatograph equipped with a 2.5 mm $\times$ 1.6 m stainless-steel column packed with Chromosorb 101, 60–80 mesh, was used. Aqueous samples (crude or purified) were acidified with 1 N HCl to pH 2 and injected directly onto the column. Gas flow rates used were: N_2 carrier gas, 25 ml/min; H_2, 30 ml/min; O_2, 150 ml/min. Temperatures were: injectors, 220°C; detectors, 240°C; and column, 215°C. Tentative identification of short-chain fatty acids in the samples was based on the comparison of their retention times with those of the authentic compounds. Quantitative determination of the acids in sample solutions was based on the comparison of their peak heights to those of the standard solutions.

Gas Chromatography–Mass Spectrometry (GC–MS)

A Finnigan series 3000 quadrupole mass spectrometer interfaced with a Varian 1400 gas chromatograph was used to confirm the presence of short-chain fatty acids in sample solutions. The mass spectrometer was used with a

sensitivity of 10^{-6} A/V; electron multiplier high voltage -2.00 kV; and electron energy -69.5 V; conditions used for gas chromatography were similar to those of the F and M instrument except that helium was used as the carrier gas.

A steam distillate was concentrated for GC-MS identification. Fifty milliliters of the 12-day-old straw extract was adjusted to pH 2 with 1 N HCl and steam distilled under reduced pressure. The first 15 ml of the distillate was collected and extracted with diethyl ether. The ether phase was separated and then added with 1 ml of distilled water. The ether was evaporated under a stream of N_2 and the remaining aqueous solution was used for GC-MS identification.

RESULTS

Column Chromatography

Fractions collected from the α-cellulose column were evaporated and the

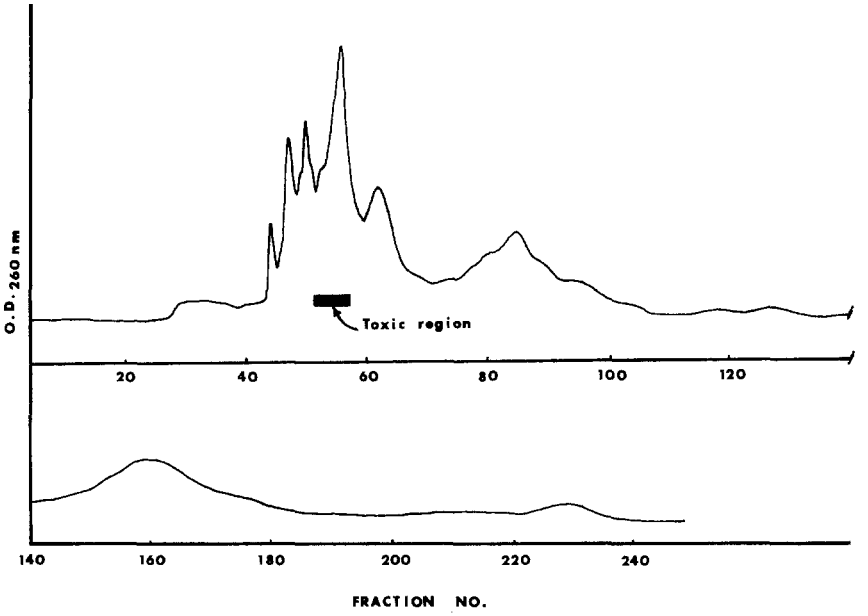

FIG. 1. Chromatogram of the phytotoxic fraction isolated from α-cellulose adsorbent on a Sephadex LH 20 column (5×100 cm). The flow rate of distilled water was 300 ml/hr, 25/ml fraction. The toxic region contains mainly salts of C_2, C_3, and C_4 acids.

TABLE 1. AMOUNTS OF ACETIC, PROPIONIC, AND BUTYRIC ACIDS IN DECOMPOSING WHEAT STRAW IN RELATION TO THEIR INHIBITION OF WHEAT SEEDLING GROWTH

Time incubated (days)	Acid (μmol/ml straw extract)[a]			Inhibition[b]
	Acetic	Propionic	Butyric	
1	Trace	0	0	0
2	Trace	0	1.2	2
4	1.7	0	4.1	4
8	12.9	0.6	5.9	4
12	20.3	1.3	5.9	5
17	11.9	1.4	4.1	5
20	8.9	0.4	1.9	4

[a] Based on the direct gas chromatographic determination of these acids in crude straw extracts.

[b] Evaluated by the penetration of seedling roots (7 days old) into the agar medium incorporated with 0.25 ml of the straw extract. Control growth usually exceeds 40 mm. From 35–30 mm = 1; 30–25 mm = 2; 25–20 mm = 3; 20–15 mm = 4; 15–10 mm = 5. Eight seedlings were used in each set of tests.

toxicity of their residues determined. Only the methanol fraction showed high inhibitory activity, and it was therefore subjected to further purification on the Sephadex LH-20 column. A chromatogram together with the position of the toxic fractions is shown in Figure 1. The toxic fractions were combined and a white, amorphous, hygroscopic precipitate was obtained. Results of the bioassay indicated that this precipitate was highly toxic to seedling root growth. Nuclear magnetic resonance spectrum of the precipitate showed no aromatic protons. A simple flame test suggested the presence of potassium.

Gas Chromatography and Gas Chromatography–Mass Spectrometry

The precipitate was brought to a pH 2 aqueous solution with 0.1 N HCl. The gas chromatogram of this solution on Chromosorb 101 showed a typical profile of short-chain fatty acids. The three major peaks had the same retention times as those of authentic acetic, propionic, and butyric acids. Fragmentation patterns of each of these peaks on GC-MS were identical to those of the authentic fatty acids. Isobutyric, pentanoic, and isopentanoic acids were also identified by GC-MS as minor components in the concentrated distillate of the 12-day-old straw extract.

Table 1 shows that amounts of the three acids increase gradually in the straw extract up to 12 days. Toxicity of the extract also increases accordingly.

DISCUSSION

The adverse effect of crop residues on the productivity of successive plantings has prompted many studies since last century (cf. Patrick et al., 1964). Although it was established that under certain conditions phytotoxic substances were produced from decomposing plant remains, the question is greatly complicated by the multiple factors involved in actual field conditions. Our present work on the phytotoxic substances isolated from rotting wheat straw was not intended to cover these various factors. Instead, we have used a simple system to generate phytotoxins, with a hope that a broad spectrum of toxic substances would be produced under these mixed aerobic (upper layer) and anaerobic (bottom layer) conditions. To our surprise, the phytotoxic fraction isolated from the 12-day-old rotting straw extract by column chromatography was found to contain mainly salts of acetic, propionic, and butyric acids. Traces of isobutyrate, pentanoate, and isopentanoate were also identified. Patulin and phenolic acids were not detected in our preparation either by solvent extraction or by column chromatography on the Sephadex LH-20 column.

The present finding of short-chain fatty acid salts as important phytotoxic compounds of decomposing straw extract in certain aspects coincides with observations reported by other researchers: The toxin(s) has been commonly described as water soluble; Guenzi and McCalla (1962) separated the ethanol extract of wheat straw into strongly acidic water-soluble, neutral, weakly acidic, and basic compounds. The strongly acidic fraction was found most inhibitory to wheat seedling root growth. The same authors also reported that increase of conductivity (salt concentration) of the residue positively correlated to the inhibitory effect. Schwartz et al. (1954) found that certain Ohio soils contained significant amounts of acetic and formic acids. Takijima (1961) reported growth inhibition and root injury to rice seedlings in water-logged soil containing readily decomposable organic matter. He believed that the injury was due to organic acids, mainly acetic and butyric, and probably other organic substances. Formation of organic acids in submerged soil treated with green manure or cellulose was studied at the International Rice Research Institute (1972). Amounts of acetic, propionic, butyric, and isobutyric acids reached toxic levels and the injurious effect on rice growth was alleviated by adding sulfur compounds such as ammonium sulfate, which presumably changed the microbial population and suppressed the production of organic acids in the soil. In a recent report (Lynch, 1977), wheat straw was subjected to aerobic and anaerobic decomposition in suspensions of soil. The products of the aerobic process stimulated the root extension of barley seedlings, whereas the anaerobic fermentation yielded products which inhibited growth. Acetic acid was the phytotoxin present in the greatest

amount, along with propionic, butyric, and hydrocinnamic acids. Phytotoxicity has been repeatedly demonstrated using straws of a wide range of plant materials, suggesting that this phenomenon may be caused by common, nonspecific microbial metabolites as well as exotic toxins. Our preliminary results have indicated that these short-chain fatty acids are also produced in the decomposing straws of rice, rye grass, and blue grass under similar conditions used for wheat. The acids reported here are well-known products formed during anaerobic fermentation. The inhibitory effect of short-chain fatty acids on wheat seedling growth has been studied by Prill et al. (1949). Among them, propionic, butyric, and pentanoic acids are more inhibitory than acetic acid. Recently, short-chain fatty acids have been found to be powerful inhibitors of gibberellin-induced amylolysis in barley endosperm (Buller et al., 1976).

As pointed out by Patrick et al. (1964), the following aspects are of importance in leading to any conclusive information on residue toxins: (1) how many of these substances are actually formed in the soil; (2) what is their chemical nature; (3) are they formed in sufficient quantities and are they sufficiently stable to affect the plant; and (4) what are their effects? These questions are mostly unanswered due to the fact that routine chemical assay of a specific toxic compound in large numbers of soil or plant residue samples is difficult, if not impossible. Since the short-chain fatty acids we have identified in this study can be easily determined with GC procedures described here and elsewhere (cf. Cochrane, 1975), we believe that the actual role of these acids as phytotoxicants in the field can be eventually assessed.

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CUTICULAR HYDROCARBONS OF THE EASTERN SUBTERRANEAN TERMITE, *Reticulitermes flavipes* (KOLLAR) (ISOPTERA:RHINOTERMITIDAE)

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Abstract—Major cuticular hydrocarbon components in several castes of *Reticulitermes flavipes* (Kollar) have been identified and quantitated. Types of hydrocarbons present include *n*-alkanes, 2-methylalkanes, 3-methylalkanes, 5-methylalkanes, an alkene, and an alkadiene, with a range in carbon numbers from C_{21} to C_{26}. This is the first report on insect cuticular hydrocarbons in which both 2- and 3-methylalkanes are present, as well as the first report of an insect with a conjugated alkadiene.

Key Words—Caste, methyl-branched hydrocarbons, alkenes, *Reticulitermes flavipes*, Isoptera, Rhinotermitidae, termite, eastern subterranean termite, cuticles, cuticular hydrocarbons, methoxy ether derivatives.

INTRODUCTION

The cuticular hydrocarbons found in insects have been the subject of a number of recent investigations (Jackson and Blomquist, 1976). The major cuticular components reported on are normal, branched, and unsaturated hydrocarbons. Of these, the branched components are of special interest structurally because they often comprise a majority of hydrocarbon material and are present in complex mixtures. Functionally, these hydrocarbons have

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been implicated in water retention, protection from abrasions, and as barriers to microorganisms (Beament, 1964), as pheromones and kairomones (Inscoe and Beroza, 1976; Jones et al., 1976), and as defense chemicals (Blum, 1974). The sole report on cuticular hydrocarbons in the order Isoptera is Moore's (1969) preliminary note on the Australian species *Nasutitermes exitiosus* (Hill). Knowledge of the structure and composition of the cuticular hydrocarbons in *Reticulitermes flavipes* (Kollar) is necessary to begin understanding their functional significance.

METHODS AND MATERIALS

Portions of four colonies of *Reticulitermes flavipes* (Kollar) were collected from fallen logs in the DeSoto National Forest approximately 32 km north of Gulfport, Mississippi, during March and April of 1976. The four sites were dominated by pine overstory and were at least 1.6 km from each other. The termites were extracted from the logs and separated into as many castes as were present. All logs yielded workers which are defined as undifferentiated larvae beyond the third instar and/or pseudergates (Miller, 1969) and soldiers; most logs also yielded first to third instar larvae and supplementary reproductives. A few logs yielded eggs and alates. In none of the logs were the primary reproductives found.

Cuticular lipids were extracted by immersing the insects in three successive portions of hexane for 5 min each. Combined samples were concentrated under nitrogen and placed on a 3-cm "mini column" of BioSil A (Bio-Rad Labs, Richmond, California)[5] in Pasteur pipettes. Hydrocarbon material was isolated by elution with hexane.

Alkenes and alkadienes were separated from alkanes by preparative TLC on 20% silver nitrate silica-gel plates developed in hexane. Bands were made visible with rhodamine 6G under UV light. Each band was scraped into a vial, 4 m of hexane was added, the vial centrifuged, and the hydrocarbon in the solvent transferred to another vial and taken to dryness under nitrogen. Straight-chain components were removed from portions of both the total hydrocarbon and alkane fraction by inclusion in Linde 5 A molecular sieves in 2,2,4,-trimethylpentane (O'Conner et al., 1962).

Gas–liquid chromatography (GLC) was carried out on the fractions using a Hewlett-Packard Model 5711A gas chromatograph (GC) equipped with a dual flame-ionization detector and a 1.8-m×3.18-mm ID stainless-steel column packed with 3% SE-30 on Gas Chrom Q. All GLC analyses

[5] Mention of trade names or companies is solely to identify material used and does not imply endorsement by the U.S. Department of Agriculture.

utilized temperature programming from 150° to 300°C at 8°/min. Retention times were compared to those of *n*-alkane standards.

Gas chromatography–mass spectrometric (GC-MS) analyses were performed on a Hewlett-Packard 5710A GC-5980A mass spectrometer operated at 70 eV and interfaced to a Hewlett-Packard 5933 data system. Hydrocarbon components were separated in an all-glass column 0.91 m×2 mm ID packed with 3% OV-101 + 0.2% Carbowax M on Chromosorb W, temperature programmed from 180° to 250°C at 8°/min prior to introduction via a membrane separator into the ion source.

The positions of double bonds in the alkene and alkadiene were located by GC-MS analysis of the monomethoxyl derivatives of the parent compounds. To prepare these derivatives, the olefin was placed in 2 ml of methanol–hexane (9:1) containing excess mercuric acetate, and the mixture was held overnight in the dark. Next, excess sodium borohydride and a few drops of glacial acetic acid were added, the samples were taken to dryness under nitrogen, and the residue was partitioned between hexane and water. Then, the hexane layer containing the methoxyl derivatives and unreacted alkenes was concentrated and subjected to GC-MS as described above (Abley et al., 1970). Authentic alkene and alkadiene samples were taken through the same process. An ultraviolet spectrum of the diene in hexane was obtained using a Cary Model 17 spectrophotometer.

RESULTS

The amount of cuticular hydrocarbons per gram of live biomass was determined for workers, soldiers, and alates (Table 1). For these three castes the cuticular hydrocarbons accounted for only a small fraction of the total biomass (0.0004–0.01%). In relative terms, however, soldiers contain approx-

TABLE 1. AMOUNT OF CUTICULAR HYDROCARBONS IN WORKER, SOLDIER, AND ALATE CASTES OF *Reticulitermes flavipes* (KOLLAR)

	Hydrocarbon (mg/g biomass)		
Caste	Mean	95% confidence limits	Number of determinations
Worker	0.21	0.11–0.30	11
Soldier	3.59	1.86–5.52	6
Alate	1.32	0.69–1.95	5

TABLE 2. PERCENT COMPOSITION[a] OF MAJOR CUTICULAR HYDROCARBONS FROM A REPRESENTATIVE COLONY OF *Reticulitermes flavipes* (KOLLAR)

Peak	Hydrocarbon	Caste					
		Egg	Worker	Nymph	Soldier	Alate	Sup. Repro.[e]
a	*n*-eicosane	Tr	Tr	Tr	7	Tr	Tr
b	*n*-heneicosane	1	Tr	1	3	Tr	Tr
c	*n*-docosane	Tr[b]	Tr	Tr	Tr	Tr	Tr
d	2-methyldocosane	Tr	Tr	1	Tr	Tr	Tr
e	*n*-tricosane	5	7	18	14	10	7
f	3-methyltricosane	4	3	5	4	20	3
g	*n*-tetracosane	3	3	3	2	3	2
h	2-methyltetracosane	48[c]	11	13	17	13	11
i	(*Z*)-9-pentacosene		25	30	15	18	34
j	*n*-pentacosane	16	20	17	15	12	12
k	5-methylpentacosane	17[d]	4	15	5	3	4
l	(*Z*,*Z*)-7,9-pentacosadiene		22	2	11	2	20
m	3-methylpentacosane	6	5	5	7	3	7
	Percent saturated		52	68	74	64	46
	Percent unsaturated		48	32	26	39	54
	Percent branched		22	29	33	39	25
	Percent *n*-alkanes	25	30	39	41	25	21
	Ratio: branched-normal		0.7	0.7	0.8	1.7	1.2
	Ratio: saturated/unsaturated		1.1	2.1	2.9	1.6	0.9

[a] All values rounded to the nearest whole number.
[b] Trace equals greater than 0.1 but less than 0.5%. In addition, unidentified hydrocarbons of greater than 26 equivalent carbon units comprised trace to 2% of the cuticular hydrocarbons in some samples.
[c] Combined value alkane and alkene (*h* + *i*).
[d] Combined value alkane and alkene (*k* + *l*).
[e] Supplementary reproductive.

imately five times more cuticular hydrocarbons than alates and approximately 20 times more than workers.

Each caste had the same 13 major hydrocarbon components, but the relative abundance of each varied from caste to caste (see Table 2 and Discussion). Six classes of hydrocarbons are present in *R. flavipes*: (1) a homologous series of *n*-alkanes, with odd-carbon-number components predominating; (2) 2-methylalkanes, predominantly of odd carbon number; (3) 3-methylalkanes, all of even carbon number; (4) one 5-methylalkane; (5) one major alkene of odd carbon number; and (6) one major conjugated diene of odd carbon number. As Figure 1 indicates, the mono olefin, (*Z*)-9-pentacosene is present in the 2-methyltetracosane peak, and (*Z*, *Z*)-7,9-pentacosa-

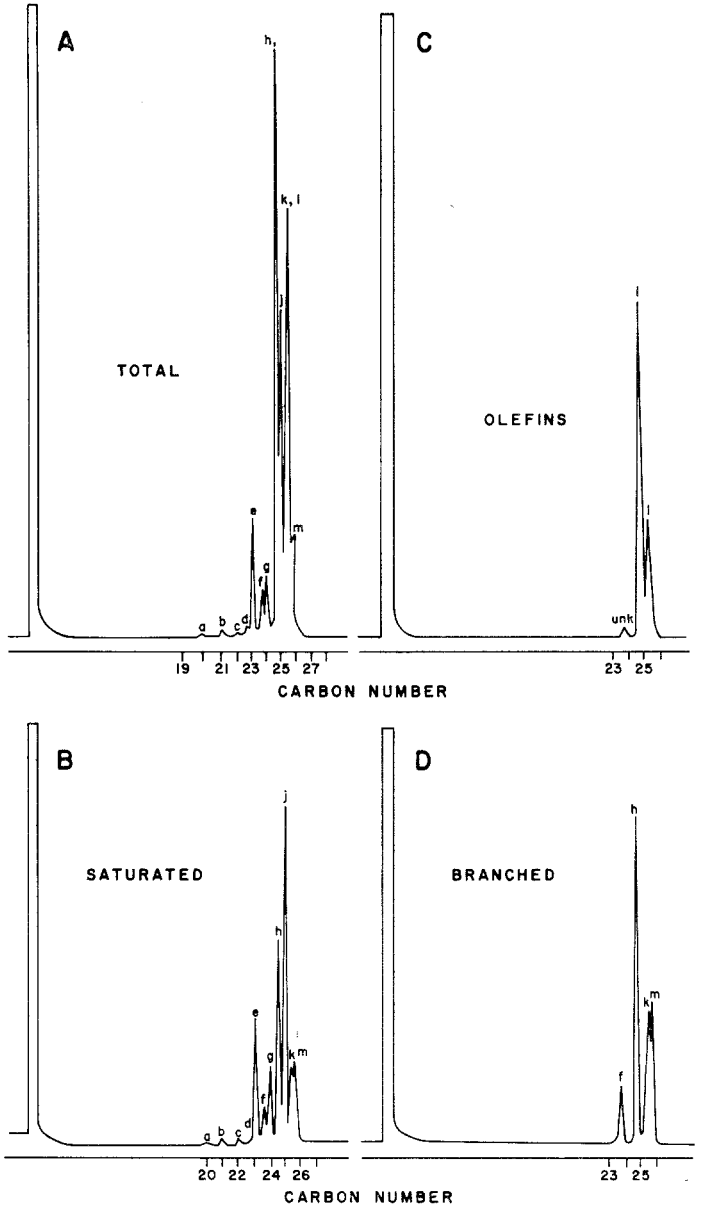

FIG. 1. GC trace of total hydrocarbons, saturated hydrocarbons, branched hydrocarbons, and olefins isolated from workers.

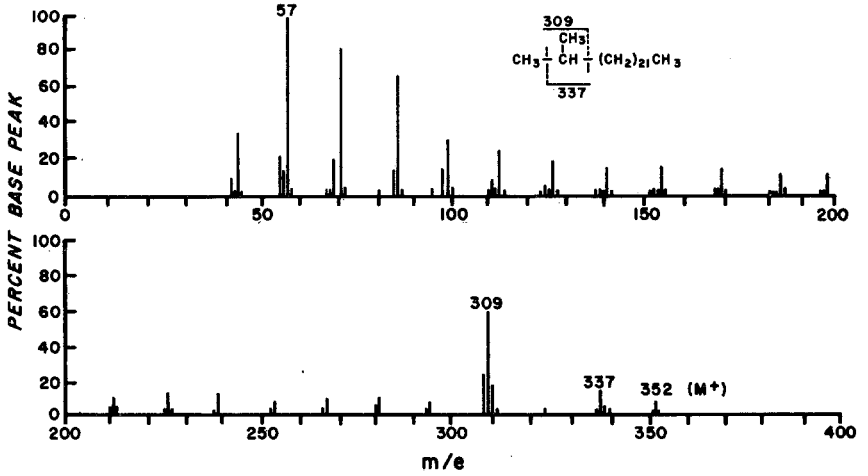

FIG. 2. Mass spectrum of 2-methyltetracosane.

diene is present in the internally branched monomethylpentacosane peak. In addition to the GC evidence, mass spectral evidence (see below) suggests that small amounts of 2-methylalkanes of even carbon number are present in the corresponding 3-methylalkane peaks of the same carbon number.

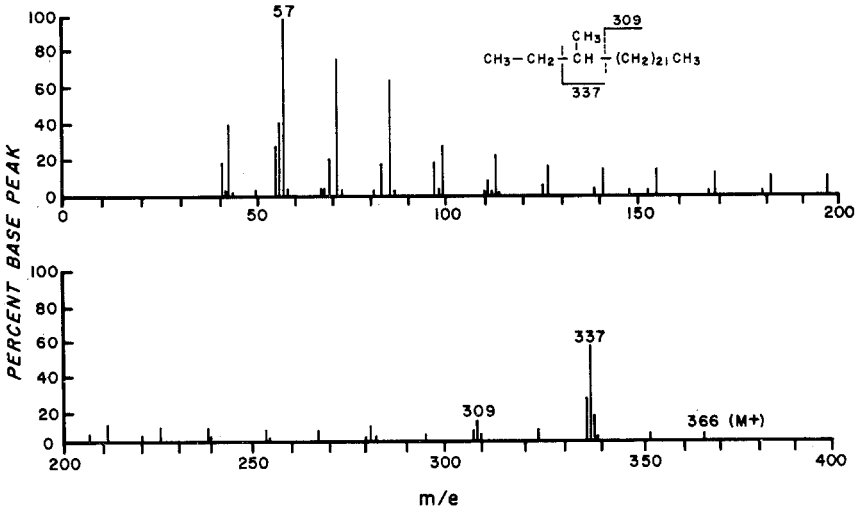

FIG. 3. Mass spectrum of 3-methylpentacosane.

The *n*-alkanes of carbon number 20–25 had retention times identical to known standards and gave mass spectra identical to those of standard *n*-alkanes. Both the major 2-methylalkanes (2-methyldocosane and 2-methyltetracosane) and the 3-methylalkanes had GLC retention times, corresponding to 0.3 carbon units less than the corresponding *n*-alkane of the same carbon number. The major 2-methylalkanes produced characteristic spectra; that is, they had strong M-15 and M-43 fragment ions corresponding to a loss of methyl and isopropyl groups, respectively (Jackson and Blomquist, 1976). The mass spectrum of 2-methyltetracosane is shown in Figure 2. The molecular ion is at *m/e* 352 with the M-15 and M-43 ions at *m/e* 337 and 309, respectively. The mass spectrum of 3-methylpentacosane is shown in Figure 3. Its molecular ion is at *m/e* 366, and the two ion fragments at *m/e* 337 and 309 represent the characteristic loss of ethyl and secondary butyl groups (Jackson and Blomquist, 1976). Careful examination of the mass spectra of each of the 3-methylalkane peaks revealed the presence of low-abundance fragment ions corresponding to M-15 and M-43 transitions, which we believe to arise from small amounts of 2-methylalkanes of even carbon number, which GC does not separate from 3-methylalkanes of the same carbon number.

The presence of 5-methylpentacosane was deduced from the small GC peak occurring approximately 0.6 carbon units less than *n*-hexacosane with its characteristic mass spectral fragmentation pattern (Figure 4) (Jackson and Blomquist, 1976). Its molecular ion is at *m/e* 366, and the major secondary ion fragment peaks at *m/e* 309 (308) and 85 (84) result from cleavage followed by

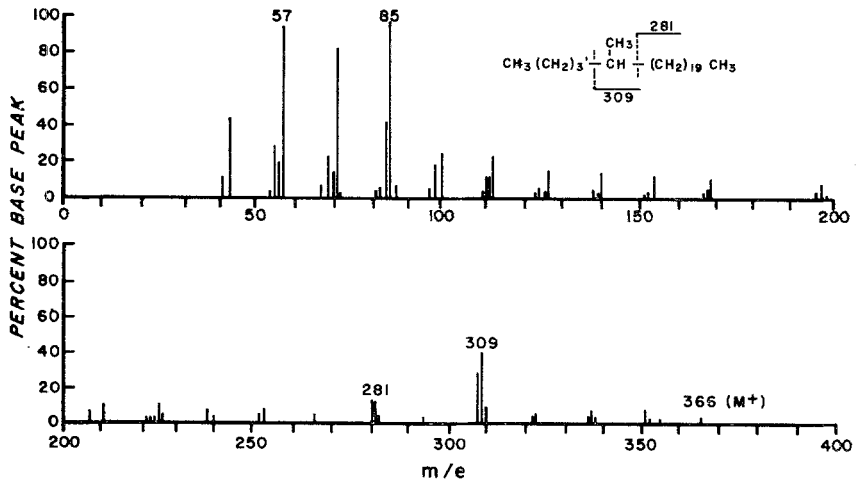

FIG. 4. Mass spectrum of internally branched monomethylpentacosanes.

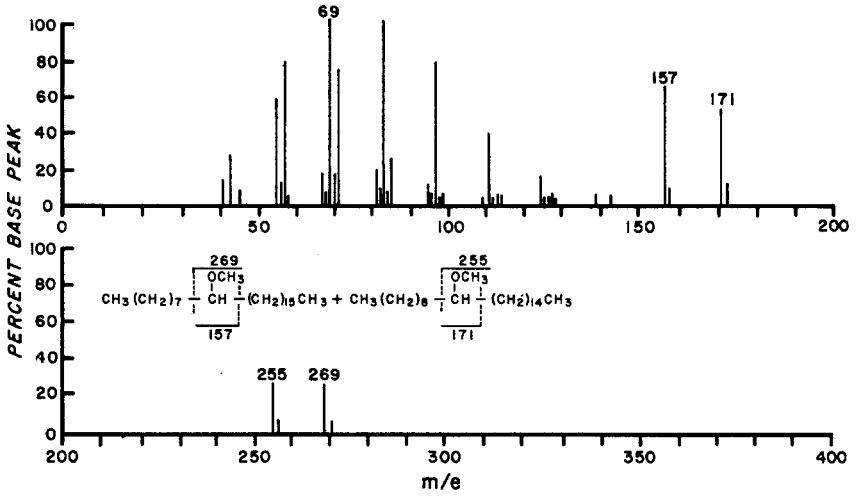

FIG. 5. Mass spectrum of methoxy derivitive of (*Z*)-9-pentacosane.

hydrogen loss on each side of the methyl branch of 5-methylpentacosane. The small *m/e* 280 (281) peaks arise from retention of the charge on the 20-carbon primary fragment.

A substantial contribution by a C_{25} alkene to the 2-methyltetracosane GC peak of the total hydrocarbon mixture was indicated by a *m/e* 350 peak in the mass spectrum. After isolation of the olefin by preparative silver nitrate TLC, infrared (IR) and MS analyses were performed. A *cis* stereochemistry is strongly suggested by the presence of a 730 cm^{-1} IR band and the absence of a 940 cm^{-1} IR band (Nakanishi, 1962). Location of the position of the double bond is not possible by direct mass spectral analysis (Budzikiewicz et al., 1967) but is readily achieved by an analysis of the fragmentation pattern of the monomethoxyl ether derivatives (Figure 5) (Abley et al., 1970). Cleavage alpha to the methoxy-containing carbons yielded four major fragment ions. The pair at *m/e* 157 and 269 arise from 9-methoxypentacosane, and the pair at *m/e* 171 and 255 arise from 10-methoxypentacosane. Mass spectral characterization of the monomethoxyl derivates of authentic (*Z*)-9-tricosene gave equivalent fragmentation patterns (major fragment ions at *m/e* 157, 171, 227, and 241). Although the derivatization did not go to completion, starting materials and reaction products separated cleanly by gas chromatography, permitting a direct GC-MS analysis of the crude reaction mixture.

The presence of a C_{25} diene was indicated by a *m/e* 348 peak in the mass spectrum of the C_{26} internally branched monomethylalkane peak of the total hydrocarbon mixture. Preparative $AgNO_3$ TLC yielded the pure

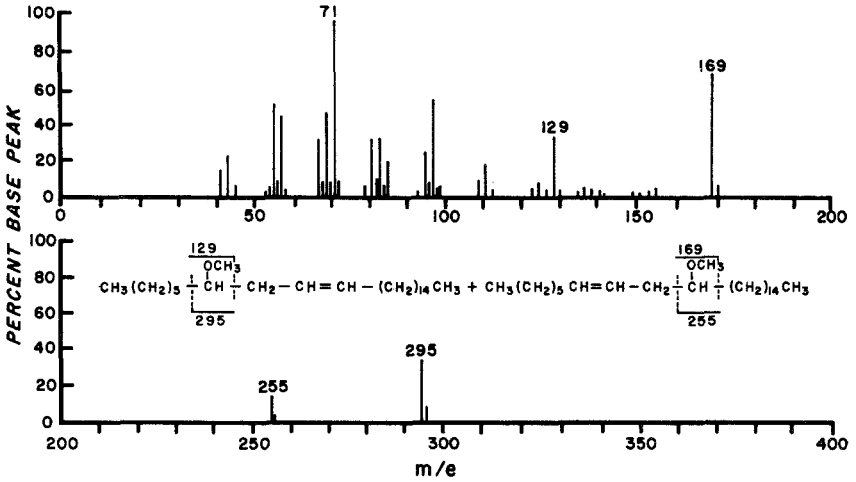

FIG. 6. Mass spectrum of methoxy derivitives of (*Z*,*Z*,)-7,9-pentacosadiene.

diene, but at a rather unusual R_f position. Under our experimental conditions, alkanes have an R_f of approx. 0.9, monoolefins of approx. 0.5, and methylene-interrupted dienes of approx. 0.1. The *R. flavipes* diene, however, had an R_f of approx. 0.35. Examination of the isolated diene by UV spectroscopy indicated a λ_{max}^{hexane} of 235 nm ($\varepsilon = 15{,}660$), which strongly suggests a conjugated diene (Scott, 1964). The positions of the double bonds were located by GC-MS of the monomethoxyl derivative. We anticipated eight major fragment ions from the four possible monomethoxyl derivatives; instead we observed only four major ions at *m/e* 129, 169, 255, and 295 (Figure 6). Such a result is explicable if a 1,4 addition to the conjugated diene occurred, generating a mixture of 7- and 10-monomethoxy alkenes (Bloodworth et al., 1976). Monomethoxy derivatives of (*Z*,*Z*)-6,9-heptacosadiene gave a different fragmentation pattern, as methoxy groups were detected at positions 6, 7, 9, and 10. There was insufficient sample of the purified C_{25} diene for an IR spectrum, but an IR spectrum of a mixture of the diene and the (*Z*)-9-pentacosene showed no evidence of a band at 970 cm^{-1}, which leads us tentatively to assign a *Z*,*Z* sterochemistry to the *R. flavipes* diene.

Quantitative GC analyses of the cuticular hydrocarbons from eggs, workers, soldiers, nymphs, supplementary reproductives, and alates were obtained, and the results from a representative "colony" are presented in Table 2. Insufficient material was available from the eggs to allow separation into normal, branched, and unsaturated components. Accordingly, for eggs only, a combined percentage value is recorded for the 2-methyltetracosane: (*Z*)-9-pentacosene peak and the 5-methylpentacosane:(*Z*,*Z*)-7,9-penta-

cosadiene peak. Table 2 also provides a comparison by caste of percent saturated, unsaturated, branched, and normal hydrocarbons, as well as ratios of branched to normal hydrocarbons and saturated to unsaturated components.

DISCUSSION

The caste-dependent cuticular hydrocarbon composition of *R. flavipes* is an example of flexible phenotypes in genotypically identical organisms. These termites have a soft, thin cuticle and are so dependent upon a nearly saturated atmosphere that they cannot be cultured in a dry environment, even in the presence of abundant food and water (Collins, 1969). Soldiers and alates, however, are sometimes exposed to desiccating atmospheric conditions for lengthy periods of time. As Table 1 indicates, both these castes possess greater quantities of cuticular hydrocarbons than do workers, thus providing the soldiers and alates with a greater barrier to water loss. This variation was likely selected for: in the event of a major catastrophe to the "nest", the soldiers remain outside attacking the intruding organisms (usually ants), while the workers close off the gallery entrances as rapidly as possible, orphaning the soldiers. Probably for similar reasons, alates also contain greater amounts of cuticular hydrocarbons than workers. That they contain lesser amounts than soldiers perhaps can be explained by their flight usually only after a rain (hence high humidity) and their remaining above ground only long enough to find a mate.[6]

Added protection from desiccation is not solely a function of the quantity of cuticular hydrocarbons, however. As Table 2 indicates, both soldiers and alates contain high proportions of saturated hydrocarbons. Some evidence (Neville, 1975) suggests that the greater the proportion of saturated hydrocarbons present in cuticular lipids, the greater the effectiveness of these lipids in prevention of desiccation. Those castes that seldom, if ever, leave the "nest"—workers, supplementary reproductives, nymphs, and eggs—might then be expected to have a lower proportion of saturates in their cuticular hydrocarbons. Indeed, as Table 2 indicates, eggs, workers, and supplementary reproductives have very similar, unsaturate-rich hydrocarbon profiles, which vary in the proportions of branched to normal hydrocarbons. The significance of branched hydrocarbons in insects has been the subject of some speculation (Jackson and Blomquist, 1976), but the function of the branching methyl groups is not clearly defined. The proportion of saturates in eggs was not obtained because of the small quantities of sample available.

[6] One reviewer suggested that an alternative explanation for this difference resides in the greater degree of sclerotization possessed by the alates. We agree that this factor could operate, but are uncertain as to its relative importance.

The significance of the cuticular hydrocarbon composition of the nymphs is puzzling. Their proportion of branched to normal components is about the same as in workers, but their proportion of saturates to unsaturates lies between those of alates and soldiers. Since nymphs can become either alates, supplementary reproductives, or (via retrogressive molts) soldiers (Miller, 1969), perhaps our measured average values reflect the proportions of nymphs destined for those castes in our colonies. This suggestion implies, of course, that if other colonies had been sampled which were in a different segment of their yearly cycle, the cuticular hydrocarbon composition of their nymphs would be different. A test of this hypothesis is in progress.

Nymphs also differ from the other castes examined in the makeup of their unsaturated hydrocarbon components. Workers possess approximately equal amounts of monoene and diene, nymphs contain almost all monoene, and supplementary reproductives have approximately a 1.7:1 ratio of monoene to diene. The significance of this caste variation in terms of water-retention properties is unknown.

Clearly, the functional significance of all the cuticular hydrocarbon components of *R. flavipes* has not been delineated in this study. It remains to be seen if the caste-dependent compositions measured in this study will remain constant over the course of a year or over a wider geographic range. All of our samples were drawn from an area dominated by pine overstory. If the cuticular hydrocarbons of *R. flavipes* should prove to be closely linked with their food resources, one might anticipate different cuticular compositions in other ecological settings.

The cuticular hydrocarbon composition of *R. flavipes* contrasts rather sharply with that of the only other termite previously examined, *Nasuititermes exitiosus* (Hill). Moore (1969) reported a homologous series of hydrocarbons from C_{24} to C_{47}, with odd carbon-number components predominating. For that species, however, unsaturated hydrocarbons were reported to make up the bulk of the sample, with degrees of unsaturation ranging from four to eight double bonds at each of the higher carbon numbers. The predominant cuticular component was identified as $\Delta^{10,16,22,28}$-nonatriacontatetraene, $C_{39}H_{72}$, with unspecified stereochemistry. As noted above, the hydrocarbons of *R. flavipes* are of a much lower carbon-number distribution, C_{20} to C_{26}.

The finding of both 2-methyl- and 3-methylalkanes in *R. flavipes* is interesting since until this year no other insect has been shown to contain both. Nelson[7] recently has found both in hemolymph of Japanese beetle (*Popillia japonica* Newm.) larvae and in pupal cuticular lipids of the tobacco

[7] Personal communication with D.R. Nelson, U.S. Department of Agriculture, Agricultural Research Service, Metabolism and Radiation Research Laboratory, Fargo, North Dakota.

budworm (*Heliothis virescens* [F.]). Only crickets (Orthoptera: Gryllidae) have been shown to have 2-methylalkanes (Hutchins and Martin, 1968; Blomquist et al., 1976). Whether *R. flavipes* biosynthesizes its branched hydrocarbons, procures them from dietary sources, or their gut tract bacteria (Kolattukudy and Walton, 1973; Albro and Dittmer, 1970), or uses some combination of the above three possibilities remains to be determined. However, other insects have been shown to biosynthesize 2-methyl-, 3-methyl-, and internally branched monomethylalkanes (Blailock et al., 1976; Blomquist et al., 1975; Blomquist and Kearney, 1976). Moreover, preliminary radiolabeling experiments suggest that *R. flavipes* is able to biosynthesize some, and possibly all, of its cuticular hydrocarbons (Blomquist and Howard, unpublished observations.

The occurrence of a conjugated diene in the cuticular hydrocarbons of *R. flavipes* is unusual. Although conjugated olefinic systems are well-known subunits of insect pheromones, they are always associated with an additional functional group (Inscoe and Beroza, 1976). We know of no other instance of the presence of a conjugated diene hydrocarbon derived from an arthropod source. Rather, most insect dienes are of the methylene or polymethylene interrupted type. Elucidation of the biosynthetic pathways to this diene would, therefore, be of great interest.

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THE "DEER LACTONE"

Source, Chiral Properties, and Responses by Black-Tailed Deer[1]

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Abstract—Urine of the black-tailed deer is the source of the "deer lactone," which is deposited on the tarsal gland tufts by "rub-urination." The enantiomer composition of the lactone from the urine of the female is 89(*R*)-(−)/11(*S*)-(+). Responses by deer were strongest toward the synthetic racemic lactone in the social test and toward the natural lactone in the choice test. In both tests, the (−)- lactone released slightly stronger responses than its enantiomer.

Key Words—Black-tailed deer, "deer lactone," discrimination, enantiomers, (*Z*)-4-hydroxy-6-dodecenoic acid lactone, *Odocoileus hemionus columbianus*, lactone.

INTRODUCTION

Sniffing and licking of the tarsal gland of conspecifics is prominent in black-tailed deer behavior (Müller-Schwarze, 1971). We have previously identified the lactone (*Z*)-4-hydroxy-6-dodecenoic acid lactone (I) in an extract of the hair tuft associated with the tarsal gland (Brownlee et al., 1969) and found

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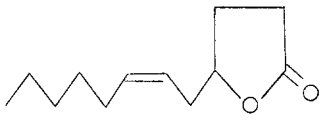
that it was the component in the extract most effective in eliciting approach and the sniffing and licking response. The actual source of the lactone was left open to question because we were aware of frequent soaking of the tuft by urine during during "rub-urination" (Müller-Schwarze, 1971).



I

Both of the geometric isomers of (I) have been synthesized and tested in the black-tailed deer, which could discriminate between them; the naturally occurring *Z* isomer elicited the stronger response (Müller-Schwarze et al., 1976). However, neither the enantiomeric composition nor the absolute configuration of the naturally occurring compound was determined because of the small amount of material available from the tarsal tuft and the unavailability of reference compounds; nor could the responses of the deer to the enantiomers be tested. These goals became possible with the synthesis of both enantiomers of (I) from optically highly pure starting materials of known absolute configuration (Ravid and Silverstein, 1978), and by the finding that relatively large amounts of (I) were available from urine.

METHODS AND MATERIALS

Urine was collected by catheterization from a sedated (xylazine, trade name Rompun), 1.5-year-old female black-tailed deer between July 29 and August 12, 1976. This female was part of a captive herd penned near Syracuse, New York, and maintained on alfalfa hay and food pellets (50% alfalfa plus barley, wheat bran, and minerals); fresh grass and forbs were available during the growing season.

The accumulated urine (1.2 liters, held at −20°C) was acidified with H_2SO_4 to pH 4–5 and extracted with ether (3×750 ml). The combined ether extract was filtered through Celite, washed (2×100 ml water), concentrated to 10 ml, and extracted with 1 N KOH (2×5 ml). The alkaline solution was shaken with ether (2×5 ml) and acidified with H_2SO_4 to pH 4, and the acidic solution was extracted with ether (3×10 ml). The ether solution was washed (2×5 ml H_2O), dried over $MgSO_4$, and the solvent was removed by distillation. The residual oil was fractionated by GC in order to collect the "deer lactone" (I) (5% SE30 on Chromosorb G 60/80 mesh, 2.4 m×6 mm OD glass, 60 cm^3 He/min, 170°C). The fraction eluting between 16.5 and 19.5 min was collected and refractionated (6% OV-17 on Chromosorb W

60/80 mesh, 2.4 m × 6 mm OD glass, 60 cm^3 He/min, 180°C). The peak that eluted between 15.5 and 17.0 min was collected.

Gas chromatography was done on the Varian Model 2740, and fractions were collected in a thermal gradient collector (Brownlee and Silverstein, 1968). Infrared spectra were obtained on samples of 40 μg in CCl_4 in a Barnes Engineering 4-μl cavity cell on a Perkin-Elmer 621 fitted with beam condensers; the solvent absorption was balanced out. Mass spectra were obtained on a Hitachi RMU-6 fitted with a flexible metal tube in which the GC collector capillary could be broken and sample thus introduced. NMR spectra in $CHCl_3$ were obtained on a Varian XL 100. Optical rotations were done on a Perkin-Elmer Model 531 polarimeter.

The bioassay was carried out with captive black-tailed deer during three seasons. In 1973–1974 and 1974–1975 the enantiomers were tested in a social situation, in 1976 on a choice apparatus.

Social test

As experimental animals, we used a group of three adult females and a second group of three male and three female fawns in 1973–1974, and a group of three male and three female fawns in 1974–1975. Thus, three adult females and twelve fawns were exposed to the enantiomers.

Tests were performed between December 2, 1973, and March 19, 1974, and between November 5, 1974, and February 1, 1975, with the following synthetic samples (number of presentations): (*S*)-(+)-lactone (47), (*R*)-(−)-lactone (47), recombined (+) and (−) lactones (25), and racemic lactone (46). The crude extract from tarsal tufts was presented 12 times, and solvent controls 34 times. All samples were presented in randomized sequence.

Samples of 2.0, 0.2, or 0.02 μg of deer lactone were dissolved in 0.25 ml petroleum ether (30–36°) (PE) and sprayed onto the outside of a hindleg of a deer with a needleless syringe. Other deer in the group approached the treated animal and sniffed and licked the sample until it was consumed. This is the typical response of black-tailed deer to fresh urine that is released on the hocks during "rub-urination". The responses of the other group members to the treated deer were observed for 15 min.

Choice Test

Seven males and three females (one year of age or older) and two female fawns were used. Since the fawns did not lick the samples and sniffed only one third of them, the data for the fawns were not analyzed. The tests were performed between October 1 and December 22, 1976, with the following synthetic samples (number of presentations): (*S*)-(+)-lactone (10), (*R*)-(−)-

lactone (12), racemic lactone (12), a mixture of 75% (*S*)-(+)-lactone and 25% (*R*)-(−)-lactone (12), and a mixture of 75% (*R*)-(−)-lactone and 25% (*S*)-(+)-lactone (12). In addition, the natural lactone, isolated from urine, was presented 22 times. All samples consisted of 2.0 μg in 0.25 ml hexane; hexane was presented 23 times and, additionally, was paired with every sample in each of the 103 tests.

The choice apparatus consisted of two horizontal 15-cm-long Teflon sticks (1 cm diam) that were placed 125 cm above ground and 90 cm apart. One stick was treated with a sample in hexane, the other with hexane alone. The sniffing and licking frequencies and durations and other responses, such as chewing or forehead rubbing, were recorded.

All behavior tests were carried out before the chiral properties of the natural deer lactone were known. Thus, observer bias with regard to specific samples was excluded. Moreover, in the choice test during the 1976 season only number-coded samples were used, and the identity of the samples was revealed by the chemist only after all tests had been completed ("double-blind test").

RESULTS AND DISCUSSION

In a reinvestigation of the tarsal gland, we found that when the tarsal gland contents were squeezed onto the freshly cleaned (ethanol and chloroform) gland surface, no "deer lactone" (I) was detected. The compound collected from the urine was identified as the "deer lactone" (I) by congruence of GC properties and spectra with those of an authentic sample (Figure 1). A total of 1.4 mg was obtained from 1.2 liters of urine. Thus, it now appears that at least one of the components that evoke the sniffing and licking of the tarsal gland is deposited on the specialized tarsal hairs (Müller-Schwarze et al., 1977) during rub-urination.

Unsuccessful efforts were made to determine the enantiomeric composition of (I) by ^{1}H and ^{13}C NMR with the chiral shift reagent, $Eu(Hfbc)_3$; large chemical shifts were obtained, but the differential shifts were too small for quantitative determinations. Pirkle's lactone reagent (Pirkle et al., 1977) was successful only with γ-caprolactone. The isolated lactone (I) gave the following specific rotations: $[\alpha]_D^{20}$ − 5.8 (c = 0.14, $CHCl_3$), −12.4° (c = 0.14, CH_3OH). The specific rotations of the synthetic lactone of *S* configuration were $[\alpha]_D^{20}$ + 6.3 (c = 0.14, $CHCl_3$), +15.0° (c = 0.10, CH_3OH). The *R* synthetic lactone gave $[\alpha]_D^{20}$ − 16.1 (c = 0.28, MeOH) (Ravid and Silverstein 1977, 1978).[4] Thus, if the highest $[\alpha]_D$ is assumed to be the value of

[4] The reasonable assumption is made that the optical purity did not deteriorate during storage for the test period under N_2 at about −20° nor during the brief exposure for the bioassay.

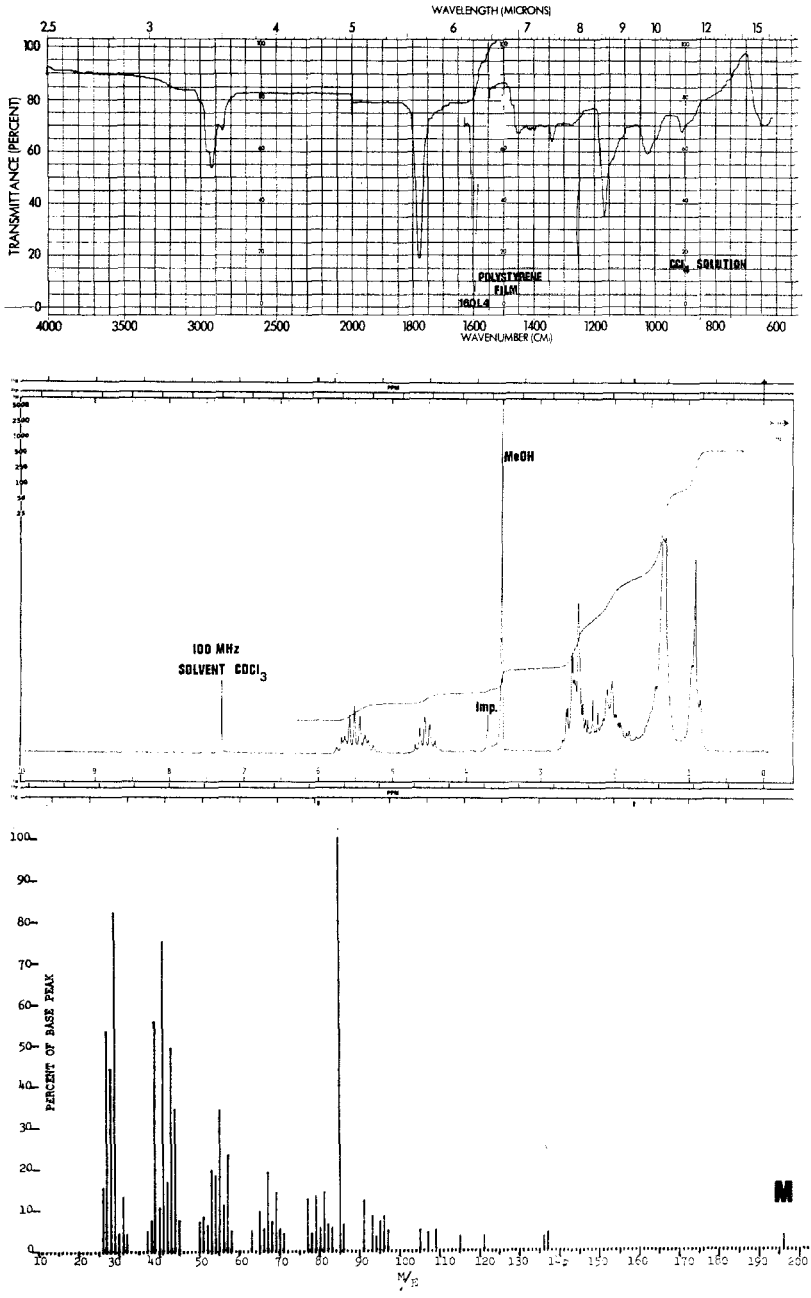


FIG. 1. "Deer lactone" (I). Infrared, mass, and NMR spectra.

the pure enantiomer, the isolated compound has an enantiomeric composition of 89(*R*)-(−)/11(*S*)-(+). The assumption is probably valid since the synthesis proceeded in the same way from the same optically highly pure starting material as did the synthesis of γ-caprolactone, which was shown to be optically pure within the limitations of Pirkle's reagent (Pirkle et al., 1977); (Ravid and Silverstein, 1978).

Isolation of (I) from urine was based on ring opening of the lactone and extraction of the salt by dilute base, and on subsequent ring closure on acidification, both steps proceeding without affecting the chiral center.

In theory, chiral receptor sites should acknowledge the spatial arrangements of enantiomers, and careful experiments have shown that some insects are capable of making the distinction. Kafka et al. (1973) conditioned honeybee drones to discriminate between a pair of enantiomers and obtained differences in responses from single olfactory receptor cells of the migratory locust and the honeybee. Lensky and Blum (1974) obtained similar results from conditioned honeybees and noted a differential response by the bees to a pair of enantiomers that could not be differentiated by humans. However, none of the compounds used with conditioned insects was a pheromone. Riley et al. (1974a, b) showed that two species of leaf-cutting ants responded to a lower concentration of the naturally occurring enantiomer of the alarm pheromone than of the other enantiomer. Wood et al. (1976) demonstrated, in both laboratory and field bioassays, a preference by a bark beetle for the naturally occurring enantiomers of two components of the aggregation pheromone. Borden et al. (1976) found that an ambrosia beetle gave the maximum response to the naturally occurring ratio of the enantiomers; neither enantiomer alone gave an appreciable response. The elm-bark beetle responds to a lower concentration of the naturally occurring enantiomer of α-multistriatin, a component of the aggregation pheromone, in comparison with the other enantiomer (Lanier, personal communication). (*S*)-(+)-α-Pinene elicits an oviposition response from the spruce budworm, whereas the enantiomer gives no appreciable response (Staedler, 1974). The (7*R*, 8*S*)-(+) enantiomer of disparlure is active toward the gypsy moth, the (−) enantiomer is virtually inactive, and the racemic form is slightly active; the enantiomeric composition of the naturally occurring material was not determined (Iwaki et al. 1974). (*S*)-(−)-Ipsidienol is more attractive to the bark beetle, *Ips grandicollis* than the (+) enantiomer, but again the composition of the natural material is not known (Vité et al., 1976). Tumlinson et al. (1977) have shown in extensive field tests that both the unnatural enantiomer and the racemic form of the Japanese beetle sex attractant are virtually inactive. Levinson (personal communication) found, in a laboratory bioassay, that the khapra beetle responds to the (*R*)-(+) enantiomer of α-caprolactone, but to neither the other enantiomer nor the racemic form. Thus in all

TABLE 1. MEANS OF DURATION OF LICKING (IN SEC) SAMPLES (SOCIAL TESTS FOR SEASONS 1973–1974 and 1974–1975)

Sample	N	2 Micrograms 1973–1974	2 Micrograms 1974–1975	2 Micrograms average 1973–1975	0.2 Microgram average 1973–1975	0.02 Microgram average 1973–1975
Crude extract	12	90.5		90.5		
Synthetic racemic	22	65.9	21.2	45.5	22.8	29.1
Recombined (+) and (−),1:1	10		20.2	20.2	23.3	16.6
Synthetic (−)	22	50.6	22.1	37.7	13.9	17.6
Synthetic (+)	22	34.0	20.2	27.8	14.4	14.1
Solvent PE	29	24.7	2.7	21.5	18.6	18.5

cases reported in which the naturally occurring enantiomer is known, the insect is more responsive to it.

Humans can discriminate between some enantiomers (Leitereg et al., 1971; Russell and Hills, 1971; Friedman and Miller, 1971) but not others (Lensky and Blum, 1974). Six people in this laboratory were unable to distinguish between the enantiomers of γ-caprolactone, γ-nonalactone, or the "deer lactone" (I). So far as we are aware, no investigations have been undertaken on the ability of other mammals to discriminate between enantiomers. Since we have shown that black-tailed deer can discriminate between the geometric isomers of (I), we challenged the deer with the enantiomers of

TABLE 2. SIGNIFICANT DIFFERENCES OF RESPONSES OF DEER TO SAMPLES AT THREE DIFFERENT CONCENTRATIONS DURING THE 1973–1974 AND 1974–1975 SEASONS (VALUES .01 AND .05 ARE P LEVELS)

Discriminant analysis	(+) lactone (μg) 2	(+) lactone (μg) 0.2	(+) lactone (μg) 0.02	(−) lactone (μg) 2	(−) lactone (μg) 0.2	(−) lactone (μg) 0.02	(+) lactone (μg) 2	(+) lactone (μg) 0.2	(+) lactone (μg) 0.02	PE (μg) 2	PE (μg) 0.2	PE (μg) 0.02	Single variables
(±)						FL.05				DL.01 FS.01	FS.05	FL.05 FS.01	
(−)										DL.05 FS.01		FS.05	
(+)										FS.05			
Solvent (PE)	.01	.05	.05	.01		.05							

(I) which were available from a recently devised general synthesis of γ-lactone enantiomers (Ravid and Silverstein, 1977).

In the social tests, the licking responses to crude extract of tarsal tufts were the strongest, followed by responses to the synthetic racemic lactone. Responses to (−), (+), and recombined (−) and (+) lactone were intermediate. Controls tended to release the weakest licking responses. In 1973–1974, the responses varied systematically with the samples, but in 1974–1975, no differences were discernible (Table 1).

Table 2 lists the significant differences. On the upper right, significance levels are given for comparisons of the single variables: duration of licking (DL), frequency of licking (FL), and frequency of sniffing (FS). The three variables were combined in a discriminant analysis. The significant differences are listed at the lower left. Synthetic racemic deer lactone and solvent (petroleum ether) differed significantly in eight comparisons. The responses to the (−) lactone were significantly stronger than those to solvent in five comparisons, whereas only one significant difference between the (+) lactone and solvent exists. Although there were no significant differences between the (+) lactone and the (−) lactone, generally stronger responses were released by the (−) lactone.

In the choice tests, males alone ($N = 7$) and males and females combined ($N = 10$) responded most strongly to the natural deer lactone with regard to duration of sniffing ($\bar{X} = 22.7$ sec), licking ($\bar{X} = 5.4$ sec), and chewing ($\bar{X} = 8.5$ sec), and the number of bouts of responses ($\bar{X} = 3.7$). The responses to the (+) lactone were the weakest, and all other samples were less active than the natural lactone. The yearling females and adult males showed a more defined tendency to respond more to the mixtures with a high proportion of (−) lactone than the male yearlings. One male "rub-urinated" (a threat behavior) in response to natural lactone, to 100% (−) lactone, but also to the 75% (+) lactone sample. Only the responses to the natural lactone and the solvent were statistically different ($P < 0.05$).

By analogy with the ambrosia beetle results (Borden et al., 1976), we might expect a maximum response to the naturally occurring ratio of the enantiomers. The results of the bioassay, however, are difficult to interpret, as the deer were only slightly more responsive to the (−) lactone than to the (+) lactone. But the response to 100% (−) lactone tended to be consistently strong, near the level of response to natural deer lactone in the choice apparatus tests. The social test yielded more consistent results, possibly because the samples were presented within their natural context. We have to consider numerous intervening variables in the field tests, such as age and individual differences of the deer, variations in weather, and possible habituation during the experiments or physiological changes during the weeks of the experiments. In light of this, the tendency to respond more to the (−) lactone cannot be

dismissed as fortuitous. We conclude that deer tend to respond more to the (−) lactone than to its (+) enantiomer, although our experimental data show many exceptions to this tendency.

The demonstrated preference for the natural deer lactone in the choice test and to a certain extent for the (−) lactone in both tests may not adequately reflect the underlying ability to discriminate. Sensory discrimination is at least as good as the behavioral preference, but may exceed it.

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BOOK REVIEW

CHEMICAL CONTROL OF INSECT BEHAVIOR: Theory and Application. Proceedings of a conference, Bellagio, Italy, May 1975. *H.H. Shorey and John J. McKelvey, Jr.* (Eds.). New York, Wiley-Interscience, 1977. vii+413 pp., illustrations, $19.50. A volume in the Environmental Science and Technology Series.

Chemical Control of Insect Behavior is the second multiauthored book discussing the chemical ecology of insects [*Pheromones*, 1974. M.C. Birch, (Ed.), American Elsevier, New York. xxi+495 pp.] and several chapters are written by the same authors. Unlike *Pheromones*, this is not a text/reference book, but results from the proceedings of a conference sponsored by the Rockefeller Foundation.

A number of the 24 chapters are philosophical—even argumentative. When one participant (Silverstein, Chapter 14) states, ". . . proponents of the 'run it up the flagpole and see who salutes' methodology have not been too successful in rationalizing biologic activity from chemical structure of homologues and analogues," while a second (Roelofs, Chapter 16) maintains, "A general hypothesis on predicting structural modifications that would affect the sensory input seems to be valid with the moth species investigated to date," one can imagine that some lively discussion resulted. I wish the editors had shared some of this intercourse with us.

The book is a collection of the formal presentations of 21 international scientists (five receive support from the Rockefeller Foundation) and, therefore, suffers (though less than in similar works) from a redundancy of introductory remarks. There is also frequent overlap in the subjects discussed; however, this presentation of varied approaches and points of view is a major strength.

The work is divided into five sections. The first contains three fine articles (Schoonhoven, Seabrook, Kaissling) on sensory physiology, although it seems odd that none of these authors commented on the theories of Wright or Callahan. The second discusses the behavioral responses of phytophagous (K. Munakata) and hematophagous (R. Galun) insects to host-produced chemicals. A particularly interesting chapter by J.S. Kennedy focuses upon the behavioral mechanisms of olfactory responses to distant odor sources and is complemented by remarks of L. Barton Browne on behavioral aspects of repellents and deterrents to feeding and oviposition.

The behavioral responses of Diptera (B.S. Fletcher), Hymenoptera (M.S.

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Blum), Coleoptera (J.H. Borden) and Lepidoptera (R.J. Bartell) to pheromones, allomones, and kairomones are presented in the third section. Also included is Professor Kennedy's reasoned plea for analytical behavioral assay methods (a recurrent theme in the book) that will illuminate the specific behavioral units and orientation mechanisms involved in gross behaviors such as attraction or repulsion.

Next are chapters by R.M. Silverstein, Y. Tamaki, and W. Roelofs, natural product chemists, who discuss many of the specific behavior-modifying chemicals with particular emphasis upon the importance of multi-component systems. These are not dry chemical treatises, but contain thought-provoking theses on the nature of chemical communication systems and the philosophies and methodologies of the investigators.

The final section consists of seven chapters presenting the status and prospects for practical utilization of behavior-modifying chemicals to control pests (F.G. Maxwell, A.A. Kahn, D.L. Chambers, W.E. Burkholder, H.H. Shorey, D.L. Wood, and A. K. Minks) and closes with a multiauthored (C.S. Koehler et al.) summary of the state of the art. Unbelievably, research on organismal biosynthesis of behavior modifying chemicals and pheromone–hormone relationships are not on the list of areas recommended for special research attention.

This is a work that all insect behaviorists will read, enjoy, and react to. It contains many controversial statements reflecting healthy wars of thought. One could spend several pages attacking Blum's attack on the term kairomone, defending Kennedy's theory of odor-conditioned, optomotor anemotaxis, or lauding Bartell's interesting discussion of adaptation and habituation.

The importance of chemical blends and the behavior-modifying effects of individual components of multichemical odor cues are recurring themes. Several authors wrestle with the terms commonly used to describe the roles of chemical stimuli and the behavioral responses they elicit. These chapters are recommended supplements to any animal behavior course designed to develop students who think. The two chapters by J.S. Kennedy should be read by all would-be bioassayists.

The work may be of lesser interest to the nonspecialist although it contains several object lessons on the value of basic research to an applied world. Frequent reference is made to the real or potential usefulness of the knowledge presented, and the final chapters summarize several pest management rationales, all of which embrace the multifaceted approach of modern pest management. The ultimate benefit of thoroughly investigating insect behavior is tersely summarized by Borden, "What is understood can be manipulated and managed."

This is a wide-ranging book, and it contains many very strong and a few

rather weak chapters. Its subjects have been examined in several other works; overall, it is an important and stimulating contribution.

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A TRAPPING SYSTEM FOR THE WESTERN PINE BEETLE USING ATTRACTIVE PHEROMONES[1]

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Abstract—A trapping system utilizing attractive pheromones is described for the suppression and survey of western pine beetle, *Dendroctonus brevicomis* LeConte (Coleoptera: Scolytidae), populations over large forested areas and for extended periods of time. Two types of compactable sticky traps are described. A large vane trap was developed for population suppression. It consists of 6 m^2 of trapping surface comprised of four sticky coated fabric panels supported with a telescoping mast. A smaller cylindrical shaped trap with 0.19 m^2 trapping surface was developed for survey purposes. Perchloroethylene cleaning devices were developed to remove insects from both traps. Methods for sample splitting and sequential counting of the subsamples were developed to estimate numbers of insects on the suppression traps. Experiments were conducted to evaluate the efficacy of both the traps and the counting procedures.

Key Words—*Dendroctonus*, pheromones, attractants, pheromone trap, trapout, survey, bark beetles.

INTRODUCTION

D. brevicomis attacks and kills ponderosa pine, *Pinus ponderosa* Laws., a tree of high commercial value in the western United States. Large numbers of these beetles are attracted to and subsequently colonize living trees by means of a complex pheromone system. The literature pertaining to this host-finding and colonization behavior has been summarized by Wood (1972)

[1] This work was supported by grants to the University of California (D.L. Wood) from the National Science Foundation/Environmental Protection Agency (NSF-6B-34718/BMS75-04223), Forest Service, U.S. Department of Agriculture and Rockefeller Foundation. The findings, opinions, and recommendations are those of the author and not necessarily those of these institutions.

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and Wood and Bedard (1977). A host terpene, myrcene, and two beetle-produced bicyclic ketals, *exo*-brevicomin (Silverstein et al., 1968) and frontalin (Kinzer et al., 1969), have been identified which, when evaporated near sticky traps, attract large numbers of these beetles. A simple, economical method to evaporate the three pheromones at reasonably consistent rates for periods in excess of 30 days was developed by Rodin and Tilden (unpublished data). Data on their efficacy as attractants was based on extensive small-scale field trials (Bedard et al., 1970; Wood, 1972). The large numbers of beetles caught during these tests plus data on flight behavior in the vicinity of pheromone release points (Tilden, 1976) provided the necessary background on beetle behaviour to undertake large-scale field tests (Bedard and Wood, 1970, 1974). The objective was to cause a measurable reduction in beetle populations. A second objective was to evaluate pheromone traps as a population survey tool. Variations in system design were tested during three consecutive years, in 1970 at Bass Lake (Sierra National Forest, Madera County, California) and in 1971 and 1972 tests at McCloud Flats (Shasta National Forest, Siskiyou County, California). A description of the 1970 test, its design rationale, and a preliminary summary have been published (Bedard and Wood, 1974).

Prior to the Bass Lake experiment most of the *D. brevicomis* pheromone research was directed toward the evaluation of the relative attractiveness of various pheromone mixtures (Bedard et al., 1970). Maximizing trap catch was not a goal of these early studies. Most traps used were sticky-coated metal fabric or hardware cloth (Bedard and Browne, 1969). In experiments with various sizes and configurations of these sticky-fabric traps a positive correlation between trap surface area and the number of beetles caught was observed (Tilden, 1976). As one of the objectives of the Bass Lake and McCloud Flats experiments was to attempt a measurable reduction of local populations directly by trapping beetles, it was suspected that a much larger trap than previously used would be required. However, considerations of durability, means of deployment and cost dictated a compromise in size.

The system that evolved for trapping and counting bark beetles during the Bass Lake and McCloud Flats studies is described in this report.

METHODS AND MATERIALS

Vane Trap

The vane trap used for population suppression in these experiments is shown in an exploded view in Figure 1. The trapping surfaces were four 203 × 76-cm vertical vanes or panels made of sticky (Stikem Special®, Michel

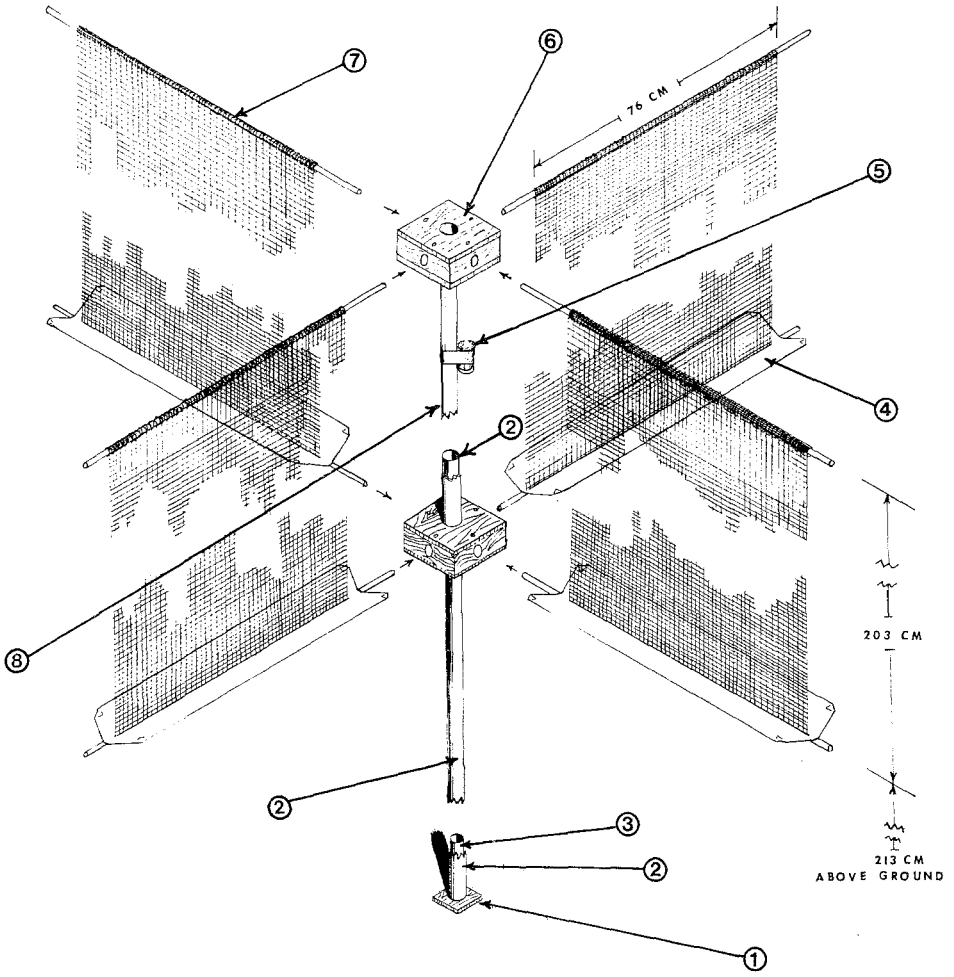

FIG. 1. Bark beetle vane trap: (1) ground plate; (2) EMT standpipe; (3) iron pipe; (4) aluminium catch trough; (5) pheromone evaporation device; (6) vane block; (7) upper vane dowel; (8) PVC vane support.

& Pelton Co., Emeryville, California) coated fabric and mounted at right angles to each other on a telescoping mast.

Masts consisted of two parts, a vane support (8 in Figure 1) which telescoped over a trap standpipe (2 in Figure 1). This telescoping assemblage allowed the trap to be raised and lowered in the field.

The vanes consisted of a vinyl-coated fiberglass (0.5-mm strands with a 5×12.7-mm mesh opening) stapled top and bottom to horizontal dowels. An aluminum catch trough was attached between the fabric and the bottom

dowel. This trough reduced the loss of insects caused by either the beetles crawling down the vanes through the sticky material or by "flowing" downward on very hot days when the sticky substrate became much less viscous. To apply the sticky material, fabric for each vane was wound around the upper dowel and laid along the bottom of the trough. Molten Stikem Special was then ladled along the top of the roll and by turning the upper dowel several revolutions a thorough application could be achieved. The fabric roll remained stuck to the bottom of the trough upon cooling, and the sides of the trough were folded around the vane to protect it. The function of the mast was to hold the vanes erect and at a sufficient height above the forest floor to reduce possible damage from grazing animals and to reduce the accumulation of debris. In addition it was hypothesized that this was a better position to intercept beetles in flight. Previous studies (DeMars et al., 1970) have shown that *D. brevicomis* initiates attacks at mid-bole on second-growth ponderosa pine.

Cylindrical Trap

The cylindrical trap designed for the survey component of the McCloud Flats experiments is shown in Figure 2. This trap consisted of a replaceable veil made of the same sticky coated fabric previously described and shaped into a cylinder (20 cm diam × 30.5 cm high) with two end plates made from corrugated paperboard (Wet Lock®, Hoerner Waldorf Corp., San Leandro, California). The trap veil was held erect in the field by a 1.8-m-high standard which passed loosely through the center of the bottom plate (E, + B, and F) and supported the top plate (A, B, and C) so that the weight of the lower plate held the veil taut.

The dies[2] used in cutting the endplate disks were "nicked" so that small parts of the disks were not cut through and all of the pieces remained in sheets. The endplate parts were punched from the sheets at the construction site. Plugs for the trap standard and evaporation device holes were not removed at first in order to prevent hot sticky material from leaking from the traps during construction. These plugs were removed when the traps were erected in the field and saved for reuse. The veil was coated with the sticky material by lifting the top end plate, inserting a ladle containing approximately 75 ml of molten sticky material through the seam, and depositing it onto the center of the horizontal bottom plate. After the ladle was withdrawn the top plate was rotated one or two turns while being lowered to the bottom plate. This process collapsed the veil, and an additional partial turn of the plates

[2] Dies for these traps, designed by the author, are the property of the USDA Forest Service and reside with the manufacturer, Hoerner Waldorf Corp, San Leandro, California.

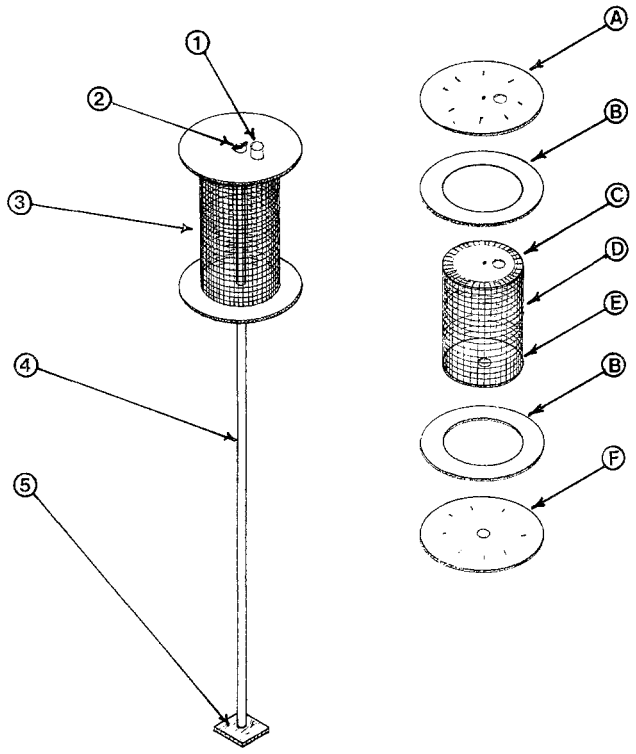

FIG. 2. Bark beetle cylindrical trap: (1) pheromone evaporation device; (2) wing nut and fender washer fastener; (3) sticky coated veil; (4) PVC trap standard; (5) ground plate; (A–F) exploded view showing veil and endplates.

while pressed tightly together (until the sticky material appeared at the periphery) completed the coating of the veil. The trap, thus collapsed, completely protected the sticky surfaces. At the end of a trapping period when traps were removed from the standards, they were twisted and pressed lightly together in a similar manner. Thus the trap was closed for transport with trapped insects safely inside.

Estimating Trap Catches

Vane Trap Cleaning. In the suppression experiments at both Bass Lake and McCloud Flats, vane trap catches were estimated in the laboratory using a sequential counting procedure. This procedure required that insects to be

counted be removed from the traps and be completely free of sticky materials.

For the Bass Lake experiment a heater-pump device was built which pumped kerosene, heated to 80°C at 40 psi, to a hand-held spray wand. Trap panels to be cleaned were unrolled along the bottom of a large tank. The hot kerosene partially dissolved the sticky material, and the high pressure

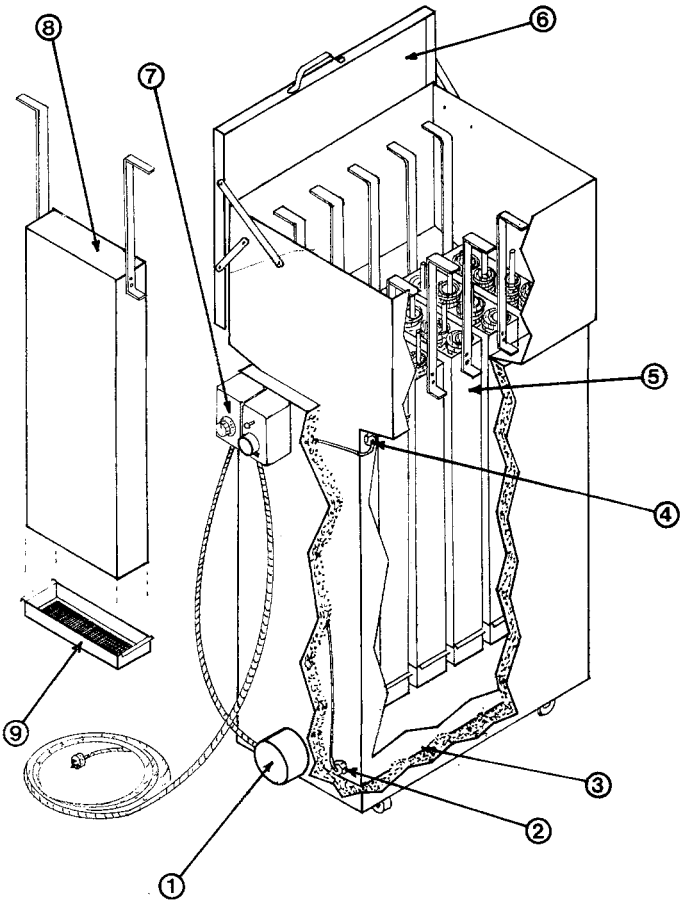

FIG. 3. Vane trap degreaser: (1) heater; (2) thermal cutout switch sensor; (3) cut-away view showing lower part of the chamber, insulation, and outer shroud; (4) thermostat sensor; (5) baskets with trap vanes in position for cleaning; (6) lid in the open position; (7) temperature controls; (8) upper portion of a vane basket; (9) a basket end cap removed to show 20-mesh screen in bottom.

spray provided sufficient agitation to dislodge insects from the trap fabric. The combination of insects and debris washed from the traps was strained out of the kerosene. Materials removed were further cleaned with trichloroethylene[3] vapors to remove the remainder of the sticky material. The trichloroethylene degreasing procedure, followed by oven drying at 150°C for 30 min, conditioned the samples so the material could be accurately subdivided and counted. Because this system was dangerous and laborious, a perchloroethylene cleaning system was developed for subsequent experiments.

A perchloroethylene degreaser designed to clean the McCloud Flats vane traps is shown in Figure 3 and includes an exploded view of one of the six baskets used to hold the trap vanes. Part of the metal shroud and insulation is cut away to show the location of the thermostat wells in the chamber wall and the baskets with trap vanes in position to be cleaned. The baskets were supported above the solvent reservoir. The lower part of the chamber which contained the solvent reservoir and vapor column was insulated on the four sides and bottom with 3.8-cm-thick fiberglass. The wider upper part of the chamber was uninsulated and served as a condenser. The chamber wall was fitted with the pipe reducer bushings in holes through the walls to provide fittings with female pipe threads (NPT) for the mounting of the heater, two sensors, and a shutoff valve. The heater and the sensor for the safety cutoff switch were located so as to protrude into the liquid reservoir. The sensor controlling the vapor height (4 in Figure 3) was located on the front wall 2.5 cm below the top of the insulation. Chamber accessories consisted of a 3000-w heater (Chromalox MTO 230 A) to vaporize the solvent, a thermal cutout switch (Chromalox ARC-540) wired in series with the heater to protect against overheating and subsequent ignition of the residue in the event of solvent depletion, and a controlling thermostat (Chromalox AR 2524) which in normal operation sensed the heat transferred upward over the trap baskets by the solvent vapor column. During periods of heavy usage the reservoir was drained daily and replenished with fresh solvent to prevent excessive accumulations of sticky material. The baskets (8 in Figure 3) for the suppression traps had removable end caps (9 in Figure 3) to catch insects that may have fallen from the traps during the degreasing process. All steel parts were zinc coated to prevent corrosion. The bottom of the end cap was 20-mesh copper screen.

A vane brushing machine (Figure 4) was developed to dislodge insects and debris from the perchloroethylene-cleaned vanes. After degreasing and cooling, the vanes were removed from the degreasing baskets one at a time and passed through the brushing machine. This machine consisted of a

[3] This procedure was suggested by B. Ewing, Department of Entomological Sciences, University of California, Berkeley, California.

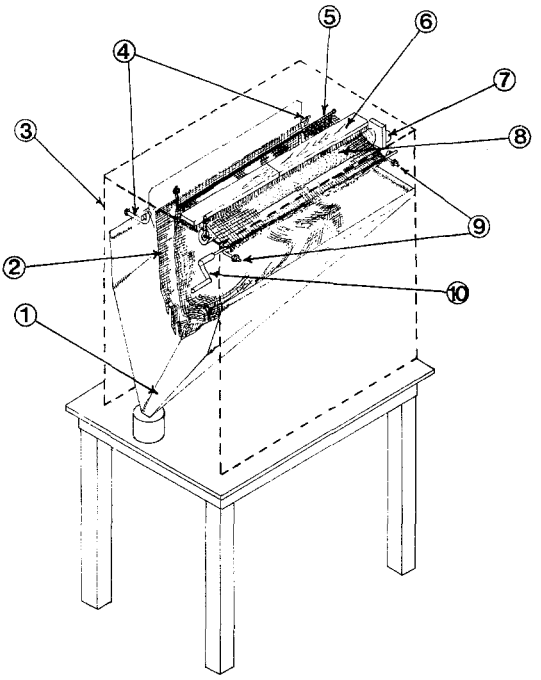

FIG. 4. Vane brushing machine: (1) spout; (2) unrolled vane fabric to be brushed; (3) plywood frame; (4) hooks holding vane trough; (5) guide rod; (6) stationary brush; (7) gear motor; (8) rotary brush; (9) hooks holding upper vane dowel; (10) winding crank.

boxlike frame 57 cm wide, 100 cm high, and 100 cm deep. In order to show the interior construction, the frame (3 in Figure 4) is depicted as being transparent and its shape is indicated with broken lines. It was constructed from 19-mm plywood, open at the front and designed to rest on a table top. The brushing assembly consisted of a stationary brush (6 in Figure 4) fastened to the underside of the frame ceiling and a rotating brush (8 in Figure 4) which was powered by a 20-rpm electric gear motor (7 in Figure 4) controlled by a foot switch. The rotating brush was mounted parallel to, slightly below, and to the right of the stationary brush. The two brushes were positioned so as to lightly contact each other. In operation the vane catch trough was first flattened, then hung in hooks mounted on the left-hand wall (4 in Figure 4). Next the fabric was unrolled, the upper dowel threaded over the guide, below the stationary brush, over the rotating brush, then drawn

across the machine and cradled in two hooks (9 in Figure 4) mounted on the right-hand wall. A hand crank (10 in Figure 4) was attached to the protruding end of the dowel. By turning the crank the fabric was drawn through the brush assembly and rewound onto the upper dowel. The vane catch trough was then removed from the wall hooks and fed through the brushes also. Upon removal from the machine, the trough was then refolded around the wound fabric making the vane ready for a reapplication of sticky material and reuse in the field. Material that was brushed from the vanes could be accurately subdivided for counting without further processing.

Sequential Counting Procedure. Insects and debris removed from the vane traps were split into a geometric progression of halved fractions prior to counting using a no. 34 Boerner Sampler (Seedburo Equipment Co., Chicago, Illinois). A sequential counting scheme for counting the samples in the reverse direction of the split was developed[4] to estimate the total number of beetles in the trapped material. Each sample of trapped material was sieved directly into the Boerner Sampler through hardware cloth (36-mm^2 opening) to remove the large debris. Material larger than 2.5 cm across could block the sampler splitting orifices and bias the split. The sampler divided the material into two equal fractions, one of which was saved and labeled while the other was reintroduced into the sampler and redivided. The geometric progression of 1/2, generated by saving one half and rehalving the other, was continued until the desired smallest fraction was obtained. The smallest fraction at Bass Lake represented 1/32 of the trap total while at McCloud Flats, because of the higher numbers caught the traps were divided to 1/128. Counting always began with the smallest fraction, then progressively larger fractions were counted until, upon completion of a given fraction, the total accumulated count was equal to or greater than 60 ($\pm 26\%$ accuracy with a 95% level of confidence). This counting process was carried out simultaneously on the subdivided material for both the western pine beetle and one of its predators, *Temnochila chlorodia* (Mann.) (Coleoptera: Trogostidae). The trap total was then computed by multiplying the number of insects counted by the reciprocal of the proportion of the trap examined.

An extensive effort was made to determine the accuracy of this sequential counting procedure by subdividing and counting the catch from 32 traps. The subsequent counts of factions were recorded separately according to whether they originated from the A or B funnel. Recording the data in this manner permitted detection of various sources of bias in the sample-splitting procedure. Several sources of bias found in the splitting procedure were corrected by (1) adjusting the splitting vanes of the sampler (vane calibration gauges are available from Seedboro Co.), and (2) sieving the material directly

[4] The mathematical description of this sampling technique will be published elsewhere by B. Ewing.

into the sampler rather than using the installed drop gate. When the drop gate was used, the fibrous material in the sample tended to clump the insects together causing greater variation in the splits. Also, the drop gate did not adaquately distribute a small sample over the sampler cone. Degreasing the sample obviated the severe clumping that would have occurred had the residual sticky material not been removed.

Field Estimates of Vane Trap Catch. At Bass Lake the vane trap design was also used for survey. Survey catch estimates were made in the field at approximately two week intervals throughout the study period. These estimates were made by removing with tweezers and counting the insects caught within a 930-cm^2 predesignated area in the center of each of the four trap vanes. These sample areas represented 6% of the total trapping surface. In order to estimate total catch from the counts of the field-sampled areas, ten survey traps were selected from various kinds of trap sites throughout the study area. On two separate occasions during the study, vanes from these traps were collected, cleaned, and the total catch for each trap was estimated with the sequential counting procedure. New vanes were installed at the time of collection. The exposure time of the vanes at the first collection averaged 138 days with the shortest period being 107 days. The replaced vanes from these same traps were collected an average of 27.4 days later. The material cleaned from these traps was subdivided to 1/32 with the minimum count limit at 60. The correlation of field counts to sample-splitter counts was made for estimates of both the *D. brevicomis* and its predator, *T. chlorodia* in order to derive a suitable factor by which to expand the field counts.

Total catch of *D. brevicomis* and *T. chlorodia* on survey traps at Bass Lake was estimated by multiplying by 10 the total number counted and removed from the four 0.37-m^2 sample areas of each trap during the biweekly visits. This factor of 10 is approximately the slope derived from the linear regression of the field count data on the laboratory estimates of whole trap catches from ten traps. For *D. brevicomis* $b = 10.34$, $r = 0.964$ and for *T. chlorodia* $b = 9.44$, $r = 0.927$.

Subsequent to the Bass Lake experiments an improved field method to sample the vane surface was developed. This procedure was tested at McCloud Flats but not used in those experiments. A 10-cm-wide vertical white stripe to delineate the count area was painted down the center of each vane, representing 13.3% of the surface area. Counts from this area were compared to counts of beetles following a perchloroethylene cleaning where all insects and debris were brushed from the vanes and subsequently subdivided using the Boerner sampler. The smallest subsample equaled one eighth of the vane total. Beetles were counted using the sequential counting procedure. However, the minimum count limit was increased to 120 in order to estimate the catch with a $\pm 18\%$ accuracy with a 95% level of confidence.

D. brevicomis counts from this sample area on each trap vane were more highly correlated with the laboratory estimate than were the counts from square sample areas on the Bass Lake traps. Total vane surface was 7.5 times greater than the area of the sample stripe. The slope of the regression for counts from the stripe on the laboratory estimate was 7.7. The increase in the correlation coefficient ($r = 0.98$) of the stripe over the square sample area ($r = 0.964$) reflects both the increase in the proportion of the vane counted and an improved sampling of the distribution. The stripe subsampling scheme tested at McCloud Flats was sufficiently accurate to estimate the total catch per trap. However, excessive labor cost would result when catches were high (one trap with approx. 30,000 beetles took one man-day to count). The need to synchronize the catch estimates for all traps within a very short time period and the development of an improved perchloroethylene cleaning system caused our rejection of this field estimation procedure.

Loss of Beetles from Traps Due to Exposure. It was anticipated that some beetles would be lost from the traps either while the trap was standing in the field or during the collecting and cleaning procedures. In addition, it was suspected that trap tenacity decreased with field exposure. In order to evaluate the fate of beetles landing on traps near the end of a trapping period, retention of beetles on "old" vanes which had been exposed in the field was compared with new, unexposed vanes. Three "old" vanes were removed from unbaited traps that had been exposed to field conditions for a period of one month. They were carefully examined in order to remove any *D. brevicomis*. These vanes were compared with 7 "new" vanes. Fifty *D. brevicomis* were either forced to fly or were tossed onto each of both "old" and "new" vanes. These vanes were then mounted on unbaited trap masts in the field for an additional exposure period of one month. Four "new" and two "old" vanes were exposed in a sunny opening in the forest canopy and three "new" vanes and one "old" vane were exposed in the shade of a group of fir trees at McCloud Flats. These vanes were exposed near unbaited check traps. These initially beetle-free check traps were used to estimate the number of beetles that were randomly trapped on the study vanes. The vanes exposed in the sunny site were located approx. 30 m from one unbaited check trap and approx. 1.2 km from a second. The vanes in the shady site were located approx. 13 km from those in the sunny site and about 5 m from a third check trap and approx. 1.2 km from a fourth. At the end of this exposure period the vanes were removed from the trap masts, individually wrapped in plastic bags, and cleaned in the perchloroethylene degreaser. All of the trapped insects removed from each vane were counted.

An average per vane of 42.4 *D. brevicomis* were recovered from all vanes after exposure in the field for one month. This was 84% of the number placed on these vanes at the beginning of the test; however, a few of the recovered

beetles may have been trapped during the exposure period. An average of 3.1 beetles per vane were caught on the two check traps nearest the shaded traps and 1.9 beetles per vane were caught on the two check traps nearest the traps exposed to the sun. A mean of 2.5 beetles were caught on all 16 check trap vanes during this period. Subtracting 2.5 beetles from the average number recovered indicates that estimates made for catches on suppression traps represents at least 80% of the beetles actually caught. Traps in more exposed sites lost a greater number of beetles than traps in the shade. A mean of 46.8 (93%) beetles were recovered from the shaded vanes and a mean of 39.2 (78.3%) beetles were recovered from the vanes in full sun. A mean of 43.5 (87%) and 39.5 (79%) of the beetles were recovered from "new" and "old" vanes, respectively.

Vane Efficiency. In the experiment to determine loss of beetles from traps some of the 50 beetles per vane were forced to fly onto the vanes. These beetles were applied in this manner in order to determine the number of beetles that could escape immediately after an encounter with the vane. Of 138 beetles that were forced to fly into five "new" vanes, 119 were trapped on the vanes, 2 were trapped in the catch troughs, 2 were on the plastic sheet beneath the apparatus, and 15 were missing. Presumably these 15 flew through the vane and beyond the plastic sheet. From these observations it appears that as many as 11% of the beetles that fly into the vanes may actually fly through them. However, some of these beetles may be caught subsequently.

Cylindrical Trap Cleaning and Counting. Cleaning. A 2500-W commercial degreaser (Dayton Electric Mfg. Co., Chicago, Illinois, model 3Z233) was modified by redesigning the cleaning basket to fit the cylindrical traps. The "parts basket" of the Dayton degreaser was replaced with a basket that held nine collapsed cylindrical traps edgewise to permit drainage of the melted sticky material. This new basket was a semicylindrical screen 36.5 cm in length with a radius of 17.3 cm and divided into 9 compartments by 20-gauge zinc-coated steel partitions. Two such baskets were constructed so one set of traps could be cleaned and cooled while another set was being counted. Traps were found to survive this cleaning process in excellent condition and could be recycled into the weekly replacement schedule. These traps were reused many times during the ensuing two years of the McCloud survey experiments.

Counting. Because of the reduced size of the cylindrical traps, the catch was greatly reduced. This, together with the improved perchloroethylene cleaning procedure, permitted the entire catch to be counted. The number of traps (225) and the frequency of trap collection (weekly) at McCloud Flats required the development of a display to monitor the progress of the collection, cleaning, and counting procedure. The survey plot grids were drawn on identical boards with holes drilled at each point representing the location

of a survey trap. Each board represented a survey week. After counting, trap contents were stored in plastic cups (30 ml) with both the trap and insect data recorded on the lid. Each cup was then placed into a hole in the display board that corresponded to that trap's position in the survey grid. These display boards greatly reduced mistakes in data recording and provided easy access to counted materials.

DISCUSSION

This trapping system was adequate for the Bass Lake and McCloud Flats experiments because it provided a reliable means of evaluating different trapping stategies. With this system it is possible to estimate the cumulative catch at varying time intervals, trap densities, and pheromone evaporation rates. However, it was not without its limitations. Although the perchloroethylene was a very efficient trap cleaner, the process removed fatty materials from the insects leaving them in a hard and brittle state. Insects with hard exoskeletons survived the process intact. Soft- and fragile-bodied insects such as Diptera and Hymenoptera were badly broken, however. It was noted that the insect bodies remained pliable when cleaned and stored in kerosene. Several attempts to accurately subsample this kerosene trap residue while wet failed, in part, due to the large amount of debris present. Various density and sieving procedures to separate insects from debris in both wet and oven-dried samples were unsuccessful. Attempts to split oven-dried samples in the Boerner sampler without the trichloroethylene vapor cleaning were not accurate because a "greasy" residue on the samples, consisting mostly of undissolved Stikem from the traps, caused clumping.

Several species of Hymenoptera and Diptera known to be associated with *D. brevicomis* within infested trees may have responded to the attractive pheromones. The presence of these insects on the McCloud Flats traps, both suppression and survey, were detected by selecting traps randomly from each set of collected traps and cleaning them in hot kerosene (Roettgering, 1973). The kerosene cleaning apparatus used has been described by Stephen and Dahlsten (1976). These traps were examined in their entirety.

During the Bass Lake and McCloud Flats experiments all traps caught in excess of 7.7 million *D. brevicomis*, based on preliminary estimates for both Bass Lake (Bedard and Wood, 1974) and McCloud Flats (Roettgering, 1973), and do not include estimates of beetles lost from traps in the field. Thus they are conservative, perhaps representing only 80% of the mortality caused by trapping. A catch of 63,232 beetles on a single trap demonstrates that trap capacity must be an important design consideration. Unbaited traps of the same size on occasion caught no beetles. Thus it is necessary

that the counting procedure provide a means of estimating both extremes with a calculable probability. At Bass Lake the survey vane traps caught an average of 435 *D. brevicomis* per week. It was felt that this large number of beetles was not essential for the purpose of survey and could effect local densities. The smaller cylindrical trap caught an average per week of 47.5 (0–1117) in 1971 and 55.0 (0–1375) in 1972 at McCloud Flats. Thus, the goal of catching large numbers of beetles on the vane traps for population suppression and few on the cylindrical traps for population survey was achieved.

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PREPARATION AND USE OF SEX ATTRACTANTS FOR FOUR SPECIES OF PINE SAWFLIES[1]

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Abstract—Sex attractants were prepared for four species of diprionid sawflies, *Neodiprion lecontei*, *N. swainei*, *N. sertifer*, and *Diprion similis*, by esterification of both synthetic and naturally occurring alcohols. For the first two species totally synthetic (racemic) compounds were attractive to males in the field. For the latter two species, none of the synthetic compounds tested were consistently attractive. For these, effective attractants were prepared by saponification and reesterification of crude extracts from female insects.

Key Words—Pheromones, pine sawflies, field trial, *Neodiprion lecontei*, *N. swainei*, *N. sertifer*, *Diprion similis*, sex attractants, 3,7-dimethyl-2-pentadecanol esters.

INTRODUCTION

Recently we reported the identification of 3,7-dimethyl-2-pentadecanol from several species of pine sawflies. Chemical and behavioral evidence indicated that several species of the economically important genera *Neodiprion* and *Diprion* use acetate or propionate esters of this alcohol as their major sex attractant (Jewett et al., 1976a). The alcohol we synthesized was a mixture of eight optical isomers, some of which could reduce the effectiveness of the synthetic material in the field relative to the pure natural product. However, in identifying the natural product, we discovered various materials, including two synthetic analogs, acetate and propionate of 3-methyl-2-pentadecanol, that worked well in the field for their respective species. Since crude solvent extracts from female pine sawflies are generally unattractive to males, these

[1] Hymenoptera: Diprionidae.

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materials, the preparations of which we report below, may offer an opportunity to experiment with chemical sex attractants for survey and control in this group of insects in the field. Below we report the details of preparation and evaluation of attractants for *Neodiprion lecontei* (Fitch), *N. swainei* (Middleton), *N. sertifer* (Geoffroy), and *Diprion similis* (Hartig). For the first two species adequate synthetic compounds were found; for the latter two species, no truly effective synthetic compounds have been found, and we report instead simple modifications of solvent extracts from virgin females which converted these inactive naturally occurring materials to highly active ones.

METHODS AND MATERIALS

Synthesis of 3-Methyl-2-pentadecanol and 3,7-Dimethyl-2-pentadecanol

3-Methyl-2-pentadecanol and 3,7-dimethyl-2-pentadecanol were prepared by reacting the appropriately substituted 2-methyltetradecanal with methylmagnesium iodide. The aldehydes were prepared by hydride reduction and subsequent oxidation of the corresponding 2-methyltetradecanoic acids. These latter were prepared by malonic ester synthesis from diethylmethyl malonate and the appropriately substituted dodecyl halide (Figure 1). Alternately, a small amount of 3,7-dimethyl-2-pentadecanol was prepared by reduction of the corresponding ketone, which was obtained by the anodic coupling of 4-methyldecanoic acid with 3-methyllevulinic acid.

2-Methyltetradecanoic Acid (*Ia*). 1-Bromododecane, diethyl methylmalonate, and sodium ethoxide (0.2 mol each) were refluxed 8 hr in 400 ml absolute ethanol. The mixture was cooled, diluted with 500 ml water and thoroughly extracted with ether to remove the product. After removal of the ether, the product was refluxed 4 hr with 40 g NaOH in 500 ml of 80% ethanol. This mixture was cooled in an ice bath and acidified slightly with dilute H_2SO_4. The dicarboxylic acid was collected by extracting twice with ether. The ether layer was washed twice with saturated NaCl solution and dried over anhydrous Na_2SO_4. After removal of the solvent, the diacid was heated on an oil bath at 150–200 °C under a gentle stream of nitrogen until no more CO_2 was evolved. The acid (Ia) so obtained was used in the next step without further purification.

2-Methyl-1-tetradecanol (*Ib*). Vitride® (165 ml, Eastman Chemical Co.) was added to 400 ml anhydrous ether in a 2-liter round-bottomed flask equipped with a magnetic stirrer and reflux condenser. 2-Methyltetradecanoic acid (Ia) (about 1.8 mol) was dissolved in 100 ml anhydrous ether and added dropwise with stirring to the Vitride solution, so that hydrogen was not evolved too rapidly. After addition was complete, the mixture was refluxed

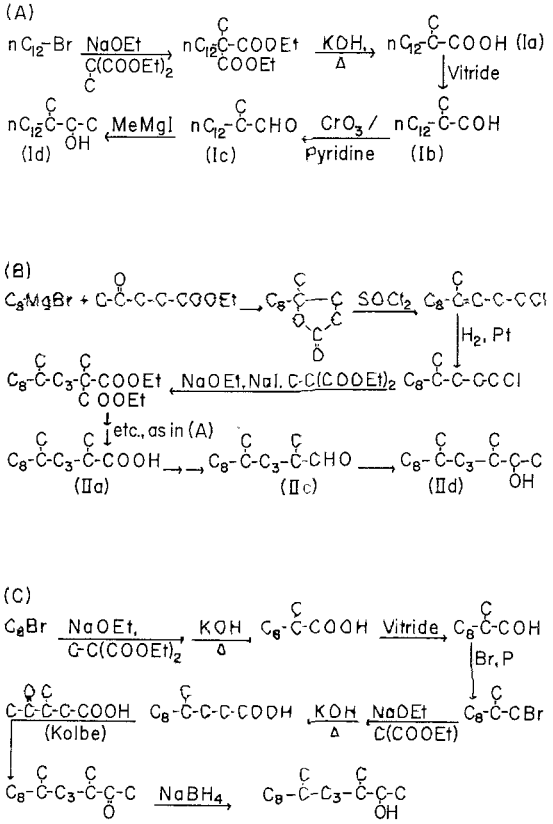

FIG. 1. Synthetic schemes for the preparation of 3-methyl-2-pentadecanol (A) and 3,7-dimethyl-2-pentadecanol, via malonic ester route (B) and 3-methyllevulinic acid route (C). There are other routes of synthesis now available: for direct synthesis of 3,7-dimethyl-2-pentadecanol from cyclohexanone see Kocienski and Ansell (1977), and for 3-methyl-2-ones see Burgstahler et al. (1977).

4 hr. The flask was cooled to 0°, and ethyl acetate was added until effervescence of hydrogen ceased. Aqueous KOH (400 ml 20%) was added, and refluxing was resumed until all of the white precipitate dissolved (about 4 hr). The aqueous layer was discarded, and the ether layer containing the product was washed twice with water and once with saturated NaCl and dried over anhydrous sodium sulfate. The alcohol (Ib) obtained after removal of the solvent was used for the next step without purification.

2-Methyltetradecanal (*Ic*). Crude 2-methyltetradecanol (Ib) was oxidized to the aldehyde by chromium trioxide–pyridine in methylene chloride. The method of Collins (1968) was followed, except that aqueous extraction of the product was avoided by the following procedure. After the reaction was complete, the solvent was removed by a rotary evaporator over a luke-warm water bath. The brown residue was stirred thoroughly with pentane, and the latter was filtered through diatomaceous earth with the aid of a vacuum. The pentane was removed by a rotary evaporator, and traces of pyridine were removed by warming the product for a few minutes under a gentle stream of nitrogen. The product was used in the next step without further purification.

3-Methyl-2-pentadecanol (*Id*). Magnesium turnings (0.11 mol) were placed in a 500-ml round-bottomed flask equipped with a reflux condenser and magnetic stirrer. The turnings were covered with a few milliliters anhydrous ether, and methyl iodide (0.1 mol) in 200 ml anhydrous ether was added with initial heating until the reaction started. After all the halide was added, the mixture was stirred for 10 min. It was then cooled to 0°, and crude 2-methyltetradecanal (about 0.05 mol) in 50 ml anhydrous ether was added dropwise. The mixture was stirred 1 hr at 0°. Excess reagent was destroyed by the slow addition of water. The alkoxide was hydrolyzed by adding 400 ml 20% ammonium chloride solution and stirring 4 hr until both ether and aqueous phases were clear. The aqueous layer was discarded, and the ether layer was washed twice with water and once with saturated NaCl solution and dried over anhydrous Na_2SO_4. The crude alcohol (Id) so obtained was esterified without further purification.

1-Chloro-4-methyldodecane. This compound was obtained by the procedure of Cason et al. (1948) for 1-chloro-4-methylnonane, except that bromooctane was substituted for bromopentane.

2,6-Dimethyltetradecanoic Acid (*IIa*). 1-Chloro-4-methyldodecane, diethyl methylmalonate, and sodium ethoxide (0.2 mol each) and sodium iodide (0.05 mol) were refluxed 8 hr in 400 ml absolute ethanol (Cason and Winans, 1950). Isolation, hydrolysis, and decarboxylation of the product to give IIa were exactly as described for Ia. The acid so obtained was used for the next step without further purification.

2,6-Dimethyltetradecanal (*IIc*). This compound was obtained by the hydride reduction and subsequent oxidation (exactly as for Ic) of the corresponding acid (IIa). The product was used for the next step without further purification.

3,7-Dimethyl-2-pentadecanol (*IId*). This compound was obtained by reacting methylmagnesium iodide with 2,6-dimethyltetradecanal (IIc) exactly as for Id. The crude alcohol so obtained was esterified without further purification.

Preparation of 3,7-Dimethylpentadecan-2-ol via 3-Methyllevulinic Acid

The scheme is shown in Figure IC. 2-Methyldecanol was obtained by reduction of the corresponding acid by using Vitride, as above. It was converted to the bromide and reacted with diethylmalonate to give 4-methyldodecanoic acid. The cross-coupling of the latter with 3-methyllevulinic acid was accomplished by the method of Finkelstein and Peterson (1960). Sodium borohydride was used to convert the ketone to the corresponding alcohol.

The identities of key synthetic intermediates were checked by thin-layer chromatography and infrared spectroscopy. The purities of the final products were ascertained by gas chromatographic analyses as described below.

Esterification

Acetates and propionates of the synthetic alcohols and of saponified natural extracts (see below) were prepared by the same method. The materials to be esterified were heated 1 hr at 80 °C with 10 vol of 2:1 (v/v) mixture of pyridine and the appropriate anhydride. The cooled mixture was dissolved in pentane and washed 3 times with water, once with 0.1 N HCl and once with saturated $NaHCO_3$ solution and dried over anydrous magnesium sulfate. The esters so produced from the saponified natural extracts were used without further purification. The esters of the synthetic compounds were purified by preparative TLC on silica gel HF_{254} (hexane–ethyl acetate 14:1, developing solvent). The esters were detected by ultraviolet light after spraying the plates with a 0.5% solution of dichlorofluorescein in 95% ethanol. The esters were eluted from the silica gel by ether and further purified by preparative gas chromatography (6-m × 1-cm column, 30% DC-11 on 80–100 mesh Gaschrom Q, nitrogen flow 100 ml/min, temperature programed to 210 °C). The largest (and last) peak (flame-ionization detector) emerging from the column was the desired product.

Preparation of a Modified Natural Extract Attractive to N. sertifer

Virgin female *N. sertifer* were obtained from cocoons from last instar larvae collected in the field at Lake Geneva, Wisconsin. Shortly after emergence the insects were frozen and stored at −20 °C. About 5000 insects were covered with methanol (about 500 ml) in a 2-liter round-bottomed flask and refluxed 24 hr. The methanol was removed and filtered. Fresh methanol was added, and refluxing was repeated for 24 hr. The second extract was filtered and added to the first in a 2-liter round-bottomed flask. The solvent was evaporated to a volume of 200 ml, and 10 g KOH was added. The mixture

was refluxed 2 hr. Most of the methanol was removed by a rotary evaporator. Hexane, 500 ml, and water, 200 ml, were added, and the mixture was swirled gently for 5 min. The mixture was transferred to a separatory funnel, and the aqueous layer removed. The solvent layer was passed through a funnel containing anhydrous Na_2SO_4. The pale yellow oil (a few milliliters) was acetylated with acetic anhydride in pyridine. The reaction mixture was dissolved in hexane and extracted with water, dilute HCl, and saturated $NaHCO_3$ solution. The hexane phase was dried over anhydrous $MgSO_4$ and adjusted to a volume of 200 ml (i.e., 10 female equivalents per 0.4 ml).

Preparation of a Modified Natural Extract Attractive to D. similis

This procedure was the same as that for the saponification and esterification of the extract from *N. sertifer*, except that the extract was prepared from virgin female *D. similis*, collected at Amery, Wisconsin, and propionic anhydride in pyridine was the reagent.

Preparation and Deployment of Traps

The synthetic or modified natural attractants were dissolved in hexane so that a known amount of attractant was contained in 0.4 ml. These solutions were applied to 2 cm lengths of cotton dental wick, and the hexane allowed to evaporate. Wicks so prepared retained their activity for a year or more when stored in a freezer. In the field the wicks were pressed firmly against the adhesive in the tops of Sectar traps (3M Company, St. Paul, Minnesota). The traps were suspended from branches 1–3 m from the ground, in areas where sawfly infestations were known or suspected: *N. sertifer*, red pine, southeastern Wisconsin, Sept.–Oct. 1975; *N. lecontei*, slash pine, Gainesville, Florida, July–Sept. 1975; *D. similis*, white pine, Amery, Wisconsin, June, 1972; *N. swainei*, jack pine, Quebec, Canada), June–July 1975.

RESULTS AND DISCUSSION

Table 1 indicates only the most effective attractant found for each species, the amounts used, and provides representative data for catches in the field.

N. lecontei. Racemic 3,7-dimethyl-2-pentadecyl acetate, corresponding to the natural sex attractant of this species, was attractive at the level of 1 mg/trap; however, it was less effective than the simpler analog, 3-methyl-2-pentadecyl acetate (Table 3). Traps baited with the latter remained effective in the field up to 4 weeks. The highest amount tested per trap, 1 mg, was more effective than smaller amounts. An attractant prepared by saponification and acetylation of a solvent extract from virgin female *N. lecontei* (as described

TABLE 1. SUMMARY OF MOST EFFECTIVE ATTRACTANTS, AMOUNTS, AND REPRESENTATIVE FIELD DATA FOR FOUR SPECIES OF PINE SAWFLIES

Species and most active attractant	Effective amount per trap	No. of traps	Total time	Total no. of males caught[a]
Neodiprion lecontei				
3-methyl-2-pentadecyl acetate	1 mg	10	8 wk[d]	315
Neodiprion swainei				
3,7-dimethyl-2-pentadecyl propionate[b]	200 μg	1	1 wk	81
Neodiprion sertifer				
Modified natural extract	10 feq[c]	62	5 wk	1068
Diprion similis				
Modified natural extract	10 feq	3	5 min	27[e]

[a] Unbaited traps caught no sawflies.
[b] Prepared by malonic ester route, Figure 1B.
[c] feq = female equivalents.
[d] Time period is not particularly meaningful since traps were monitored at odd periods. No specific indication of exactly when males were attracted was obtained except for the *D. similis* short-term traps.
[e] An air condensate, approximately 10 feq, collected from virgin female *D. similis* (Casida et al., 1963) attracted 13 males in 5 min under identical field conditions.

for *N. sertifer*) was attractive at about 10 female equivalents. However, the comparative ease with which 3-methyl-2-pentadecanyl acetate can be synthesized makes it, at present, the attractant of choice for this species.

N. swainei. For this species 3,7-dimethyl-2-pentadecyl propionate was attractive at 200 μg, the highest level tested. A trap baited with 40 μg of the same material caught 58 males during the same period (Table 3). Traps baited with 3-methyl-2-pentadecyl propionate were much less attractive, even at levels as high as 10 mg/trap, in contrast to the results of *N. lecontei.* The corresponding acetates have not yet been tested in the field, since electrophysiological evidence indicates that the natural sex attractant for *N. swainei* is probably a propionate (Jewett et al., 1976a).

N. sertifer. The material prepared by the saponification and acetylation of solvent extract from virgin female *N. sertifer* was attractive at 10 female equivalents per trap, the highest level tested. The corresponding propionate was active but did not compete with the acetate. Traps remained attractive for at least 4 weeks. Traps baited with 1 female equivalent were less effective. Synthetic 3,7-dimethyl-2-pentadecyl acetate obtained by the malonic ester route was most attractive at about 4 μg trap (1 μg/trap for that obtained via 3-methyllevulinic acid), but did not compete (at levels of up to 10 mg) with

the modified natural extract, virgin females, or with the highly purified acetylated natural alcohol. Further, when a wick containing 4 μg of the synthetic compound (via malonic ester) was placed next to a wick containing 4 μg of the purified acetylated natural alcohol, the attractiveness of the latter was decreased. This indicates that one or more of the eight optical isomers in the synthetic compounds are inhibitors (Vité et al., 1976) or that specific ratios of two or more enantiomers are required for an optimal activity (Borden et al., 1976). Similarly, 3-methyl-2-pentadecyl acetate was not competitive. A crude ether extract of virgin female *N. lecontei* was also unattractive. Thus the modified natural extract is the only practical attractant for this species at the present time.

D. similis. The material prepared by the saponification and propionation of a solvent extract from virgin female *D. similis* was attractive at 10 female equivalents. Although the data from only three replicates are presented in Table 1, similar results were obtained on many other occasions. The corresponding acetate was inactive, but the corresponding isobutyrate was somewhat active. 3,7-Dimethyl-2-pentadecyl propionate (prepared by route C, Figure 1, 1 μg/trap) and 3-methyl-2-pentadecyl propionate (50 mg/trap) were attractive at times, but the results were erratic. 3,7-Dimethyl-2-pentadecyl propionate prepared by route B, Figure 1, was unattractive to *D. similis*. We were able to isolate octadecyl isobutyrate from this species (unpublished results). While this and analogous esters of short-chain acids with long-chain (C_{12}–C_{18}) alcohols sometimes attracted male *D. similis*, again the results were erratic.

TABLE 2. NUMBERS OF MALES CAPTURED WITH VARYING AMOUNTS OF ATTRACTANT OF *Diprion similis* AND *Neodiprion sertifer*

Species	Pheromone source	Amounts of pheromone (μg/trap)						
		0.1	0.3	1	3	10	100	800
D. similis[a]	Synthetic[c]	0	0	0	0	0	0	0
D. similis[a]	Synthetic[d]			2	1	6	1	1
N. sertifer[b]	Synthetic[c]	0	0	2	0	0		
	Acetate of natural alcohol	0	2	10	0	1		

[a] Results from two seasons (August 1974, 1975) in Amery, Wisconsin. Virgin females attracted an average of 2 males.
[b] Assays conducted in Walworth County, Wisconsin (September, 1974).
[c] 3,7-Dimethyl-2-pentadecyl propionate synthesized by the malonic ester route (Figure 1B).
[d] 3,7-Dimethyl-2-pentadecyl propionate synthesized by anodic coupling of 3-methyl-levulinic acid and 4-methyldodecanoic acid (Figure 1C).

TABLE 3. NUMBERS OF MALES CAPTURED WITH VARYING AMOUNTS OF ATTRACTANT OF *Neodiprion lecontei* AND *N. swainei*

Species	Pheromone Source	Amounts of pheromone (μg/trap)						
		<1	1–2	8–10	40–80	80–100	200–250	750–1000
N. lecontei[a]	Synthetic[d]	0	0	0	0	0	3	47
N. lecontei[b]	Synthetic[e]				0	1	3	8
N. swainei[c]	Synthetic[f]	1	2	13	58		81	

[a] Traps placed in a stand of slash pines near Gainesville, Florida, over a two-month period in January through March 1974.
[b] Traps placed in the same vicinity in March through April 1975.
[c] Traps placed in Quebec, Canada, in a stand of jack pines (June 27 through July 4, 1975).
[d] 3-Methyl-2-pentadecyl acetate.
[e] 3,7-Dimethyl-2-pentadecyl acetate obtained by route B, Figure 1.
[f] 3,7-Dimethyl-2-pentadecyl propionate obtained by route B, Figure 1.

The most intriguing aspect of field bioassay of sawfly pheromone is that optimum amounts of pheromone are required to attract the maximum number of males in some species (Table 2), whereas in other sawflies no such relationships were observed within the ranges tested (Table 3). In the case of *N. sertifer* and *D. similis*, critical concentrations for optimal response were found for both the purified natural compounds and the synthetics. (Data for *D. similis* were not included in Table 2 because the absolute amount of natural pheromone was not known at the time the experiments were done.) The behavior of *N. lecontei* and *N. swainei* to their own purified natural pheromones has not yet been studied. The requirement for critical optimal concentrations correlates with the practical effectiveness of (racemic) synthetic compounds in the field; i.e., *D. similis* and *N. sertifer* have such requirements and are not attracted in large numbers by any of the synthetic compounds tested. Similar requirements have not been found for *N. lecontei* and *N. swainei* where effective synthetic attractants are known. This may be coincidental; studies on more species are needed.

In contrast to the field results, no critical optimal concentrations were found for electroantennograms, where the dose–response curves for all species tested were approximately logarithmic within the ranges tested (Jewett et al., 1976b).

For *D. similis* and *N. sertifer* in the field, the sharpness of the maxima in the dose–response curves depended on the purity of the material. Before chromatographic purification, such maxima were not apparent. Amounts of crude material varying over a range of several orders of magnitude attracted

males. After final gas chromatographic purification, it was necessary to make careful dilutions not to miss the response optimum completely.

So far we have been able to show that an effective attractant for a given species of *Diprion* and *Neodiprion* can be prepared by saponification and reesterification of a solvent extract from virgin female insects of the same species. Identification, on the basis of electroantennograms, of the sex attractants for several species from these genera as esters of 3,7 dimethyl-2-pentadecanol (Jewett et al., 1976a) provides the rationale for this procedure. We discovered that at least for *N. lecontei* and *N. sertifer*, virgin female insects, upon emergence, contain a relatively large amount of the free alcohol, which is apparently esterified at the time the insect releases the attractant. This procedure would convert the inactive alcohol to an active sex attractant. We do not know whether saponification of the crude extract is an indispensable step to obtaining an active pheromone. In *D. similis*, though, the crude ether extract from virgin females contains an unidentified compound(s) which acts as an inhibitor. This compound has been tentatively identified as an ester (unpublished results). Thus, at least for *D. similis*, saponification may be necessary to destroy the inhibitor.

Side-by-side comparisons of the chemical attractants with virgin female insects have been inconclusive until now because of the variability of the latter and because too few females were available at the right time. However, field tests at different times and in different locations indicate that the best synthetic attractant for each of the 4 species is comparable in effectiveness with virgin females. The unmated insects remain attractive for about a week, whereas traps baited with the appropriate synthetic compounds or modified extracts have remained attractive for as long as 4 or 5 weeks. Further, the chemical lures are easier to handle and deploy at the appropriate time.

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ALLELOPATHIC POTENTIAL OF *Ambrosia cumanensis* H.B.K. (COMPOSITAE) IN A TROPICAL ZONE OF MEXICO

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Abstract—Several experiments were carried out to confirm the allelopathic potential of *Ambrosia cumanensis* H.B.K. (Compositae) in a tropical zone of Mexico. The aqueous extracts of leaves and roots inhibited growth of several species used in bioassays. The wash-water of the leaves inhibited some species and stimulated others. The aqueous solutions of soil, in reference to those collected in July, were significant in their general inhibitory action. Decomposition of leaves and roots, in pots where several species were growing, was highly inhibitory to some of the seedlings; microorganisms have a major role in this process.

Key Words—Allelopathy, competition, aqueous extracts, leaf wash-water, soil solutions, organic residues, ruderal species, secondary vegetation.

INTRODUCTION

Most ragweeds are distinguished by their wide ecological tolerance, a generalized distribution, and more flexible habitat requirements than many other plants. This is because the total sum of habitat conditions exposed to strong perturbations have compelled ragweeds to develop structural adaptations, physiological reactions, and growth strategies which allow them to adjust simultaneously to different aspects of their environments, such as intra- and interspecific competition, predation, physical conditions of the environment, etc.

The success of the strategies developed by ragweeds in order to confront potential competitors depends on several ecological and biological factors. In

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several studies concerning the colonizing plants of the Compositae family, such as *Parthenium argentatum* (Bonner and Galston, 1944), *Artemisia absinthium* (Funke, 1943), *Helianthus annuus* (Wilson and Rice, 1968), *Helianthus rigidus* (Curtis and Cottam, 1950), *Chrysanthemum morifolium* (Kozel and Tukey, 1968), *Ambrosia cumanensis* (del Amo and Anaya, 1971), *Ambrosia psilostachya* (Neill and Rice, 1971), and *Ambrosia artemisiifolia* (Raynal and Bazzaz, 1975; Jackson and Willemsen, 1976), it has been shown that these plants may produce substances which are toxic to the germination and growth of other plants as well as to the development of certain soil microorganisms, a fact which could be important in explaining the success in the establishment of these colonizing species.

The *A. cumanensis–A. psilostachya* complex was studied from the biosystematic point of view; results obtained from this study led to the identification of all populations of this complex in Mexico as belonging to a single species: *Ambrosia cumanensis* H.B.K., with broad genetic, chemical, and morphological variations (del Amo and Gómez-Pompa, 1976).

Ambrosia cumanensis is a ragweed which is widely distributed in the semiarid and warm–humid zones of Mexico. It exists as very dense and practically pure populations, a fact which suggested that this species might have developed mechanisms for producing substances which hinder potential competitors and provide the weed with advantages for its subsistence within the community.

Some studies carried out in the Recovery of Tropical Rain Forest's Project[3] were made to determine the allelopathic potential of some plants of the secondary vegetation of the Tuxtlas region, in Veracruz (Anaya, 1976). Here, *Ambrosia cumanensis* was found as an important part of the ruderal vegetation of the zone, growing vigorously and in almost pure masses. Because of this, we decided to continue with allelopathic tests for this species, planning to evaluate this phenomenon as a determining factor for the structure of the community, as well as for changes in secondary succession.

METHODS AND MATERIALS

In order to detect allelopathic substances produced by *A. cumanensis*, and to prove their inhibitory effects on the growth of other plants, four types of experiments were done:

1. Aqueous extracts of leaves and roots were tested in order to detect the part of the plant which produced or accumulated the allelopathics.
2. The wash-water of the leaves was tested, based on the fact that rain is

[3] Project 029, Consejo Nacional de Ciencia y Tecnología.

very abundant in this zone and is the principal mechanism of transport of allelopathics produced by the aerial parts of the plants.

3. Aqueous solutions of the soil, where *A. cumanensis* grows, was tested in order to detect accumulations in the soil of allelopathics produced by *A. cumanensis*.
4. The organic residues of leaves and roots of *A. cumanensis* were added to pots containing sterilized and nonsterilized soil wherein other species were planted, to detect whether roots and leaves of *Ambrosia*, during their decomposition, produce allelopathics and to further observe the role of the soil's microorganisms in such process.

In order to choose the species to be used in the biological tests, we looked for those belonging to secondary vegetation which would develop naturally in the zone and which had a high viability and a short germination period. Seven representative ruderal species were chosen: *Mimosa pudica* L. (Leguminosae), *Achyranthes aspera* L. (Amaranthaceae), *Bidens pilosa* L. (Compositae), *Crusea calocephala* D.C. (Cruciferae), *Mimosa somnians* H.B. (Leguminosae), *Cassia jalapensis* Bitt. (Leguminosae), and *Crotalaria sagittalis* L. (*Leguminosae*). Another species was chosen from a more advanced stage of the succession: *Ochroma lagopus* Sw. (Bombacaceae).

The selection of these two groups of species, representing two different successional stages, was made in order to establish differences in the effect of the allelopathic substances of *A. cumanensis* on them and, at the same time, to detect if there is an allelopathic specificity of *Ambrosia* over the species that live with it during the first stages of succession.

Test with Aqueous Extracts of Leaves and Roots

For these tests, fresh tissue was chosen from both organs and dried in air. Two grams of leaves and 15 g of roots, were weighed, blended separately with 100 ml of distilled water, filtered, and the osmotic pressure determined with a freezing-point osmometer in order to prevent the concentration of extracts from exceeding the species' tolerance limit to osmotic pressure (25 mosm/liter approx.) (Anaya and Rovalo, 1976). A 1% agar medium was prepared with these extracts, put in Petri dishes, and maintained at 27 °C with a photoperiod of 12 hours. Thirty seeds were set in each of three dishes.

Test with Wash-Water of the Leaves

Since rainwater—which is so abundant in this zone—acts as the principal transport mechanism of allelopathics from aerial parts of the plants to the ground, we washed the aerial parts of *Ambrosia*, simulating the action of rain to see if the water removed inhibitory substances. A 1% agar medium was

prepared with this water and put in Petri dishes in which 30 seeds were placed in each of three dishes with conditions the same as in the previous experiment.

Tests with Aqueous Solutions of Soil from A. cumanensis Stands

This experiment was designed to detect possible allelopathic substances produced by *Ambrosia* and accumulated in soil. The procedure was as follows:

Some samples of soil were taken to a depth of 10 cm, from an *A. cumanensis* stand, excluding the layer of litter. These samples were homogenized and solubilized in distilled water in a 2:1 proportion of soil–water. The solutions were shaken for several minutes and left to stand for 24 hr; after this period, they were filtered and combined with 1% agar in which 30 seeds were planted in each of three dishes with conditions the same as in the previous experiments.

The sampling of soils and biological tests were done during two different times of the year: one in December, when *Ambrosia* is in the vegetative state, and another in July, when it is flowering. This was done in order to see if the phenological state of the plant had an influence on the presence or concentration of inhibitors in the soil. The control soil was taken from a recently cut hillside which had no vegetation growth, but was close to the place were *Ambrosia* populations grew. In this experiment, utilizing the soil's aqueous solutions, the control represented a problem since in this zone of study it was difficult to obtain an area not covered by vegetation. Thus it was decided to take advantage of the nearby cut hillside, in order to obtain a surface sample of the soil which, being weathered and without vegetation, could hardly contain secondary metabolites originating from the plants.

Tests with Organic Residues of Leaves and Roots of A. cumanensis Added to Sterilized and Nonsterilized Soil

Allelopathics are produced not only in living leaves and roots (and liberated through an active secretion or by the mechanical leaching of water), but may also be liberated or produced during the decomposition of the organic residues of the plant. We added organic remains of leaves and roots separately to sterilized and nonsterilized soil in order to determine the effects of microorganisms on this process. We prepared pots containing 500 g of soil and 20 g of leaves or roots, using soil collected under a population of *Ambrosia*, and set 25 seeds directly in the soil of each of three pots. The pots were placed in a greenhouse and kept there for one month.

In the first three experiments, the following data were recorded: germination percentage, stem length and root length; in the last experiment we recorded dry weight of aerial parts and dry weight of roots.

All data were analyzed statistically by an F test.

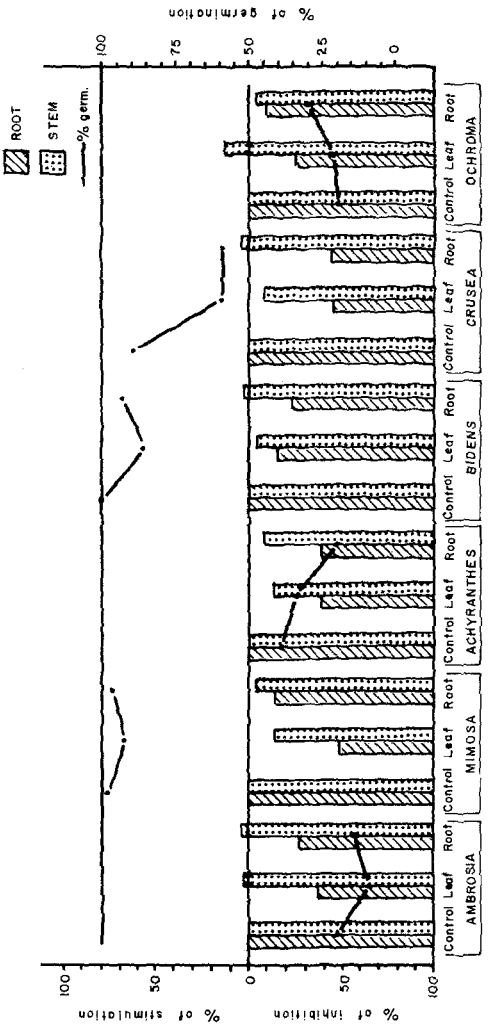

FIG. 1. Germination and growth of six species with the aqueous extracts of leaves and roots of *A. cumanensis*.

TABLE 1. PERCENTAGE OF INHIBITION OF 8 SPECIES WITH SEVERAL TREATMENTS OF *Ambrosia cumanensis*[a]

	Ambrosia cumanensis		*Mimosa pudica*		*Achyranthes aspera*		*Bidens pilosa*	
Treatment	Root	Stem	Root	Stem	Root	Stem	Root	Stem
Leaf solutions	38.76**	−3.57	48.68**	12.32*	38.76**	12.37*	16.03	4.41
Root solutions	27.44**	−4.28	13.39*	3.67	38.46**	9.95	24.22**	−1.34
Wash-water of the leaves	−10.41	10.40	11.31	22.41**	4.61	2.91	−27.87**	2.78
Soil sulutions								
July	8.57	11.04	41.64**	36.41**	27.50**	50.62**	37.95**	30.19**
December	−21.09*	−27.48*	−29.05**	7.01	15.60**	24.65**	0.13	21.84**
Pots								
Sterile soil with leaves	23.70**	−3.11	−48.10**	−8.36			−6.80	−148.18**
Sterile soil with roots	−0.25	32.71**	−32.32**	−24.40**			−52.34**	−48.84**
Nonsterile soil with leaves	61.44**	−74.54**	−20.81**	−49.57**			−38.00**	−119.61**
Nonsterile soil with roots	59.76**	43.61**	22.52**	17.06*			33.33**	50.55**

[a] The numbers shown pointed out the growth of the roots and stems treated as indicated; 100% corresponds to the growth of roots and stems (controls) in agar or in soil without any further addition. *Significant at 5% level. **Significant at 1% level as determined by an F test.

RESULTS AND DISCUSSION

All the results are summarized in Table 1, and the percentages of inhibition due to the different treatments can be compared.

Tests with Aqueous Extracts of Leaves and Roots

Growth of all species was affected by the leaf extract as well as by the root extract (Figure 1). The growth inhibitors present in aqueous extracts of *Ambrosia* leaves inhibited *Mimosa* more than did root extracts.

Ochroma lagopus, an arboreal species growing in late stages of succession, showed opposite growth responses in root and stem; root growth was inhibited by the leaf extract and stem growth was stimulated by it.

It is also interesting to point out the effect that these extracts had on *Ambrosia* itself: root growth was inhibited by root and leaf extracts, while stem growth was not affected by either treatment.

Achyranthes aspera was greatly inhibited by the aqueous extract of leaves as well as by the root extract, while solutions of *Ambrosia* leaves and roots inhibited only root growth of *Bidens*. Root growth of *Crusea* was clearly inhibited by the aqueous solution of *Ambrosia* leaves and roots.

The germination percentage was affected in *Achyranthes*, *Bidens*, *Mimosa*, *Ambrosia*, and *Crusea*; root growth of all species was inhibited to a greater extent than stem growth by both treatments.

TABLE 1 (*cont.*)

Crusea calocephala		*Ochroma lagopus*		*Mimosa somnians*		*Cassia jalapensis*		*Crotalaria sagittalis*	
Root	Stem	Root	Stem	Root	Stem	Root	Stem	Root	Stem
45.66**	9.84	25.18**	−17.08**						
44.80**	−4.25	10.00	4.42						
18.77**	0.53	−12.96*	2.98						
9.79	5.03								
7.94	8.98								
		100**	100**	100**	100**	82.76**	64.25**	26.01**	−8.30
		61.30*	0	51.92**	94.34**	42.24**	45.53**	−34.00**	−50.42**
		−347.34**	−38.17**	5.66	−94.12	65.52**	61.45**	34.50**	23.61**
		−76.99**	1.61	100**	100**	47.41**	26.65**	38.75**	9.31

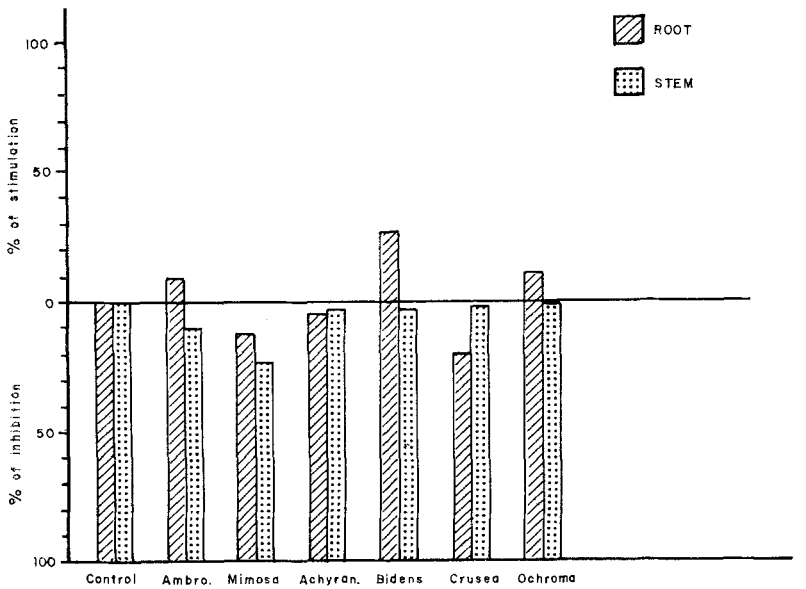

FIG. 2. Growth of six species with the wash-water of the leaves of *A. cumanensis*.

Tests with Wash-Water of the Leaves

Three of the species used in these biotests displayed some type of growth inhibition (Figure 2): *Mimosa pudica, Crusea calocephala,* and *Ambrosia cumanensis.* The first species showed significant inhibition of root and stem growth; the second one only showed root growth inhibition, while stem growth was not affected.

In *Ambrosia* the wash-water of the leaves inhibited stem growth of young plants and slightly stimulated the root.

Wash-water of the leaves stimulated three species: *A. cumanensis* was moderately stimulated in its root growth; *Bidens pilosa* showed significant root-growth stimulation, but its stem was not affected; and *Ochroma lagopus,* showed only stimulation of root growth. The wash-water of the leaves of *A. cumanensis* affected all species used in these biotests in some way or another, with the exception of *Achyranthes aspera.*

Test with Soil Solutions

All species used in the biotests showed considerable growth inhibition by the solutions of soil collected in July, when *Ambrosia* is in bloom (Figure 3). Solutions of soil collected in December produced diverse effects on growth. It stimulated two of them significantly, *Mimosa pudica* and *A. cumanensis* itself, and inhibited the others.

Solutions of soil collected in July produced around 50% growth inhibition of *Mimosa,* while the December solution stimulated its growth. This difference in growth may be intimately related to the phenological state of *Ambrosia,* which, as has been shown, determines a significant difference in the content and proportion of the chemical constituents of the plant (Amo and Gómez-Pompa, 1976). Soil solutions affected *Ambrosia* in the same manner they affected *Mimosa.* The July soil solution inhibited its growth and the December solution stimulated it. Again, this was according to the phenological state of the plant and to the changes it produces in the quality and quantity of metabolites.

Soil solutions also inhibited *Achyranthes,* particularly those of soil collected in July. This agreed with results obtained from tests done with other species. Soil solutions affected *Bidens* considerably: the solution of soil collected in July inhibited the root more than the stem, while the December solution inhibited the stem more than the root. Both December and July soil solutions inhibited growth of *Crusea* seedlings.

Tests Done in Flowerpots with Leaves and Roots of Ambrosia cumanensis Added to Sterilized and Nonsterilized Soil

Growth of Ambrosia cumanensis. In general, growth of *Ambrosia* seedlings

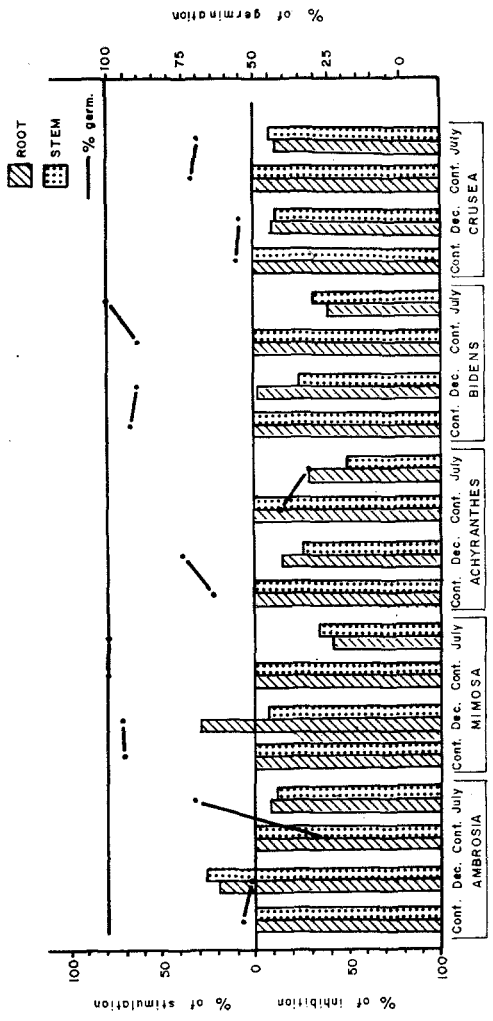

FIG. 3. Growth of five species with *A. cumanensis* soil solutions collected in December and July.

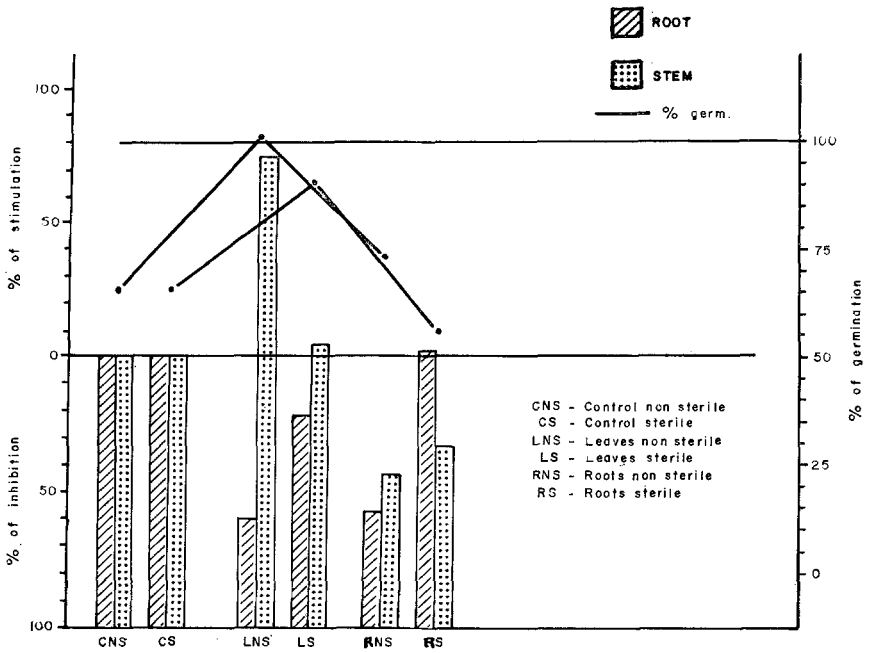

FIG. 4. Growth in pots of *A. cumanensis* in sterile and nonsterile soil with leaves and roots of *A. cumanensis*.

was inhibited by sterile and nonsterile soil with roots, while leaf residues produced opposite reactions in roots and stems of young plants (Figure 4). They inhibited the roots and stimulated the stems, independently of soil sterilization. The germination percentage was increased by leaf residues in sterile and nonsterile soil.

Although the organic residues inhibited the young plants, a strong stimulation of stem growth was observed with the leaf residues in nonsterile soil. This same treatment with leaf residues caused the strongest inhibition of root growth. Comparatively speaking, root residues inhibited young plants more than leaf residues, independently of soil sterilization.

These results suggest that *Ambrosia* has a double allelopathic effect over seedlings of its own species: the one produced by live organs and the other one produced by the decomposition of organic residues. This effect may be related to the population's self-regulating mechanisms.

Growth of Mimosa pudica. Like *Ambrosia*, *Mimosa pudica* is a typical ruderal species and, in some zones, they have been found growing together.

In general, soil with leaves stimulated root and stem growth of young plants, independently of soil sterilization (Figure 5). Sterile soil with roots

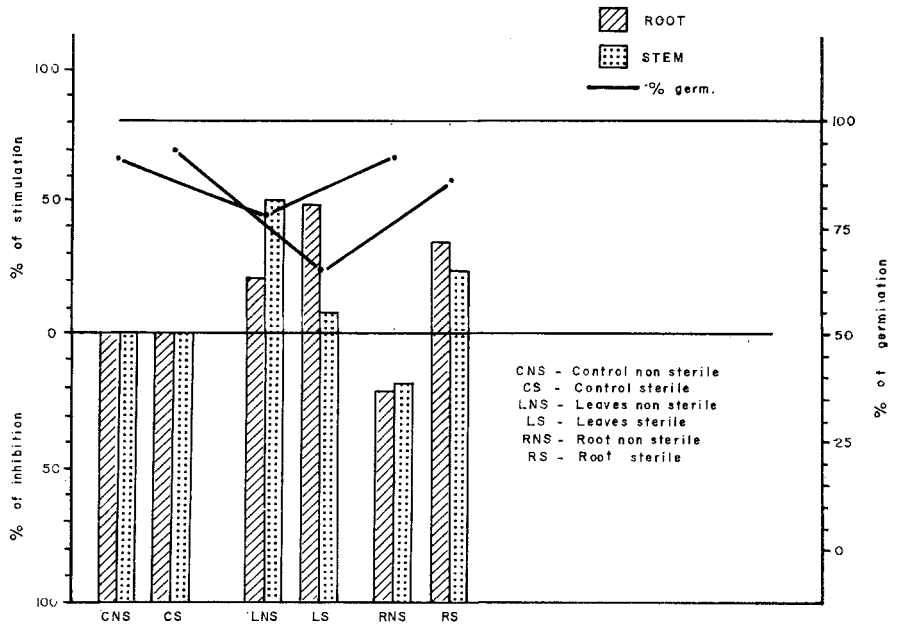

FIG. 5. Growth in pots of *Mimosa pudica* in sterile and nonsterile soil with leaves and roots of *A. cumanensis*.

stimulated growth, while nonsterile soil inhibited it. The germination percentage was diminished by leaves in sterile and nonsterile soil, while soil with roots did not affect it.

Tests done with organic residues in pots revealed that the decomposition of *Ambrosia* leaves is a process which does not inhibit *Mimosa*'s growth but, on the contrary, produces a significant stimulation. On the other hand, organic residues of roots were stimulatory in sterile soil, and inhibitory in nonsterile soil. All seedlings growing in pots with sterile soil did not show the typical growth of bacterial nodules in the roots, while those that grew in nonsterile did have them, independent of the type of organic residues that were present in the soil. This indicates that the allelopathics produced during the decomposition of organic residues do not affect *Mimosa* and do not inhibit growth of nodule-forming bacteria. On the other hand, Rice (1965, 1968) reported that some allelopathic species, including *Ambrosia psilostachya*, inhibit the growth of nitrogen-fixing and nitrifying bacteria.

Based on the above all evidence seems to indicate that *Ambrosia cuamensis* may interfere with the growth of *Mimosa pudica* in the natural environment due to the production of allelopathics coming from live organs, particularly leaves, during the season when the plant is in bloom. The allelopathics are

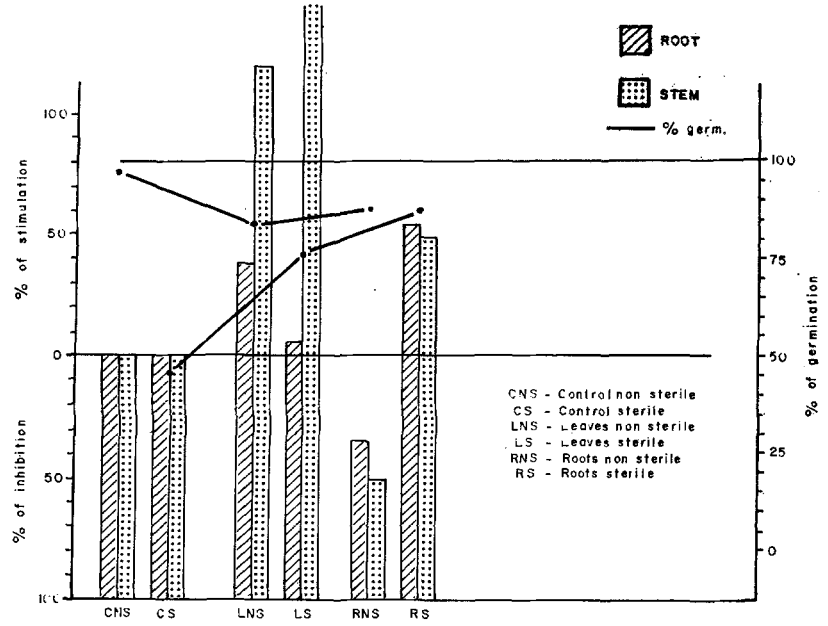

FIG. 6. Growth in pots of *Bidens pilosa* in sterile and nonsterile soil with leaves and roots of *A. cumanensis*.

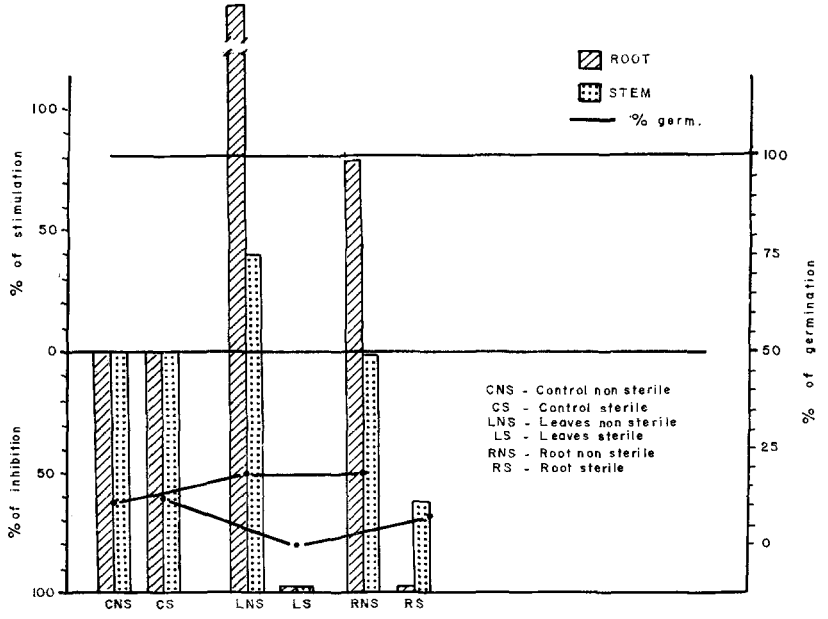

FIG. 7. Growth in pots of *Ochroma lagopus* in sterile and nonsterile soil with leaves and roots of *A. cumanensis*.

washed to the ground by rain water, especially during this season, when precipitation is more abundant.

Growth of Bidens pilosa. Bidens pilosa is a ruderal species which is not found growing together with *Ambrosia* in the region under study. Sterile and nonsterile soil with *Ambrosia* leaves strongly stimulated stem growth (Figure 6). On the other hand, the effects of soil with roots depended on sterilization: roots were stimulatory in sterile soil and inhibitory in nonsterile soil.

This could have been determined by the absence of microorganisms in the process. Root residues in nonsterile soil produced a strong inhibition of growth in general, but root residues in sterile soil stimulated *Bidens*' growth significantly.

Leaf residues produced a conspicuous stimulation, independent of soil sterilization. This indicates that *Ambrosia*'s allelopathic action over *Bidens* is particularly centered on the decomposition of *Ambrosia* root residues which, by the action of soil microorganisms, liberate large amounts of inhibitors into the environment. These inhibitors may be the cause of the inability of *Bidens pilosa* to compete, and hence share living space, with *Ambrosia.*

Growth of Ochroma lagopus. This is the only arboreal species used in the tests, and it belongs to a more advanced stage of succession. Not only the

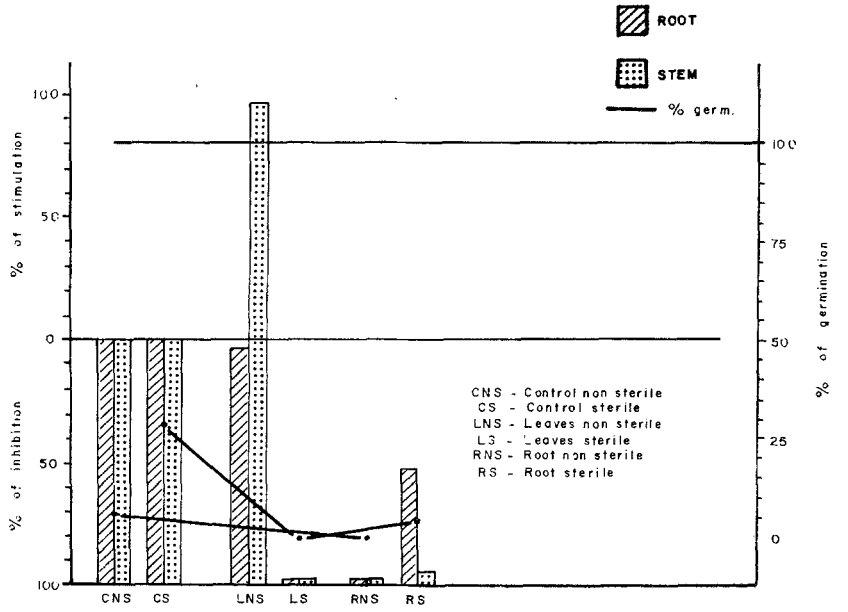

FIG. 8. Growth in pots of *Mimosa somnians* in sterile and nonsterile soil with leaves and roots of *A. cumanensis.*

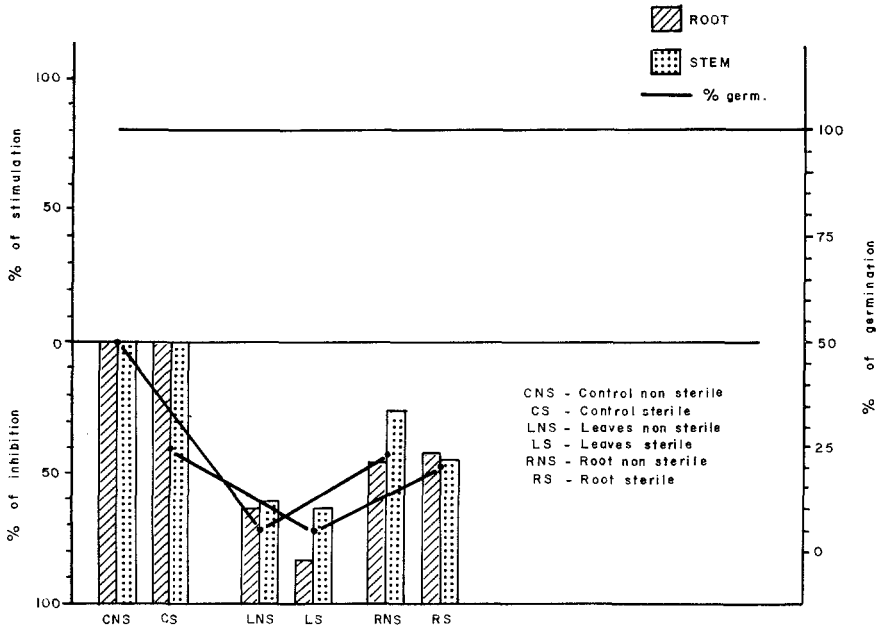

FIG. 9. Growth in pots of *Cassia jalapensis* in sterile and nonsterile soil with leaves and roots of *A. cumanensis*.

leaf but also root residues of *Ambrosia* produced an extraordinary inhibition of *Ochroma* in sterile soil (Figure 7). Without the normal soil microorganisms, these organic residues stimulated it significantly. This result clearly demonstrates the important part soil microorganisms play in allelopathic processes.

Mimosa somnians. With *Mimosa somnians*, *Cassia jalapensis*, and *Crotalaria sagittalis*, the experiment was carried out only in flowerpots. *M. somnians* was strongly inhibited by *Ambrosia* roots, especially in nonsterile soil (Figure 8). When leaves of *Ambrosia* were added to the soil, they inhibited *Mimosa* completely in sterile soil and stimulated it in the nonsterile one. The germination percentage was generally low.

Cassia jalapensis. This species was inhibited by all treatments and especially by the leaves of *Ambrosia*, which was the only treatment also affecting germination (Figure 9).

Crotalaria sagittalis. This species was inhibited, especially in its root growth, by the leaves and roots of *Ambrosia* in nonsterile soil; on the other hand, its growth was generally stimulated in sterile soil (Figure 10). The germination percentage was highest in the sterile control soil; the other treatments showed no significant difference among them.

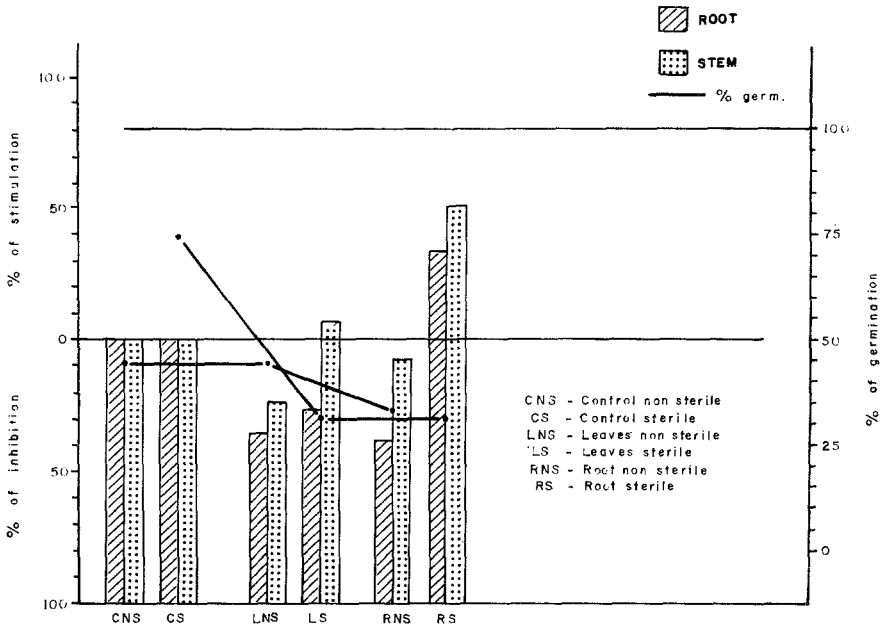

FIG. 10. Growth in pots of *Crotalaria sagittalis* in sterile and nonsterile soil with leaves and roots of *A. cumanensis*.

In conclusion, the bioassays used to test the allelopathic potential of *Ambrosia* confirmed the presence of growth inhibitors produced both by the normal metabolism of the plant and by the decomposition of organic residues. This allelopathic mechanism allows *Ambrosia* to compete advantageously with other ruderal species and, at the same time, contributes to the autocontrol of its population by preventing the growth of seedlings of its own species. Another important thing that became evident was the part played by microorganisms in the liberation of allelopathics to the environment. The sterilization process was fundamental for the manifestation of the allelopathic effect of organic residues and the demonstration of the evident specificity of the liberated compounds in the presence and absence of soil microorganisms, on the species used for the biotests. These results open a vast field of possible studies on the role of chemical interactions in community dynamics.

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EFFECT OF SOME SESQUITERPENIC LACTONES ON THE GROWTH OF CERTAIN SECONDARY TROPICAL SPECIES

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Abstract—Results of biotests carried out with several sesquiterpene lactones produced by *Ambrosia cumanensis* are presented. These compounds showed different effects on the germination and growth of several species of the secondary vegetation of a tropical zone of Mexico.

Key Words—sesquiterpenic lactones, allelopathy, terpenic inhibitors, Compositae.

INTRODUCTION

In our work on the allelopathic potential of *Ambrosia cumanensis* H.B.K. (Compositae) in a tropical zone of Mexico, it was evident that this species possesses allelopathic mechanisms which allow it to compete with other ruderal species and, at the same time, to autoregulate its population (Anaya and Amo, 1978).

The allelopathic properties of certain plant exudations are due to an extensive variety of extracts and volatile substances such as terpenes, phenols, and others which have a very harmful effect on cellular metabolism, especially on respiration and mitosis.

Higher plants produce a great variety of terpenoids, but little is known about their role in allelopathic mechanisms.

Monoterpenoids are the principal components of the essential oils of many plants; they are also the principal terpenic inhibitors which have been identified in higher plants (Asplund, 1968; Anaya, 1976). Evenari (1949) suggests that monoterpenoids and aromatic aldehydes may be mainly responsible for the inhibitory activity of essential oils. Müller and Müller (1964) mention

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several volatile, inhibitory components produced by *Salvia leucophylla*, *S. apiana*, and *S. mellifera*. Moral and Müller (1970) proved the allelopathic effects of the essential oil of *Eucalyptus camaldulensis*.

In general terms, the Compositae have been systematically searched for sesquiterpenes. Sesquiterpenic lactones of *Ambrosia* have been studied by several investigators (Miller et al., 1968; Geissman et al., 1969; Romo et al., 1970); although a large number of these lactones has been found, few works refer to their inhibitory effects. Neill and Rice (1971) proved the existence of volatile inhibitors in the leaves of *A. psilostachya*; the chemical structure of these inhibitors probably corresponds to that of sesquiterpenes.

The principal object of this work was to determine the effect of some sesquiterpenic lactones, obtained from *A. cumanensis*, on the germination and growth of diverse secondary species from a tropical zone in Veracruz, México, and to see if these substances contribute to the allelopathic potential of this species.

For a better understanding of this work, it is important to point out one of the chemical characteristics of *Ambrosia cumanensis*—the modification of sesquiterpenic lactones as a result of changes in altitude (Amo and Gómez-Pompa, 1976), which allows us to divide them into two groups (Figure 1): (1) those with lactone-ring junctions at position 6 and which coincide with populations of *A. cumanensis* living at low altitudes, and (2) those with lactone-ring junctions at position 8 and which coincide with populations living at high altitudes.

METHODS AND MATERIALS

In order to observe the effect of sesquiterpenic lactones on the germination and growth of several species, tests were done with seven of them;

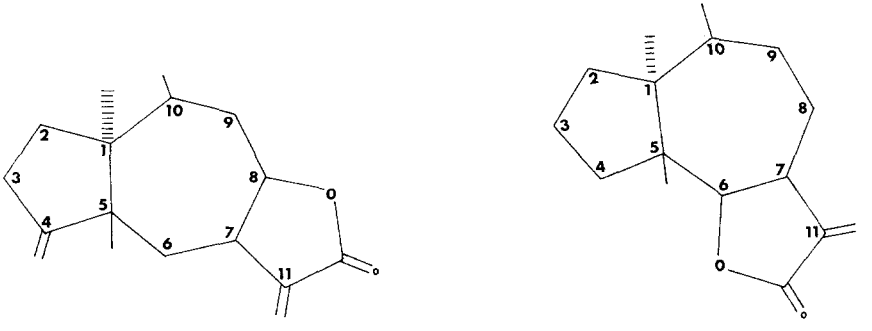

FIG. 1. Closing of the lactonic ring.

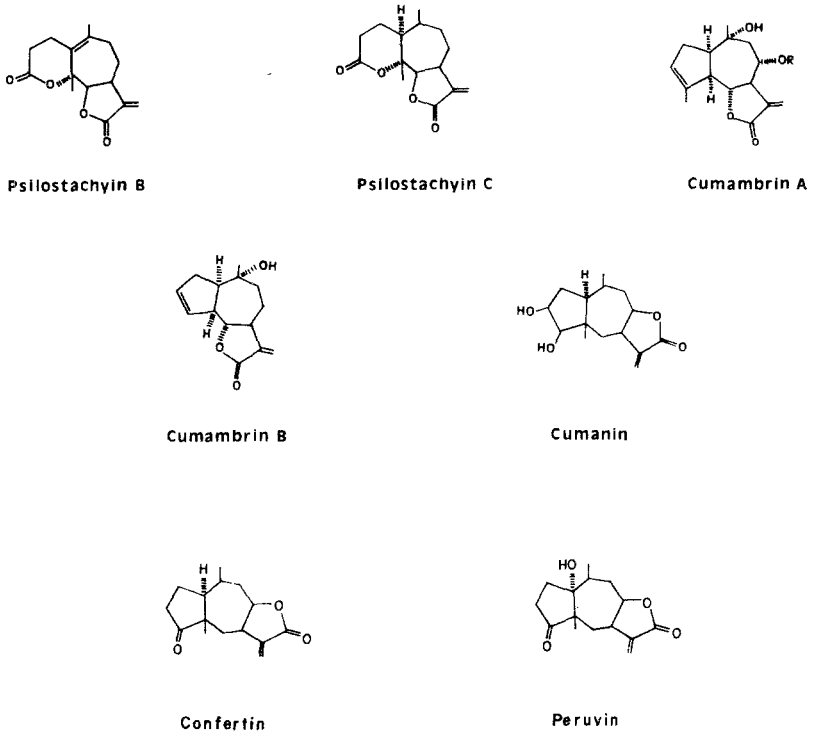

FIG. 2. Sesquiterpene lactones isolated in the cumanensis–psilostachya complex.

psilostachyn B, psilostachyn C, cumambrin A, and cumambrin B, which have lactone-ring junctions at position 6; peruvin, cumanin, and confertin, which have them at position 8 (Figure 2).

The concentrations used were 100 and 250 ppm; the solutions were placed in Petri dishes with filter paper, on which seeds of the chosen species were set to germinate, at a temperature of 27 °C, exposed to a daylight photoperiod of 12 h. Twenty-five seeds of each species with three replications were set for each treatment.

The duration of the experiments varied according to the species as followed: *Ambrosia cumanensis* H.B.K. (Compositae), 168 hrs; *Mimosa pudica* L. (Leguminosae), 72 hr; *Achyranthes aspera* L. (Amaranthaceae); 168 hr; *Bidens pilosa* L. (Compositae), 168 hrs; and *Crusea calocephala* D.C. (Rubiaceae), 168 hr. All of these species are part of the ruderal vegetation of a tropical zone in Mexico located in the region of Los Tuxtlas, Veracruz.

The data recorded at the end of the experiment were the following:

length of root, length of stem, and germination percentage. All results were valued statistically by means of an F test.

RESULTS AND DISCUSSION

It is important to point out that in all populations of *A. cumanensis* living at low altitudes sesquiterpenic lactones of the psilostachyn B and C types, and cumambrin A and B types prevail; these will necessarily be of greater interest for our study, which refers precisely to this type of population. However, it is important to mention that the chemical contents of these plants is not a constant characteristic and that a great number of factors exist which may alter it. Therefore, one must take into consideration the possibility of the existence of other lactones, like cumanin and peruvin, which, if present, could cause inhibition. Confertin requires special analysis, since it is a lactone obtained from another species—*A. confertiflora*—which is distributed throughout dryer and higher zones.

Ambrosia cumanensis

In Figure 3 we see the results of the effect of sesquiterpenic lactones, at concentrations of 100 and 250 ppm, on the germination and growth of *A. cumanensis*. In general, we can clearly see the diverse effects produced by the different lactones, as well as by the concentrations used in the tests.

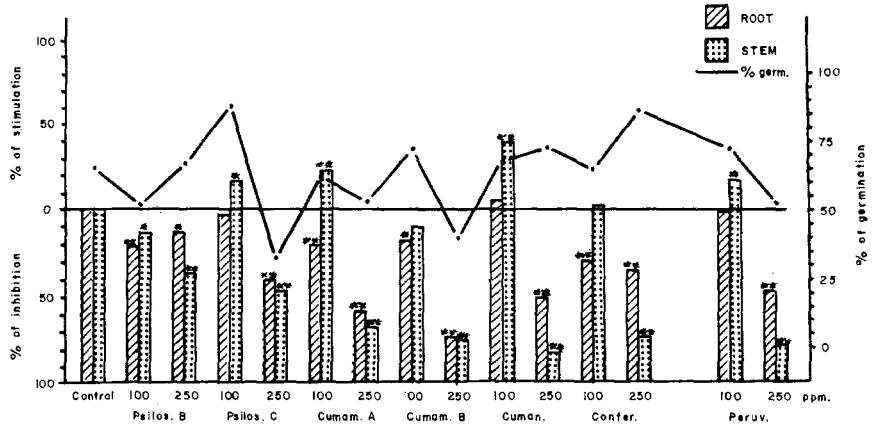

FIG. 3. *Ambrosia cumanensis:* germination and growth with two concentrations of sesquiterpenic lactones. *significant 5%, **significant 1% as determined by F test.

Psilostachyn C, cumambrin A, cumanin, and peruvin produced stem stimulation at 100 ppm, without affecting the root (with the exception of cumambrin A, which inhibited it significantly). On the other hand, at 250 ppm, the four lactones produced a severe inhibition of root and stem growth, and the stems were affected most in all four cases. Cumambrin B inhibited the root as well as the stem at 100 ppm and 250 ppm. Confertin inhibited the root significantly at 100 ppm, without affecting the stem, but inhibited both at 250 ppm. Finally, psilostachyn B greatly inhibited the stem as well as the root at both concentrations. It is interesting that all lactones affected the root more at 100 ppm, while at 250 ppm they affected the stem more. This showed a completely different growth response for both organs in the presence of this type of chemical stimulus.

Germination of *A. cumanensis* varied considerably under lactone treatment. Psilostachyn C, at 100 ppm, and confertin, at 250 ppm, produced a clear stimulation of germination, while psilostachyn C and cumambrin B—both at 250 ppm—caused a decrease in the germination percentage. Besides these effects on the growth and germination of *A. cumanensis* seedlings, some lactones produced certain irregularities in the young plants, such as a considerable increase in the formation of radicule ramifications, caused by peruvin at 100 ppm, cumambrin A at 250 ppm, and cumambrin B at 100 ppm; a great increase in root fragility, caused by cumambrin B at 100 ppm; and an increase in the development of the piliferous zone, caused by confertin at 100 ppm. These results suggest a multiplicity of lactone actions—particularly of cumambrin B, which was one of the lactones causing

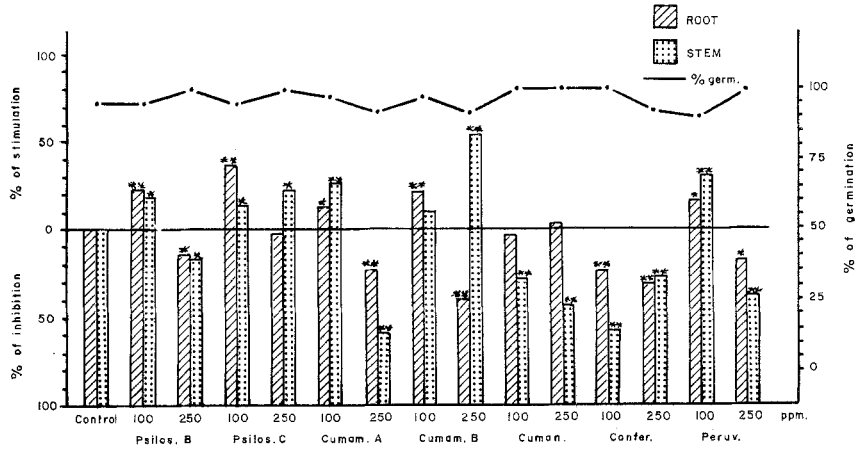

FIG. 4. *Mimosa pudica:* germination and growth with two concentrations of sesquiterpenic lactones. *significant 5%, **significant 1% as determined by *F* test.

the greatest diversity of responses—and open a vast field for research on the effects at the physiological and cellular levels.

Mimosa pudica

Confertin was the only lactone which inhibited *Mimosa* significantly at both concentrations (Figure 4); it inhibited stem growth as well as root growth. Cumambrin B stimulated *Mimosa* significantly at 100 ppm but, at 250 ppm, it inhibited the root and stimulated stem growth. Peruvin, psilostachyn B, and cumambrin A inhibited *Mimosa's* root and stem growth notably at 250 ppm. On the other hand, psilostachyn B, psilostachyn C, cumambrin A, cumambrin B, and peruvin—all at 100 ppm—stimulated *Mimosa*'s root as well as its stem. Germination was not greatly affected by any of these treatments. Irregularities displayed by *Mimosa*'s seedlings with the lactone treatments were an increase in pigmentation of the root and a slight putrefaction, produced by confertin at both concentrations.

Achyranthes aspera

This species was greatly affected by all the lactones, but especially by confertin, which inhibited it strongly at both concentrations (Figure 5). In cases in which *Achyranthes* was inhibited, its root growth was generally more affected than its stem growth, particularly by psilostachyn B and C at 250 ppm and cumambrin A and confertin at both concentrations. The root was never stimulated; on the other hand the stem was stimulated by psilostachyn

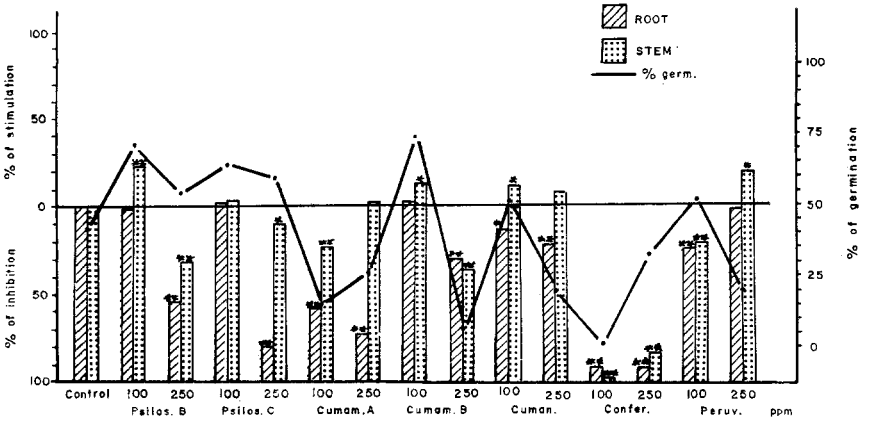

FIG. 5. *Achyranthes aspera:* germination and growth with two concentrations of sesquiterpenic lactones. *significant 5%, **significant 1% as determined by *F* test.

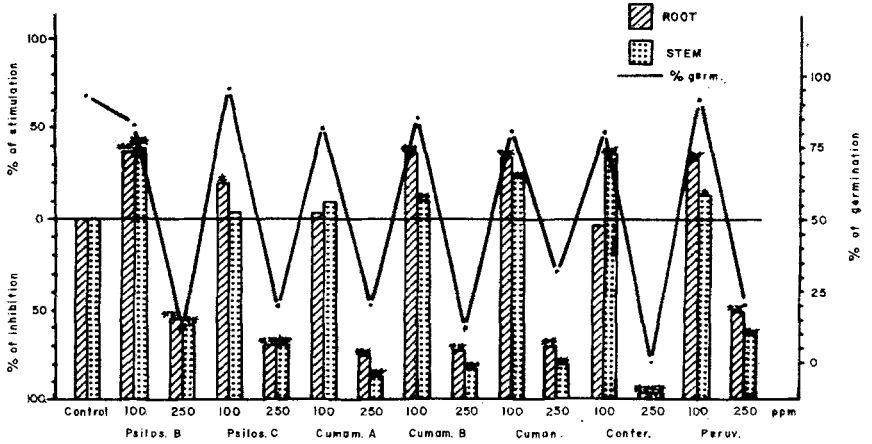

FIG. 6. *Bidens pilosa:* germination and growth with two concentrations of sesquiterpenic lactones. *significant 5%, **significant 1% as determined by *F* test.

B, cumambrin B, and cumanin at 100 ppm and peruvin at 250 ppm. Peruvin's effect on *Achyranthes* is worth mentioning because it stimulated its growth at the higher concentration and inhibited it at the lower one.

Abnormalities displayed by *Achyranthes* seedlings were a darkening of root and stem with the cumambrin B treatment at 250 ppm.

Achyranthes' germination was stimulated by psilostachyn B and C at both concentrations and by cumambrin B at 100 ppm. On the other hand, it was strongly inhibited by cumambrin A and confertin at both concentrations and by cumambrin B and cumanin at 250 ppm.

Bidens pilosa

This is the only species which underwent a significant stimulation of stem growth, root growth, or both, with all treatments at 100 ppm, but its germination was not affected (Figure 6). Similarly, we can see that *B. pilosa* stem and root growth were greatly inhibited by all lactones at 250 ppm, and so was its germination percentage. No other growth response or abnormality of seedlings was observed in this species.

Crusea calocephala

Confertin was the strongest inhibitor of root and stem growth, at both concentrations, but especially at 250 ppm (Figure 7). Peruvin was also an inhibitor but a weaker one. On the other hand, psilostachyn C stimulated

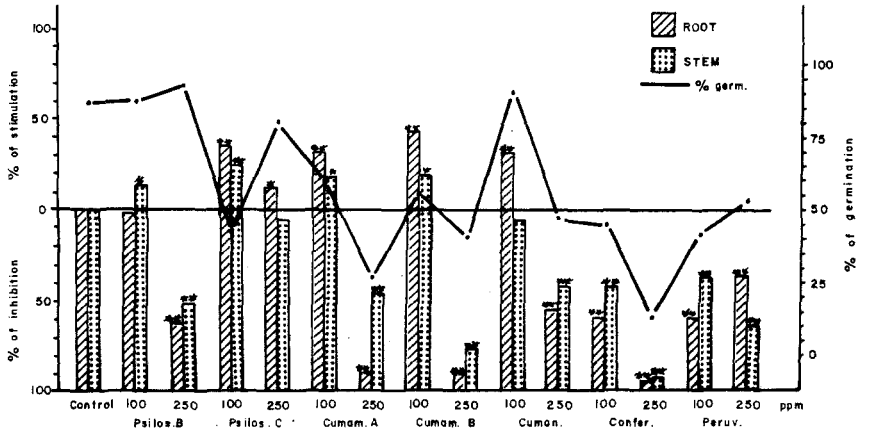

FIG. 7. *Crusea calocephala:* germination and growth with two concentrations of sesquiterpenic lactones. *significant 5%, **significant 1% as determined by *F* test.

both root and stem at 100 ppm and stimulated the root, without affecting the stem, at 250 ppm.

Psilostachyn B, cumambrin A, cumambrin B, and cumanin stimulated *Crusea*'s growth at 100 ppm but inhibited it at 250 ppm. *Crusea*'s germination varied considerably and was particularly diminished by peruvin and confertin at both concentrations and by psilostachyn C, cumambrin A and B, and cumanin at 250 ppm. *Crusea* seedlings also displayed some abnormalities such as abundant root ramifications caused by psilostachyn B at 250 ppm, peruvin at both concentrations, and cumambrin A at 100 ppm. The roots were very fragile under the confertin treatment at 100 ppm. Most ramifications produced in *Crusea* were due to the death of the principal root and the development of secondary ones.

The diverse effects obtained in these biotests were due exclusively to the individual action of each lactone on the test species. This does not allow us to make general extrapolations with respect to *Ambrosia cumanensis*' allelopathic potential. However, the results show that these lactones have a specific biological action which depends on concentration, on the type of substance used, and on the species over which it acts.

Acknowledgments—We appreciate the help of Dr. J. Romo (deceased) in obtaining all of the lactones used in these experiments. This investigation was sponsered by Project 1417, Consejo Nacional de Ciencia y Tecnología.

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ISOLATION, IDENTIFICATION, AND FUNCTION OF THE DUFOUR GLAND SECRETION OF *Xylocopa virginica texana* (HYMENOPTERA: ANTHOPHORIDAE)[1]

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Abstract—The Dufour's gland secretion of *Xylocopa virginica texana* possesses short-term repellency for conspecifics when applied to passion flowers. This secretion contains a number of straight-chain hydrocarbons. The two major components are the methyl esters of palmitic and myristic acid. A mixture of the two esters and two of the available hydrocarbons were as effective as the Dufour's gland extract in eliciting a response in females to the passion flower, *Passiflora incarnata*, to which the extract was applied.

Key Words—*Xylocopa virginica texana*, bee, passion flower, repellency, Dufour's gland, hydrocarbons, methyl palmitate, methyl myristate, esters, scent marking, avoidance.

INTRODUCTION

Scent marking of plants by solitary and social bees has been reported by several workers (Frank, 1941; Haas, 1952, 1967; Kullenberg and Bergström, 1975; Lindauer and Kerr, 1960; Raw, 1975). Reports of the glandular source of the scents have differed. The mandibular gland is responsible for scent marking in workers of *Trigona* (Lindauer and Kerr, 1960; Blum et al., 1970), *Centris* males (Raw, 1975), and *Bombus* (Haas, 1952). Kullenberg and

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Bergström (1975) indicated that labial glands are responsible for marking secretions in males of *Bombus* and *Psithyrus*. Scent marking by males of *Xylocopa* has also been reported as being accomplished by a mandibular gland substance (Velthuis and Camargo, 1975a). However, in a later paper Velthuis and Camargo (1975b) questioned their earlier interpretation that the mandibular gland was responsible for scent marking. Wheeler et al. (1976) reported *cis*-2-methyl-5-hydroxyhexanoic acid lactone as the major constituent of the mandibular gland of *Xylocopa hirutissima*.

Scent marking of passion flowers was recently reported by females of *Xylocopa virginica texana* Cresson in Texas (Frankie and Vinson, 1977). These authors stated that females of this species approaching flowers recently visited by other female *X. v. texana* would turn away before alighting on these flowers. It was also indicated that Dufour's gland was responsible for this short-lived avoidance. In the present paper the chemical composition of Dufour's gland and the biological activity of some of the major components are described.

METHODS AND MATERIALS

Females of *Xylocopa virginica texana* were collected in Brazos County, Texas, and transported to the laboratory at College Station where they were cooled for several hours at 4 °C to reduce their activity. Females were pinned upside down in a dissection dish and the abdomen opened under cold Pringle's saline (Pringle, 1938). The Dufour's gland was removed with forceps and placed in nanograde hexane (Burdick and Jackson Labs. 1953 S. Harvey St., Muskegon, Michigan 49442) for storage. The sample was later extracted in methylene chloride. Methylene chloride extracts were analyzed on the GC-MS LKB-9000 gas chromatograph–mass spectrometer utilizing a 180-cm column of 1% OV-17 (Figure 1). Temperature was programed at 10 °C/min from 60 to 300 °C. The identities of the compounds were determined by comparison to known compounds utilizing GC-MS.

Two major ester components were obtained, and their biological activity was determined in the field along with two of the available hydrocarbons. Two other esters were picked at random and used for comparison. The identified compounds from Dufour's gland and several related compounds were dissolved in nanograde hexane and diluted to 1% solutions for testing. Dufour's gland extracts from field-collected bees were freshly prepared each day. One sample consisted of the four available compounds identified from Dufour's gland mixed equally at a total concentration of 1% in hexane. These solutions were applied with small paint brushes to the corona of selected flowers of *Passiflora incarnata* L. in the field. Treatments began at

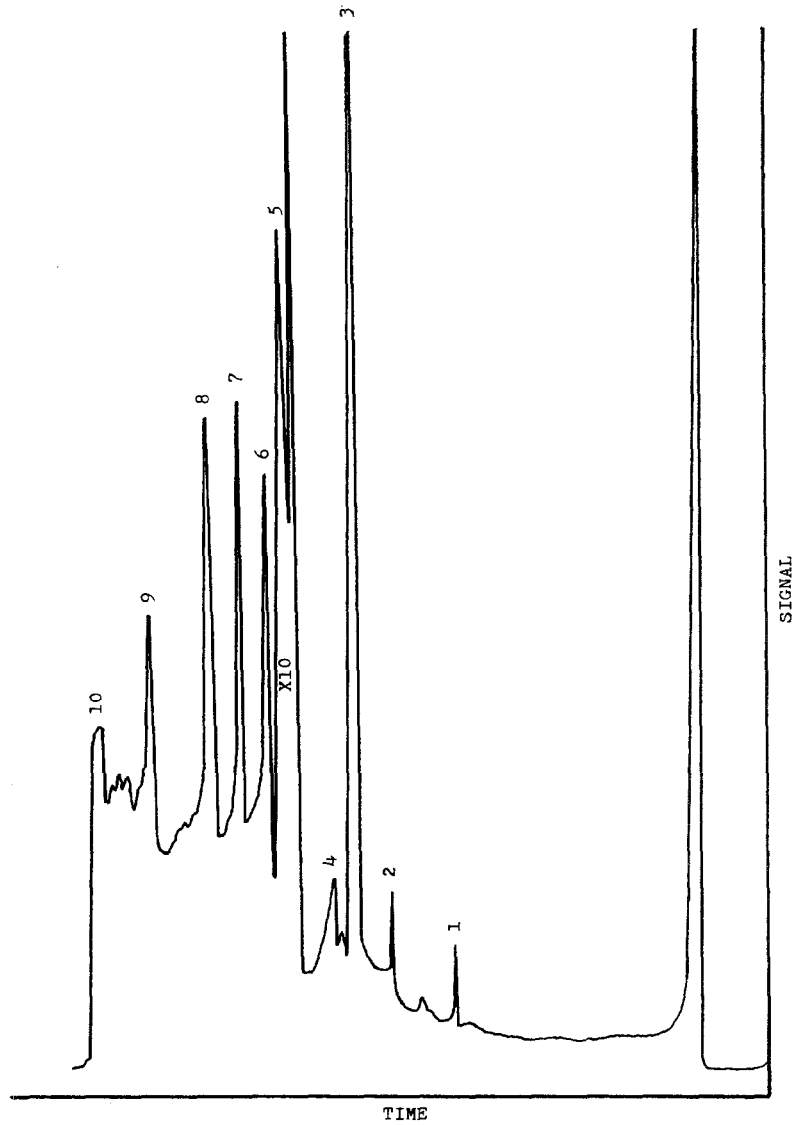

FIG. 1. A gas chromatograph tracing showing the relative proportion of the major components of the Dufour's gland secretion of *Xylocopa virginica texana*. Scale begins at 60° (0 min) and ends at peak 10 (240° or 18 min) from a 1% OV-17 column.

13:00 central standard time (approximately when the flowers in the area opened) and were re-treated every 12 min for 5 recharges. Observations continued for an additional hour.

The general arrangement of the flowers of the test was similar to that described by Frankie and Vinson (1977). These authors reported that xylocopid females made two types of flower visits. The most common type of visit was referred to as a nectar visit, which involves landing on the flower and the withdrawal of nectar. The second type of visit can best be described as an examination visit. In the latter case, the female bee approaches a flower but then turns abruptly away from the flower just prior to landing. This later behavior can best be described as an avoidance. Hexane extracts of Dufour's gland were found to mimic this avoidance behaviour (Frankie and Vinson, 1977).

The foraging behavior, nectar visits, and examination visits were recorded on tape along with the respective times of the various activities. The data were transcribed, and the number of nectar and examination visits was recorded. Because females making nectar visits marked the flowers and the marked flowers repelled other females for approximately 4–7 min (Frankie and Vinson, 1977), females making examination visits within 5 min of a nectar visit were removed from the analysis. This was done in an attempt to reduce the obvious bias due to the material deposited on a treated flower by a female making a nectar visit. Thus, females making a visit within 5 min of another female would be expected to make an examination visit due to the marking residue on the flower deposited by the first female. The number removed from the analysis is indicated in Table 2. The number of nectar visits was compared by a one way analysis of variance (Kruskal and Wallis, 1952).

RESULTS

The compounds identified in the Dufour's gland extract are listed in Table 1. Methyl myristate and methyl palmitate were the major components along with the C_{25} saturated hydrocarbon. The C_{25} saturated hydrocarbon was unavailable for testing. The C_{15}, C_{21}, C_{23}, and C_{27} saturated hydrocarbons were also present in substantial amounts; only the C_{15} (*n*-pentadecane) was available for field testing. The remainder, including *n*-hexadecane which was available for testing, were present in trace amounts.

Results of application of the three major compounds identified from Dufour's gland, *n*-hexadecane, and several other chemicals tested are presented in Table 2. The high percentage of flower nectar visits by females of *X. v. texana* did not differentiate from the untreated flowers compared to the hexane control of the *n*-hexadecane-treated flowers, indicating no effect of the

TABLE 1. ELUTION TEMPERATURES AND MASS SPECTRAL DATA FOR COMPOUNDS IDENTIFIED IN DUFOUR'S GLAND SECRETION OF *Xylocopa virginica texana*

Component	Compound	Elution temperature[a] (°C)	Mass spectral data[b] (*M/e*)
1	*n*-Pentadecane[d]	130	212
	Pentadecene	130	210
	n-Hexadecane	140	226
2	*n*-Heptadecane	150	240
3	Methyl myristate[c]	160	242, 213, 211, 199, 185, 171, 157, 143, 129, 101, 87, 74(bp), 71, 69, 59, 57, 55, 43, 41
4	*n*-Nonadecane	165	268
	Nonadecene	165	266
5	Methyl palmitate[c]	175	270, 241, 239, 227, 213, 199, 185, 171, 157, 143, 129, 101, 97, 87, 74(bp), 71, 69, 59, 57, 55, 43, 41
6	Heneicosane (C_{21} ane)	185	296
	Heneicosene (C_{21} ene)	185	294
7	Phthalate	195	149
8	Tricosane (C_{23} ane)	200	324
	Tricosene (C_{23} ene)	200	322
9	Pentacosane (C_{25} ane)[d]	230	352
	Pentacosene (C_{25} ene)	230	350
10	Heptacosane (C_{27} ane)	240	380
	Heptacosene (C_{27} ene)	240	378
11	Nonacosane (C_{29} ane)	270	408
12	Heneicontane (C_{31} ane)	280	436

[a] 1% OV-17, 10°/min from 10 °C.
[b] See Budzikiewicz et al. (1967) and Silverstein et al. (1974).
[c] Major components of the gland.
[d] Secondary components of the secretion.

solvent on the bees. The Dufour's gland extract and a combination of four components identified from Dufour's gland were the most effective in reducing nectar visits. The effect of the mixture of four components were not significantly different from that of the Dufour's gland extract. However, the number of nectar visits between the two groups was significantly different from each other at the 0.1 level. The esters, methyl myristate and methyl palmitate, identified from Dufour's gland, were the most effective of the single compounds in reducing nectar visits.

Methyl stearate and cholesterol myristate were applied to flowers to

TABLE 2. NECTAR VISITS OF FEMALE *Xylocopa virginia texana* TO FLOWERS OF *P. incarnata* TREATED WITH VARIOUS COMPOUNDS, INCLUDING SEVERAL IDENTIFIED FROM DUFOUR'S GLAND OF FEMALE BEES

Compound	Total number of "visits"[a]	Adjusted number of "visits"[b]	Total visits that were nectar visits (%)[c]
Untreated	54	48	77.1
Hexane (control)	56	40	72.5
n-Hexadecane	26	22	77.3
n-Pentadecane	57	47	55.3
Methyl myristate	55	51	25.5a
Methyl palmitate	60	56	23.2a
Combination (4 above)	54	48	12.5a
Dufour's gland extract	48	41	14.6a
Methyl stearate	19	18	44.4
Cholesterol myristate	18	12	41.6

[a] Visits included both nectar visits and examination visits.
[b] Because female bees making a nectar visit mark the flower resulting in a short-term repellency, females making an examination visit within 5 min of a nectar visit were removed from the analysis. The number removed is shown by subtracting the adjusted number of visits from the total number of visits (see text).
[c] The number of nectar visits indicated by a letter "a" following the percentages were found to be significantly less than the others at the 0.1 level using the one-way analysis of variance of Kruskal and Wallis (1952). The methylstearate and cholesterol myristate were not included in the analysis due to insufficient replications.

determine if the presence of esters or unusual compounds on the flowers might act as repellents. The results (Table 2) show that these two compounds reduced the percentage of nectar visits compared to the untreated flowers but as a group were significantly less effective than either of the esters from Dufour's glands or the combinations of the gland components.

DISCUSSION

The chemical components of Dufour's gland has been best studied in the ants. The gland contents of ants are often very complex. Bergström and Löfqvist (1971) identified 39 out of 50 volatile compounds present in the Dufour's gland of *Camponotus ligniperda* Latr. Hydrocarbons are found in the Dufour's glands of several families of Hymenoptera such as the Braconidae (Guillot et al., 1974), Mutillidae (J. Schmidt, personal communication), and Formicidae (Brand et al., 1972; Ayre and Blum, 1971), and thus it may not be surprising to find a number of hydrocarbons in the Antho-

phoridae. The presence of two methyl esters of relatively common fatty acids as the major components of these glands in *X. v. texana* is of special interest. This finding is in contrast to the identification of the more complex esters, all-*trans*-farnesyl hexanoate and geranyl octanoate in various species of *Andrena* bees (Tengö and Bergström, 1975).

The Dufour's gland secretion of female Hymenoptera often possess an important pheromonal function. Weseloh (1976) reported that the contents of the Dufour's gland elicited sexual behavior in braconid males. Vinson (1977) and Vinson and Guillot (1972) found that the Dufour's gland from the braconid *Cardiochiles nigriceps* is the source of a host-marking pheromone that reduces attack by conspecific females and elicits close range sexual excitement of the male. Guillot and Vinson (1971) reported that chemicals from the Dufour's gland from the ichneumonid *Campoletis sonorensis* also served as a host marking pheromone.

The function, like the chemical constituents of Dufour's gland, has probably been best studied in the ants. The first function analyzed for the Dufour's gland was the source of the trail pheromone in *Solenopsis saevissima* (Hymenoptera: Formicidae) (Wilson, 1962). The chemicals of these glands have been reported to function in alarm (Maschwitz, 1964), aggregation (Ayre and Blum, 1971), recognition (Bergström and Löfqvist, 1971), defense (Ayre and Blum, 1971), trail (Hölldobler and Wüst, 1973), and as a sex pheromone (Hölldobler and Wüst, 1973). As pointed out by Blum and Brand (1972), there is increasing evidence for widespread pheromonal parsimony among the Hymenoptera. Thus, a pheromone may serve several functions in the species, depending on the context of its perception.

The function of Dufour's gland secretions in bees is not well established, although Butler (1965) reported that the soil from the nest site of *Andrena flavipes* was attractive to male bees and suggested a secretion from Dufour's gland might be responsible. Bergström and Tengö (1974) suggested that the unesterified compounds, i.e., alcohols, hydroxy acids, and acids in the Dufour's gland of several species of *Colletes*, *Halictus*, and *Andrena* may function for making a hydrophobic coating of the nest cell and galleries. Frankie and Vinson (1977) reported that the Dufour's gland of *X. v. texana* is responsible for marking behavior exhibited by this species with regard to the passion flower, *P. incarnata*. As far as we know, this is the first report demonstrating a function for the Dufour's gland secretion in bees. The results of this study suggest that the methyl esters of myristic and palmitic acid are responsible at least in part for the flower-marking behavior. However, it is recognized that these compounds may have additional functions.

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RESPONSES OF REINDEER TO INTERDIGITAL SECRETIONS OF CONSPECIFICS

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Abstract—Adult captive European "forest" reindeer, *Rangifer tarandus* L., were exposed to hindfoot interdigital (ID) secretion placed on the ground. The animals were tested with blanks and interdigital secretion from themselves and male and female group members, and secretion from excised glands of male and female "mountain" reindeer. Responses to the stimuli consisted of sniffing, licking, and olfactory searching on the ground. With forest reindeer secretions, each sex responded more to its own ID secretion than to that of the opposite sex. Of the mountain reindeer samples, male ID secretion released stronger responses. The responses did not vary systematically from June to October. The functional significance of the ID secretion in free-ranging reindeer is discussed.

Key Words—Cervidae, interdigital secretion, olfactory communication, pheromones, *Rangifer tarandus*, reindeer, scent glands, scent marking, sniffing, tracking.

INTRODUCTION

Reindeer often sniff the ground and are capable of following tracks of conspecifics (Müller-Schwarze et al., 1978). A traditional Lapp view holds that the interdigital (ID) glands serve in laying an odor track on the ground. Reindeer and caribou (both *Rangifer tarandus*) have large interdigital glands, which are more highly developed on the hindfoot than the front foot (Camper,

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1782; cited in Schaffer, 1940; Quay, 1955). In the caribou, a behavior possibly involving the hindfoot interdigital glands has been described by Pruitt (1960). It is the so-called "excitation jump": an animal rears up and leaves deep foot prints. This was interpreted by Pruitt as an alarm response. The excitation jump has been observed in wild reindeer (*Rangifer tarandus fennicus*) in Finland by Helle (personal communication). Male roedeer (*Capreolus capreolus*) kick the ground with their hindfeet ("Hinterlaufschlag") during threat and when marking branches with their forehead. It is assumed that an important olfactory mark is deposited on the ground. Bucks fled from experimentally presented male ID secretion (Kurt, 1964, 1966, 1968).

Because direct observation and analysis of track-following behavior in the wild is not practical, a bioassay was developed to assess the responses of reindeer to the secretion of the hindfoot interdigital glands of conspecifics. This experiment was part of an investigation of the functions of skin glands in forest reindeer at the University of Umeå. Mountain and forest reindeer represent two different types of *Rangifer t. tarandus* L. The former migrates between mountains and coastal forests, while the latter stays year-round in the woodlands and tends to be larger. The mountain reindeer is considered the ancestor of the domesticated reindeer, and both have shorter skulls, noses, and diastemas than the forest reindeer (Siivonen, 1975).

METHODS AND MATERIALS

Eight reindeer (*Rangifer tarandus*) of the forest variety, ranging in age from one to four years at the start of the experiments, were kept as one group in two enclosures 50 × 100 m large, with the pens rotated. The ages in years of the males were: 3 (δ_A and δ_G), 2 (δ_L), and 1 (δ_M); those of the females: 4 (φ_C), 3 (φ_B), and 2 (φ_H and φ_K).

The experimental pen was located outside the holding pens. The 2-m-high fence was covered with canvas to minimize distraction of the test animal.

Twenty-four Teflon rods (10 cm long, 1 cm diameter) served as scent carriers. To collect secretion, each rod was held by tongs and inserted into a hindfoot ID gland of a captive reindeer and slowly rotated. Then the rods were set into vertical holes in wooden pegs in the ground, arranged in a hexagon of 12 rods with six arms of two rods projecting outwards from each corner. The rods were 65 cm apart. The array covered most of the pen, so that the test animal was likely to encounter rods as it walked around, and was able to sniff consecutive rods as an artificial "reindeer track" (Figure 1). Of the 24 rods, 4–16 carried samples, and each sample was applied to 4 rods in a continuous row. Treatment conditions were assigned randomly by

FIG. 1. The bioassay for interdigital secretion. Five white Teflon rods in the ground can be seen at lower left and center. A 2-year-old female sniffs toward, but not at, a sample.

drawing numbered slips. The remaining 8–20 rods served as controls, including both blanks and solvent samples. The animals were exposed to the rods for 15 min. After each test, the rods were cleaned with water and with methylene chloride.

The experiments were carried out between June 1 and September 20, 1975, and from October 11 to November 4, 1976. For statistical evaluation, the chi-square test was used.

The following stimuli were presented to the reindeer: (1a) blanks only: test animal taken from group ("social condition"); (1b) blanks only: test animal had been isolated for 66 hr ("isolation condition"); (2) ID secretion from responding animals themselves; (3) ID secretion from familiar males and females; and (4) ID secretion from excised glands of slaughtered mountain reindeer (males and females).

RESULTS

Responses to Samples

Blanks Only. First the general level of sniffing blanks and the ground was determined. In the original blank test six clean glass vials were placed on the ground 2 m apart in a hexagonal pattern. Sixteen trials were carried out with one trial immediately after removing each individual from the group ("social" condition) and one trial after each individual had been isolated for 66 hr from the group. (Isolated from the group in the afternoon of one day, it was reunited with them in the morning of the third day. Testing and observations were performed at midday; the next animal was isolated in the afternoon.) The test animal was kept in a separate pen with vision blocked by burlap fences, but with auditory and long-distance olfactory contact with its group mates ("isolation" condition). Their pens were not adjacent, and the shortest distance between the pens was about 5 m.

The responses of the reindeer to blanks, segregated by sex, are listed in Table 1. The animals sniffed the blanks more often after having been isolated ($\bar{X} = 27.1\%$) than when directly taken from the group ($\bar{X} = 10.4\%$; $P < 0.02$). They also sniffed the ground more often after isolation ($\bar{X} = 29.5$ times/15 min) than under the "social" condition ($\bar{X} = 18.7$; $P < 0.01$).

Responses to Own ID Secretion. The results of 16 trials (104 rods) in which each animal was tested with its own secretion are given in Figure 2. The test was performed between June 6 and 11, 1975, and October 11 and November 4, 1976. The reindeer sniffed the ID samples more often than the blanks, with the difference greater in the females. The "baseline" of the frequency of sniffing the blanks is similar to that in the blank tests (Table 1),

TABLE 1. RESPONSES OF 8 REINDEER TO BLANKS

	N	Rods sniffed (number)	%	Average frequency of sniffing ground
Social condition				
Males	24	2	8.3	19.8
Females	24	3	3	17.5
Total	48	5	10.4	18.7
Isolation condition				
Males	24	8	33.3	30
Females	24	5	20.8	29
Total	48	13	27.1	29.5

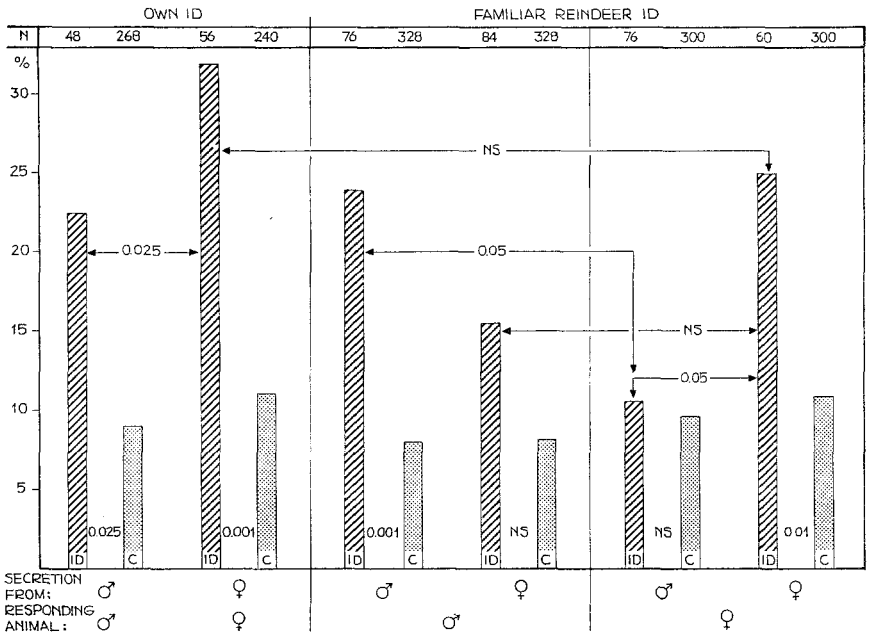

FIG. 2. Responses of forest reindeer to own ID secretion and that of familiar males and females. N: number of samples (rods) presented. ID: interdigital secretion; C: controls. Numbers represent *P* values; NS: no significant difference.

namely 9.3% in the males, and 11.7% in the females. The females sniffed the ID samples more often than the males (32.1% vs. 22.9%, $P < 0.025$).

Responses to Familiar Individuals' ID Secretion. In a total of 48 trials, each male and each female was exposed to ID secretion of all familiar males and females. The tests were carried out between June 9 and 23, 1975, and between October 11 and November 4, 1976. The strongest responses are by females to female secretion (25%) and males to male secretion (23.7%). The response differences are the same as those to the animals' own ID secretion.

Figure 3 summarizes all responses to ID secretions from forest reindeer, described in this and the previous section.

Responses to ID Secretion of Mountain Reindeer. In a total of 39 tests with 232 rods, the responses to ID secretions of mountain reindeer were tested. The experiments took place in July 1975 (male secretion only) and with male and female secretion in September 1975 and from October 13 to November 4, 1976. Figure 4 shows the results. The strongest responses were those by females to male secretion and by males to male secretion.

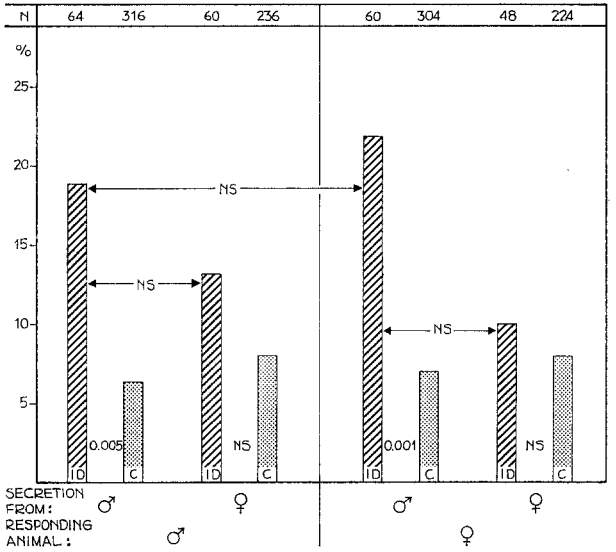

FIG. 3. Responses of forest reindeer to ID secretion of male and female mountain reindeer.

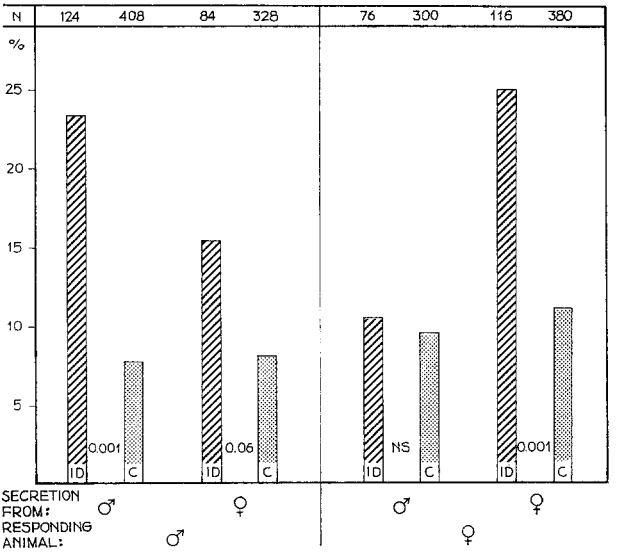

FIG. 4. Summary of responses to ID secretion from forest reindeer.

TABLE 2. SNIFFING OF DISTILLATE AND RESIDUE OF ID SECRETION AND GROUND SNIFFING BY FEMALES

		Sniffing and ground sniffing		Sniff, "sniff near," and ground sniffing			
	N	Number	%	Number	%	Total ID (%)	Control (%)
Total	60	19	31.7	20	33.3	100	1.89
Distillate	60	6	10	13	21.7	65.2	1.23
Residue	52	5	9.6	10	19.2	57.7	1.09
Control	188	24	12.8	33	17.6	52.9	1.00

Responses to Distillate and Residue of ID Secretion. The ID secretion was separated into distillate and residue at 80°C by a precolumn heater (Andersson et al., 1975). In August 1975, both males and females were exposed to the distillate (males: 16 rods; females: 60 rods) and the residue (males: 16 rods; females: 52 rods) of ID secretion from male mountain reindeer. Table 2 summarizes the results. The responses selected were: sniffing the sample, sniffing within 50 cm of the sample ("sniffing near"), and sniffing the ground. As in the tests with whole secretion, ID samples were sniffed more often than controls ($P < 0.005$). The responses to distillate and residue were intermediate, with higher responses to distillate. With the sample size used, this latter difference was not significant. Table 2 expresses the response intensities in percent of both blanks and total ID secretion.

Behavior after Sniffing Samples

Sniffing alone is a poor indicator of pheromonal activity. A reindeer's sniffing of a chemical stimulus on the ground can lead to or be part of several functional systems. It can be related to feeding, tracking, or examining urine or a bedding site. For the possible role of the interdigital secretion as a trail pheromone, it is important to record the behavior following the olfactory encounter with an ID sample. Since the reindeer sniffed only two consecutive samples at most, (and not a whole "trail"), we recorded all other behaviors following the sniffing of a test sample or blank. In a sample of 136 two-pattern sequences, eat/walk and walk/eat were most frequent (27.9%); walk/sniff ground and sniff ground/walk accounted for 22.8%, sniff sample/walk and walk/sniff sample for 7.4%, alert/walk and walk/alert for 7.4%, and eat/alert for 2.9%. Alert/sniff ground, run/walk, stand/alert, alert/sniff air, stand/sniff ground, and walk/defecate occurred at a rate of 1.5% each. The remaining 22.4% constituted miscellaneous two-pattern sequences.

TABLE 3. RESPONSES OF MALE AND FEMALE REINDEER TO ID SECRETIONS AND CONTROL ODORS

		Sniffing		Ground sniffing after sniffing sample	
Stimulus	N	Number	%	Number	%
ID	192	48	25	14	29.2
Tar oil	32	4	12.5[a]	2	50
Coffee	32	6	18.8[b]	0	0
Blanks	672	51	7.6	17	33.3

[a] Significant difference from ID ($P < 0.01$).
[b] No significant difference from ID.

Control Odors

If interdigital secretion is deposited in the tracks of reindeer and conveys information that conspecifics use in tracking, the behavior after sniffing an ID sample should differ from that which follows sniffing of a blank or some other nonreindeer odor. To examine whether ID secretion possibly serves as a trail pheromone, the occurrence of sniffing the ground and walking after sniffing ID samples, blanks, and two control odors were compared. Ground sniffing and walking may occur simultaneously or, more frequently, in sequence. The two control odors were tar oil (used in Sweden as mosquito repellent) and black coffee. The results are summarized in Table 3. Tar oil was sniffed less often than ID secretion ($P < 0.01$) and coffee slightly less often (not significant). Sniffing coffee was never followed by ground sniffing, but this is not statistically significant. Thus, the reindeer tended to show more interest in ID secretion than in the two control odors.

DISCUSSION

The purpose of the described experiments was to find clues to the possible significance of ID secretion in laying tracks and track-following by reindeer. The possibility of track odors originating from the interdigital glands of artiodactyls has been suggested many times. It has even been suggested that dogs may be able to detect the direction of a moving roedeer (*Capreolus*) either by the different interdigital odors of fore- and hindleg or even by odor differences between the front and rear sides of the hind leg (Schumacher, 1934; Schaffer, 1938, 1940).

According to Schaffer (1940), the ID glands of *Rangifer* are large, smaller in size only than those of *Axis*, *Muntjacus*, and *Odocoileus virginianus*. Among the cervids, most genera of the Telemetacarpalia, such as *Alces*, *Capreolus*, *Mazama*, *Odocoileus*, *Pudu*, and *Rangifer* have well developed ID glands. Some of the Plesiometacarpalia, on the other hand, tend to have less-developed ID glands. This applies to several *Cervus* species and *Elaphurus*, while *Muntjacus*, *Dama*, *Rusa*, and *Axis* have moderate or large ID glands. Thus, most or all of the cervids are potentially capable of leaving ID scent in their tracks. Schaffer (1940) judges that even the little-developed ID glands of red deer (*Cervus elaphus*) are sufficient for producing an odor track.

The main result is that reindeer do show interest in ID secretion, indicated by the more frequent sniffing than in response to blanks or control odors. It should be noted, however, that other, nonreindeer odors are also sniffed at a rate that is higher than that in response to blanks. A second main result is the sex specificity: males sniff male ID secretion more often, and females respond more to female ID secretion than that of males. A possible function of this difference may be the aiding of single animals in finding the way back to the group. Histologically, however, ID glands in cervids show little or no sex differences (Schaffer, 1940; Quay and Müller-Schwarze, 1970, 1971). The third finding, that isolated individuals sniff the ground more often, lends support to the ID secretion functioning in group cohesion. The annual cycle of reindeer behavior has been known to the Lapps for many centuries. There are four phases when groups exist that are composed of the same sex: from January on, when the bulls have shed their antlers and grow new ones, they keep together in male bands that separate from the females. During spring migration, the pregnant cows move first, and the bulls are the last to follow, although nowadays the Lapps force the bulls to migrate with the females. On the calving grounds, the sexes are again separated. After mixing on the summer range (but maintaining the ties of subgroups), the sexes separate again during the rut. Only one bull stays with the harem, while the other males form bachelor groups. During fall migration, the sexes move together and they stay together on the winter range until the bulls drop their antlers. It would seem that ID secretion, if it is used for tracking, would serve in helping dispersed individuals to find their way back to their group. The experiments were carried out in early summer, when the males tended to separate from the females, and during the rut, when the females stay together in the harem and all but one male in a bachelor group. Therefore, it is likely that the demonstrated sex specificity of responses reflects a social organization as it occurs in the wild. These considerations would render unlikely the use of ID secretion in long-range migration, as this would require heterosexual preferences for the spring migration (males follow the

females), and is not pertinent for the fall migration where the sexes move together. The Lapps have even believed that the tracks left by reindeer during fall migration are preserved under the snow and can be used by reindeer for orientation during the following spring migration back to the mountains. Recent direct observations of reindeer during spring migration yielded no direct evidence for the use of such tracks by reindeer (Müller-Schwarze, 1978).

The stronger responses by both sexes to the male secretion in mountain reindeer differ from the more sex-specific responses to forest reindeer secretions. The mountain reindeer glands were collected in September during the pre-rut, and hormonal or dietary factors may account for this difference. It should also be noted that the samples may have been considerably stronger than those deposited in a natural track, but the amounts left in the latter are not known.

It is likely that reindeer can discriminate between fresh and older tracks since even to the human nose the vinegar-like odor of freshly harvested ID secretion disappears after 15 min, and the secretion assumes a cheesy odor. This is due to evaporation of the fatty acids with shorter chains, such as acetic acid. Brundin and Andersson identified several volatile fatty acids in ID secretion of reindeer. This will be reported elsewhere. Evaporation of certain components during seconds or minutes would also enable a conspecific or predator to determine the direction of travel of the track-laying individual. The lower level of responses to the ID secretion of mountain reindeer may reflect an already existing behavioral barrier correlated with taxonomic distance. Banfield (1961) considers the mountain reindeer (*Rangifer t. tarandus*) and the forest reindeer of Finland (*Rangifer t. fennicus*) at the level of two incipient species.

In any discussion of pheromonal communication, one must keep in mind the changes that domestication has brought with regard to social organization and density. Before domestication, small bands lived widely dispersed, and trail pheromones may have been used both within and among groups. Nowadays the herds are large (several hundred to one thousand individuals) and are often artificially kept in small areas; bells are used to facilitate group coherence during migration.

ID secretion may not be used for tracking under conditions of captivity, due to the proximity of other reindeer and holding pens that are saturated with excrement. The high frequencies of feeding, sniffing ground, and walking after sniffing samples does not permit us to distinguish between tracking and food-searching behavior. Free-ranging reindeer may nevertheless use ID secretion for tracking, albeit under special conditions. The fact that the animals sniffed only some of the rods may mean that reindeer sample this way and obtain all the information they need.

No seasonal variation of response intensity was found in the experiments that were carried out during the months of June through November, although seasonal variation of the relative amounts of three ketones in the ID secretion was demonstrated by Brundin. On the other hand, no sexually correlated difference in the chemical composition of the ID secretion could be demonstrated, although there are sex differences in behavior.

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THE ALLELOPATHIC MECHANISMS OF DOMINANCE IN BRACKEN (*Pteridium aquilinum*) IN SOUTHERN CALIFORNIA[1]

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Abstract—Bracken, *Pteridium aquilinum*, exerts a strong dominance over associated plants throughout much of its worldwide range. Associated plants are often severely inhibited or even excluded from dense stands of the fern. This study investigated the various aspects of herb suppression in bracken stands and assessed the contribution of the various forms of interference between plants to the establishment and maintenance of bracken dominance. It was shown that competition for soil moisture, light, and nutrients could not account for the lack of herbs in bracken stands. Further, uniformity of soil pH, texture, water-holding capacity, and organic matter content ruled out variability in physical factors as a cause. Baiting and trapping experiments showed that the higher concentration of animal activity inside the bracken stands contributed significantly to the pattern of herb suppression, but only against select species. The maintenance of this pattern in the animal-free Santa Cruz Island stands indicates the importance of another factor, allelopathy. It was found that phytotoxins leached from the dead, standing bracken fronds with the first few rains of the wet season were largely responsible for herb suppression. These toxins were isolated in raindrip and from soil inside the fern stands. Removal of the fronds from the stand before the rains could leach them resulted in reinvasion by the herbs after several seasons, and,

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conversely, placing fronds over the herbs in the grassland brought about herb inhibition. A number of known allelopathic chemicals were tentatively identified from bracken leachates. The importance of the interaction of allelopathy with other factors of plant interference is illustrated by bracken.

Key Words—*Pteridium aquilinum*, bracken, allelopathy, inhibition, phytotoxins, animal grazing, competition, leachates, phenolic acids.

INTRODUCTION

In many areas where bracken (*Pteridium aquilinum*)[3] grows, dense stands of the fern exhibit strong dominance over associated plants. This dominance is observable throughout much of bracken's worldwide range, extending from the northern limits of the boreal forests to the tropics. References to bracken causing problems as an unwanted weed have been made from such diverse areas as Rhodesia, New Zealand, Costa Rica, British Columbia, Scotland, and several places in the United States (see Braid, 1959, for review). In all of these locations, associated plants appear to be severely inhibited and often excluded from bracken stands. Most investigators have implicated competitive interference (for light, water, or nutrients) as being causative in the observed patterns of dominance, but as early as 1917 Jeffries demonstrated active inhibition of associated plants by breakdown of bracken litter. In addition, as has been found in investigations of other plants (Muller, 1969), inhibitory compounds actively produced by the plants and washed out of the fronds in rain, fog, or dew drip (Gliessman and Muller, 1972) may aid in maintaining bracken's dominance. While the morphological heterogeneity of *Pteridium aquilinum* does not diminish its apparently widespread phytotoxic effectiveness, the mechanism of toxin release differs under the various climates in which the several varieties of this species grow (Gliessman, 1976). The purpose of this study was to observe this dominance in one geographic situation, to determine how the fern affects the environment, and to determine what interaction of factors may be involved.

METHODS AND MATERIALS

Study Areas

The sites chosen for most intensive study are in the San Marcos Pass area (elevation 2200 feet) of the Santa Ynez Mountains, approximately 10 miles northwest of Santa Barbara, California. Additional sites were examined on Santa Cruz Island, off Santa Barbara, one mile west of Mt. Diablo at an

[3] Nomenclature throughout follows Munz and Keck (1959).

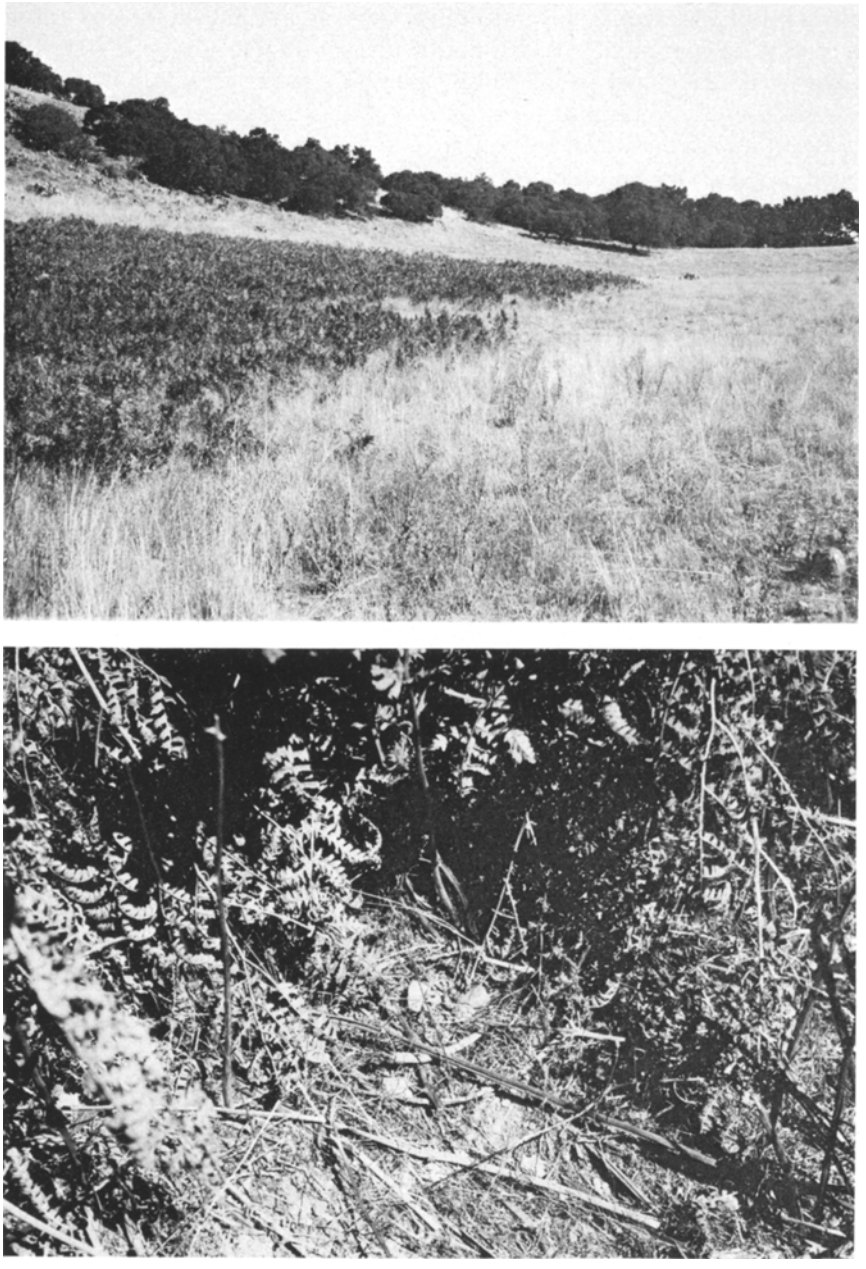

FIG. 1. (a, top) Typical bracken stand in a meadow on Santa Cruz Island, Santa Barbara County, California. (b, bottom) Close-up of interior of bracken stand in San Marcos Pass showing lack of herb cover and abundance of exposed soil surface.

elevation of 2300 feet. The annual rainfall averages 25 in. with a wet season during the winter months of November through March. In areas otherwise covered by dense oak woodland (Figure 1a), meadows occur which are vegetated by a diverse mixture of annual grasses and dicotyledonous herbs. In some of these meadows, bracken has established dominance and drastically altered species composition (Figure 1b). Apparently the meadows have existed since before white man settled the area, probably being maintained by a combination of factors, not the least being periodic fires. The deep, well-developed soils of the meadows, derived from a phase of the Coldwater sandstone series (Dibblee, 1966), have also favored maintenance of stands of the deep-rooted ferns. Except for soils derived from a different phase of the same series, environmental factors encountered in the island stands are similar to those on the mainland.

Vegetation Pattern

In order to verify and quantify the pattern of dominance observed, species counts were carried out at two proximate meadows in San Marcos Pass at intervals throughout the growing season (late October 1968 to early May 1969). Using a 10-cm-diameter circle, ten plots in each meadow were sampled inside the fern stand and in the open grassland on ten separate sampling dates. On the initial sampling date, only five plots were counted at each site. Data from both meadows were pooled, giving a total of 190 samples in each vegetation type. Based on species composition alone, there is essentially no difference between the fern-covered areas and the open grassland. Of the 35 species encountered, only two infrequent ones occur in the grassland and not in the fern stands. Upon comparing the number of individuals of each species, the differences become evident. Of the 35 species, 23 have a higher average abundance in the grassland controls, with only 12 species more numerous in the fern stands. The average total number of individuals in each plot is significantly higher in the grassland (161.66) than in the fern stands (35.27), there being a more than fourfold difference.

Dry weights of the more common herb species (Table 1) dramatically illustrate differences in plant growth. This table demonstrates a positive correlation between greater abundance of each species in the open meadow and higher average dry weight. In the cases of *Montia perfoliata* and *Cerastium viscosum*, both of which show greater abundance inside the stands, yields are much higher in the open grassland. *Pholistoma auritum*, a shade- and moisture-requiring plant, was one of the few species that showed both greater abundance and higher yields inside the fern stands.

The pattern that bracken itself exhibited was very similar to that described for bracken in other areas of the world (Watt, 1945; Cartledge and

TABLE 1. AVERAGE SPECIES NUMBERS AND DRY WEIGHTS PER INDIVIDUAL OF THE MORE COMMON ASSOCIATED PLANT SPECIES[a]

	Count/plot (number)		Dry wt/individual (mg)	
Species	Fern	Grass	Fern	Grass
Bromus mollis	2.36	65.25	1.6	2.4
Festuca megalura	15.72	29.27	1.4	2.1
Bromus rigidus	0.06	2.77	11.6	23.9
Hypochoeris glabra	0.38	15.44	2.7	8.0
Cerastium viscosum	4.62	1.83	2.0	2.9
Clarkia purpurea	0.13	2.10	1.0	2.2
Lotus subpinnatus	0.10	0.48	3.0	6.1
Pholistoma auritum	0.46	0.38	10.2	2.5
Montia perfoliata	2.13	0.44	6.7	8.1
Trifolium repens	0.19	10.31	2.5	11.9
Thysanocarpus laciniatus	0.01	2.88	1.0	3.6

[a] In 10-cm-diameter circular plots in the fern stands and in adjacent grassland. Species counts are the average of 190 plots. Dry weights are the average of 10 plots collected mid-April after most herb growth was completed (dried at 70°C for 24 hr).

Carnahan, 1971). Most of the stages described by Watt (pioneer, building, mature, and degenerate) were observed at the San Marcos Pass study sites. Watt (1947) found that the deep rhizomes invaded into the grassland as much as 75 cm/year, and then the fronds emerged forming the pioneer phase. The building phase was just inside the margin of the stands, indicated by an increase in density and vigor of the fronds. This increase reached a peak in the mature phase when the dominance of bracken was most obvious and the reduction of growth and occurrence of associated species was most evident. Finally, as the stands aged, the fern began to enter the degenerate phase where frond density and vigor dropped and associated plant species reinvaded, at least in limited quantities.

In this study, average frond density, expressed as percent cover on four different 10-m transects, from the margin to the interior of the stands was $52.2 \pm 5.1\%$. Average percent cover of the first meter of the transects, mostly in the building phase, was $36.5 \pm 6.0\%$. The average per meter in the well-developed mature phase of the interior of the stands was $58.5 \pm 14.5\%$. For some of the same reasons discussed by Cartledge and Carnahan (1971), such as fire, low rainfall, and disturbance by man, the stands examined in this study did not clearly possess a pioneer phase, nor have they been allowed to progress undisturbed to the degenerate phase.

Factors of the Environment

Soils. Soils were investigated for factors that might cause the observed vegetational pattern. Soil profile pits were dug to a depth of 1 m in four locations each in the grassland and fern stands. Due to homogeneity at lower depths and the lack of interaction between herbs and fern at such depths, the herb roots all being concentrated in the upper 20 cm of the soil, soil was sampled for analysis only above 60 cm. In each soil pit two samples were taken at depths of 0–10, 10–20, 20–40, and 40–60 cm, a total of eight samples being analyzed from each soil depth in the grassland as well as the fern stands.

Initially, each layer was examined for differences in soil texture (after methods of Bouyoucos, 1936) that could account for the pattern of plant distribution. The comparison between depths revealed similar particle size distribution, as would be expected for a soil that had once supported grassland before being invaded by the fern. The greatest difference in texture occurred in the 10- to 20-cm layer, with an average particle size distribution of 43.0% sand, 36.0% silt, and 21.0% clay in soils from the fern stands and an average of 40.4% sand, 39.6% silt, and 20.0% clay in the grassland soil. In all other layers, percentages did not differ by more than 2%. Considering that there was at least 2% difference between replicates within any single layer sampled, certainly differences in soil texture cannot account for the observed vegetation pattern.

Differences in the hydrogen ion concentration of the soil could strongly affect the vegetation cover. Therefore, pH determinations on the soil layers were made with an IL model 265 Labomatic pH/mn Electrometer with a glass electrode, using a 1:1 soil-to-water paste which was allowed to stand for 1 hr prior to analysis (Bear, 1964). The pH ranged from a minimum of 5.7 to a maximum of 6.5 in all of the samples tested. The greatest difference between grassland and fern stand samples in any one layer was only 0.5 at the 40- to 60-cm depth. At all other levels, differences averaged less than 0.3, differences that cannot be considered biologically significant and cannot account for the pattern of herb exclusion from the bracken stands.

Loss on ignition, as an estimate of total organic matter in the soil, was determined by subjecting the soil samples to 30 min at 700°C in a muffle furnace (Bear, 1964). Duplicate samples for each level in both vegetation types showed no significant differences. Percent loss in the 0- to 10-cm horizon in the grassland averaged slightly higher (8.0%) than the bracken soil (7.4%), probably due to the greater concentration of roots and litter in the upper layers of the grassland profiles. Annual grasses and herbs tend to concentrate their root activity in the upper 20 cm of the soil. Bracken rhizomes were found at a depth of more than 1 m, but were concentrated in the

TABLE 2. LEVELS OF NUTRIENTS IN SOIL IN STUDY AREAS EXPRESSED IN PARTS PER MILLION (FIGURES REPRESENT A MEAN OF 4 SAMPLES EACH AT TWO SITES)

Soil	Total nutrients	PO_4	K	Mg	Ca	Fe	Zn	Mn	Cu
Bracken									
0–10 cm	3215	125	270	258	1305	72	30	320	34
10–20 cm	2858	67	235	227	1265	63	34	80	26
Grassland									
0–10 cm	3162	110	190	230	1418	118	49	360	36
10–20 cm	3148	80	198	230	1370	88	25	90	24

20- to 40-cm layer of the profiles. Losses on ignition in the two sample depths below 20 cm were essentially the same in both vegetation types, all averaging $4.8 \pm 0.1\%$.

To test for possible competitive interactions involving soil nutrients, extensive analyses for representative essential elements were carried out. Because roots of grasses and herbs are concentrated near the soil surface, especially during germination and early growth, complete analysis of only the 0- to 10-, and 10- to 20-cm layers was attempted. Total nitrogen was determined using the Micro-Kjeldahl technique (Jackson, 1958). Cations were extracted using an ammonium acetate exchange modified from Bear (1964) and Chapman and Pratt (1961), and analyzed on a flame spectrophotometer. Anions were extracted with sodium acetate after Chapman and Pratt (1961), and analyzed on an auto analyzer. Results are listed in Table 2.

Although some of the differences may be statistically significant, the differences are probably not large enough to suggest competition for nutrients as a causative factor (Russell, 1961). In general, nutrient levels are higher in the upper 10 cm of soil, probably due to the greater concentration of roots and organic matter. Regardless, in no case does it appear that bracken is reducing the level of any of these nutrients sufficiently to exclude herbs from the interior of the stands. Higher levels of the major nutrient elements, especially phosphate and potassium, would, in fact, be thought to stimulate more vigorous herb growth.

It might be expected, especially in a region with a Mediterranean-type climate, that competition for available soil moisture could be important. However, several factors indicate that this is not so. Rainfall is mainly confined to the winter months, and it is within this time that many of the annuals complete most of their life cycle, especially germination and early growth. The pattern of exclusion of herbs from bracken stands is initiated with the

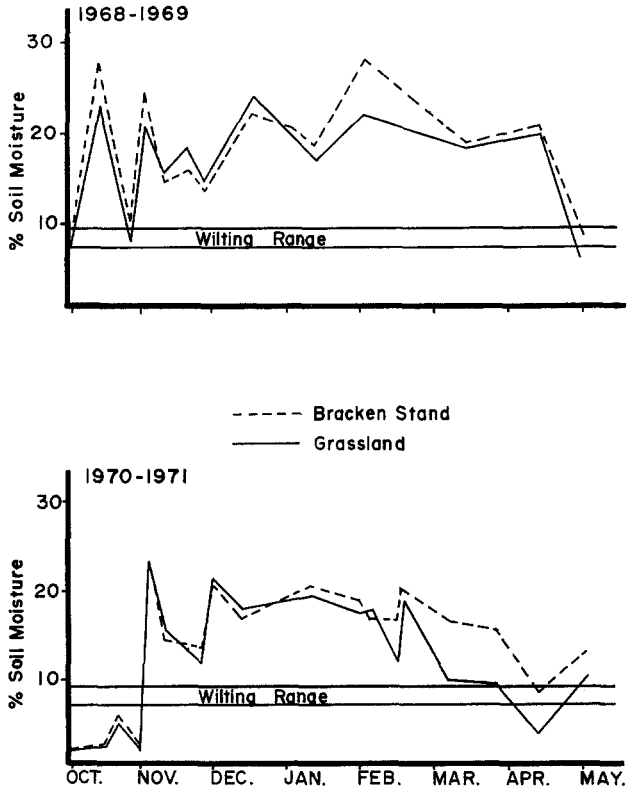

FIG. 2. Soil moisture during the 1968–1969 season, a very wet year, and the 1970–1971 season, a dry year. Each point represents the average of three replicates, all from the upper 10 cm of the soil.

first rains, usually late October or early November, when soil moisture is at or near field capacity. In addition, bracken itself is in a dormant state and remains so until soil temperature begins to rise in late February or early March. By this time the annuals would have completed a good deal of their growth. The distribution of bracken rhizomes well below herb roots would also tend to prevent competition for soil moisture.

To test the possibility that differential soil moisture levels were the cause of some of the observed patterns, gravimetric determinations of available moisture were made throughout the growing season. Because the herbs exploit the upper layers of the soil horizon, especially during the early season when the pattern of exclusion is established, only the upper 10 cm of

soil were sampled. The results are presented in Figure 2. In general, it can be seen in both graphs that initial levels of soil moisture are very similar in both grassland and inside the bracken stands. Because of the erratic occurrence of rains in the early season, soil moisture often approaches the wilting range, but because of the relatively low temperatures at this time, damaging droughts rarely occur. From pressure-plate determinations, the wilting coefficient was shown to range between 7 and 9% in both the grassland soils and in the bracken soils. This is illustrated in Figure 2 as well. The graphs also show that moisture levels inside the bracken stands in the late part of the growing season are consistently higher than in the grassland. This correlates well with the frequent occurrence of dry winds at this time. The standing crop of dead fronds and litter in the stands acts as an insulation against both wind and full sun, reducing moisture loss. Wind-driven clouds and fog are frequent in the mountain pass where the study was located, especially during the wet season. The fronds trap moisture, providing an additional source of moisture for the soils inside the stands.

Several of the plant species that are more abundant inside the stands, such as *Cerastium viscosum* and *Montia perfoliata*, are species that are usually found in relatively moist conditions (Munz and Keck, 1959). Their ability to do well inside the stands could, therefore, be explained at least partially by the higher levels of available moisture. But the moisture regimes illustrated in Figure 2 certainly do not explain the great reduction of most other herb species inside the fern stands.

Light. The factor most often invoked to explain the dominance of bracken is competition for light, the aspect of dense stands of green fronds suggesting they might "smother" or shade out any associated plants. But the fronds are not always green; during the winter months in some locations, or during the dry season in others, the fronds are dead. In this state, although the fronds often remain standing, they shrivel and cast very little shade. Much bare ground is visible inside the stands, presumably offering excellent sites for herb establishment and growth.

Light readings in foot candles were made at intervals throughout the growing season with a Weston Illumination Meter model 756. Ten readings were taken at random at ground level inside the stands and in adjacent grassland, averaged to obtain a mean value for each vegetation type, and the results presented in Figure 3. At the beginning of the wet season with the first rains (early November), growth of the annuals was initiated. At this time the grasses and dicots from the previous season were dry and formed a dense cover of standing plant material. The fronds were usually almost completely dried and shriveled although still intact and standing. Light levels in the bracken stands averaged about 50% of full sunlight at this time, and in the grassland they averaged less than 35%. Any fronds not senescent because of

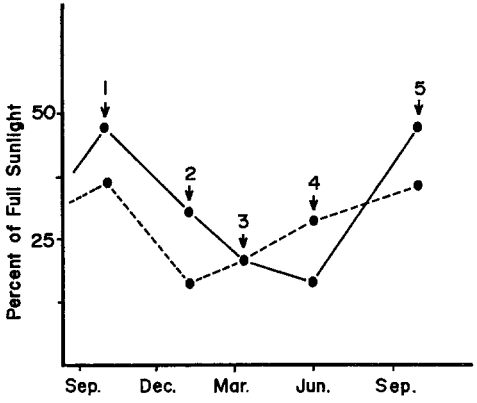

FIG. 3. Light intensities at ground level inside bracken stands (solid line) and outside in open grassland controls (broken line), expressed as percent of full sunlight taken at midday: (1) beginning of wet season and initiation of herb growth; (2) peak of herb growth; (3) initial emergence of fronds; (4) peak of frond cover; (5) end of dry season.

the drought stress encountered by the end of the dry season were killed by the first frosts that often follow the initial rains. By mid-February, before the new fronds began to emerge, the herbs were approaching full development. There was a pronounced drop in average light intensities at this season because of the increase in standing herb cover, more evident in the open grassland due to greater herb densities. Early in March the bracken fronds began to emerge and light levels fell lower in the stands, until by mid-May the fronds had fully emerged and the deepest shade was encountered (less than 15% of full sunlight). It is important to note that light readings reached their lowest levels in the open grassland several months before an equivalent reduction took place in the fern stands. Therefore, by the time shading by bracken fronds could become a factor, from April to June, most of the associated herbs had completed their life cycles and set seed. As the dry season progressed the fronds and the herbs eventually died and light levels rose, reestablishing the seasonal cycle.

In addition to these measurements, a field experiment was designed to test the shade tolerance of grassland species. Devices were constructed to provide artificial reduction of light: two 3×3 m wooden frames whose upper surfaces were 0.6 m above the ground. A layer each of 3/8-in. hardware cloth, fiberglass window screen, and aluminum window screen was stretched

over each frame. The shades were placed in the open grassland adjacent to the bracken stands during the season of October 1969 to June 1970, providing about 70–75% reduction in light intensities with the sun directly overhead. This degree of shading approximates that which occurs inside the bracken stands at the peak of herb development.

Herb species numbers were monitored throughout the growing season. A population sample taken after herb growth was well advanced (mid-December) showed very similar mean totals of individuals per 10-cm-diameter circular plot (305.3 shaded vs. 181.4 open controls). These figures are even in excess of the total reported for the grassland in Table 1. In fact many species show much greater abundance per plot under the shade frame, such as *Bromus mollis* (122.8 shaded vs. 88.8 control) and *Festuca megalura* (36.9 shaded vs. 24.8 control), aided probably by the better moisture regimes encountered under the shade frames. This is the reverse of what would be expected if shade were an important factor limiting the establishment and growth of herbs inside the bracken stands.

Herbivorous Mammals. Bracken stands may create an environment that would support large populations of herbivorous mammals that might, by their selective grazing behavior, be largely responsbile for the paucity of herb species inside the stands. The fronds may offer cover that is lacking in the open grassland for both nesting sites and protection from predatory animals.

To estimate the numbers of herbivorous mammals, a total of 255 trap nights were run over a two-week period in early December 1971, a time when the herb distribution pattern is just becoming most evident. Sherman and Longworth live traps were set in the open grassland, at the edge of the bracken stands, and in the interior of the stands. Only one animal was captured in the grassland, 4 at the edge of the fern, and 17 animals inside the stands. Approximately a third of the traps were left out during the day, but only one animal was captured and that in the interior of the stand. Thus it appears that populations are much higher inside the fern stands. Seven of the captured animals were *Microtus californicus* and the remainder were *Peromyscus maniculatus*. Both of these species are known to be herbivores, *Microtus* feeding primarily on leaves and stems, while *Peromyscus* feeds primarily on seeds (Ingles, 1965).

To determine if these populations were having an effect on herb distribution, two types of baiting experiments were performed. The first involved placing dishes in the field containing known quantities of seed of several herb species occurring in the study areas. Glass petri dishes with 1-cm-high sides and a diameter of 20 cm were filled to a depth of 3 mm with screened grassland soil. After seeds were placed in the dishes, they were covered with another 1 mm of soil. The experiment was repeated twice, the

first time using 50 seeds per dish and the second 100 seeds per dish. Half of the dishes were covered completely with small exclosures of 3/8-in. mesh hardware cloth. The exclosures were 50 cm on a side and 20 cm tall, with complete top and sides, and were attached to the ground in such a way that rodents could not enter without tunnelling. The other half had the same exclosures over them, but elevated 10 cm on metal stakes to allow easy access to small animals. One set (closed and open) was placed in the grassland and another in the bracken stand. The experiment was set up in late May after all the herbs had fully matured and begun dropping seed. It ended with the first rains of the winter. Thus the experiment was in progress during the time of the year that seed-gathering animals would probably have their most noticeable effect.

The results shown in Table 3 indicate effective animal predation. There was a certain amount of removal of seed from dishes by such natural causes as wind and rain. Considerable *Avena* seed was lost in the closed cages, probably due to the long awns which, because of their hygroscopic qualities, enabled the seed to "crawl" out of the dishes. Otherwise, the results show that animals do not touch *Festuca*, the most common herb species occurring inside the fern stands. The same is true for *B. mollis*, a species less common inside

TABLE 3. NUMBER OF SEEDS LEFT IN DISHES AFTER A FULL SEASON EXPOSED TO SEED-GATHERING ANIMALS[a]

	Grassland		Fern	
Species	Open	Closed	Open	Closed
Bromus rigidus				
1	35	45	2	47
2	81	90	3	84
Bromus mollis				
1	47	44	40	50
2	100	90	95	100
Festuca megalura				
1	50	50	50	50
2	98	85	95	97
Hypochoeris glabra				
1	—	—	—	—
2	80	92	48	90
Avena fatua				
1	3	11	1	10
2	17	32	2	11

[a] Experiment 1 used 50 seeds per dish, 100 seeds per dish in experiment 2.

the stands. *Hypochoeris*, a species virtually excluded by bracken, was taken only lightly. *Bromus rigidus*, on the other hand, was severely reduced inside the stands. There seems to be the same tendency for *Avena*, but due to seed loss in the experimentally caged dishes, the data are not significant.

The second set of experiments was designed to test for grazing of green shoots by small animals. Small plastic greenhouse trays, $20 \times 30 \times 5$ cm, were half filled with a water-retaining greenhouse soil mix and planted with at least 30 seeds each of the species in Table 3 (with the exception of *Hypochoeris*). The trays were kept in the greenhouse for about two weeks until the shoots were 5–10 cm high. They were then moved to the field on November 27, 1971, approximately one month after the rains had begun. The herbs in the field averaged 5–10 cm at this time as well. Half of the trays were placed inside the bracken stands and the other half in adjacent grassland controls. Within two days, animals had begun to clip and remove both *Avena fatua* and *Bromus rigidus* from the trays in the ferm stands. There was some preference for *B. rigidus* at first. By the 8th day inside the stands, most of the *Bromus rigidus* and *Avena* had been completely removed, but almost none of the *Festuca megalura* or *Bromus mollis* had been touched. At the end of the experiment on the 15th of December, all of the *B. rigidus* and almost all of the *Avena fatua* had been removed from the trays in the stands, but none had been removed from the trays in the open grassland. *Festuca* and *B. mollis* were hardly touched in either location.

To examine further the grazing effects of small mammals, a bracken stand was studied (Figure 1a) on Santa Cruz Island, 19 miles off the coast southwest of Santa Barbara. This island is well known for its lack of rodents. There are no rabbits, no woodrats, and almost no mice except for small populations of *Reithrodontomys megalotis* and *Peromyscus maniculatus*. While they are abundant both around ranch buildings and at the ranch dump, these island mice occur in small numbers in other habitats which on the mainland usually yield large numbers (Bills, 1969). They are almost never found in grasslands or meadows. To check this, eight Sherman live traps were set continuously from June 8, 1971, until June 11, 1971, in the bracken stand and in adjacent grassland. They failed to capture a single animal, and none of the traps was sprung. These same traps were used with positive results on the mainland. Also, glass plates baited with seeds of *Bromus rigidus* and *Avena fatua*, usually collected readily by animals on the mainland, were untouched after 5 days in both the fern stand and open grassland. The lack of rodent runs or burrows, very common in the stands on the mainland, is further evidence for the lack of rodents in the island stands. It is believed that the very abundant and omnivorous island fox, *Urocyon littoralis*, has been largely responsible for the lack of rodents (L. Laughrin, personal communication).

TABLE 4. SPECIES COUNTS AND DRY WEIGHTS INSIDE ISLAND FERN STANDS AND ADJACENT GRASSLAND

	Count/plot (number)		Dry wt/individual (mg)	
Species	Fern	Grass	Fern	Grass
Bromus rigidus	24.6	19.6	11.8	19.2
Silene gallica	0.2		1.0	
Bromus mollis	0.1	32.5	2.6	4.7
Avena fatua		11.1		21.1
Plagiobothrys nothofulvus		1.0		6.2
Erodium cicutarium		2.1		8.3
Eremocarpus sp.		1.3		4.2
Lupinus bicolor		1.6		16.8
Erodium botrys		0.3		5.4
Average total count/plot	24.9	69.5		
Average total wt/plot[a]			290.96	470.28

[a] Average biomass calculated by summing the multiples of the average number of individuals per plot and the average dry wt per individual.

The island stands have not been studied in as much detail as the mainland stands to determine what other factors of the environment might be responsible for the pattern. The following observations and analysis, however, support the belief that competition for light, water, and nutrients play only minor roles if any. Each vegetation was sampled on June 7, 1971, by taking 20 circular plots 10 cm in diameter. Each species was counted and average dry weight in milligrams computed; the results, expressed as average number of individuals per plot and average dry weight per individual, are given in Table 4. The same pattern of herb exclusion is found, except in the case of *Bromus rigidus*. This grass very rarely occurs inside stands on the mainland (Table 1) but on the island is very uniformly distributed inside the stands as well as out. An average of 24.6 individuals per 10-cm-diameter plot was encountered inside the stands. *Bromus rigidus* is essentially the only herb inside the island stands, except for scattered individuals of other species. The grassland, besides containing large numbers of *B. rigidus*, has considerable *Avena fatua* and *Bromus mollis* and an assortment of other species common to the grassland on the mainland. Most of the grassland species that are reduced to low numbers in the interior of the mainland stands are similarly reduced in the island stands, despite the lack of mammals in the island stands. *Bromus rigidus* is still affected by conditions inside the stand, because on a dry-weight basis (Table 4), individual plants average 7.4 mg less inside than

outside in the grassland controls. Over-all biomass per plot inside the fern stands is similarly reduced.

This suggests that species like *Bromus rigidus*, which almost never occur in bracken stands on the mainland, are kept out by animals. Other species, such as *Festuca megalura*, are not grazed much by herbivores and are able to grow fairly well in the presence of bracken. Species such as *B. mollis* or *Hypochoeris glabra* occur much less frequently inside the stands yet are seldom grazed. A species such as *Avena fatua*, although possibly grazed in the mainland stands, is almost totally excluded from island stands even in the absence of small herbivores. Therefore, herbivorous mammals play a role in the exclusion of herbs from the interior of the bracken stands, but some other factor is more important in maintaining the complete pattern.

Allelopathy

One remaining factor that could be responsible for the ability of bracken to inhibit so strongly the development of associated plants is allelopathy. The release of phytotoxins from the fern may be limiting development of other plants. The phytotoxin potential of bracken *in vitro* has already been demonstrated (Gliessman and Muller, 1972; Stewart, 1975).

Water extracts were made from live and dead fronds collected from the study areas by soaking 65 g of intact fronds in 1500 ml of glass-distilled water

TABLE 5. RADICLE GROWTH (mm) OF *Bromus rigidus* IN WATER EXTRACTS OF BRACKEN FRONDS[a]

Test	Control	×	4×
Green fronds	25.6(100)	25.3(98.5)	25.5(99.5)
Yellow fronds	22.3(100)	22.6(101)	18.3(81.7)[b]
Dead fronds tested before rains:			
1967	21.8(100)	18.6(85.4)[b]	8.3(38.1)[c]
1968	15.4(100)	13.1(85.0)[b]	5.3(34.4)[c]
1969	23.1(100)	17.6(76.2)[b]	5.0(21.6)[c]
1970	15.2(100)	9.6(63.0)[c]	6.0(39.7)[c]
Dead fronds tested after 1 wet season	22.1(100)	18.1(82.0)[b]	15.5(70.0)[c]
Dead fronds tested after 2–3 wet seasons	20.1(100)	19.9(99.0)	19.9(99.0)

[a] $N = 30$ for all means; numbers in parentheses are percent of simultaneously run distilled water controls; extracts are either original strength (×) or concentrated four times by evaporation (4×).
[b] t test significant at the 5% level.
[c] t test significant at the 1% level.

for 2 hr. The extract was filtered and a portion was then concentrated in a Buchler flash-evaporator at 49°C to four times the original concentration. Both concentrations were tested for toxicity using "standard sponge bioassays" described by McPherson and Muller, (1969). Preliminary experiments indicated that *Bromus rigidus* was one of the species less sensitive to the bracken toxins, but due to ease of handling and collection, as well as uniformity of germination and growth, it was employed in the bioassays. Besides, an extract significantly capable of reducing the growth of *B. rigidus* would very likely be even more effective against other species growing in the field situation.

As shown in Table 5, extracts of green fronds proved to be nontoxic. Yellow fronds beginning to show signs of senescence exhibited slight toxicity for the concentrated extract. Dead fronds were collected, over a series of successive growing seasons, before any leaching by rainfall had occurred. Extracts of these fronds proved to be highly toxic even without concentration. After exposure to one rainy season, toxicity was reduced, especially in the concentrated extract, and after several seasons standing in the field, the toxic principle appeared to have been completely removed.

As a check to be certain that the osmotic concentrations of the extracts were not solely responsible for the observed inhibition, seeds were planted in mannitol solutions made up to osmotic concentrations slightly in excess of those encountered in the extracts. Appreciable osmotic concentrations were evident only in the extracts from dead fronds collected before any leaching by rainfall had occurred. The maximum value for the unconcentrated extract was 28 mosmol, and 115 mosmol for the concentrated extract. Other frond types listed in Table 5 yielded osmotic values less than 30 mosmol, even when concentrated. Therefore, mannitol solutions of 30 mosmol and 120 mosmol were used to irrigate seedbeds prepared according to the standard sponge method cited, with glass-distilled water used as a control. Sixty seeds of *Bromus rigidus* were planted in each treatment. Mean radicle lengths after a 48-hr incubation period, in the 30 mosmol solution, were not significantly different from the distilled-water control (compared with Student's t test). The more concentrated mannitol significantly reduced radicle elongation to 60% of controls. But as shown in Table 5, the concentrated extracts of newly dead fronds reduced radicle growth to an average of less than 35% of controls. Therefore, osmotic concentrations may play a part in the bioassay inhibition observed, but there remains what can be considered organic inhibition, or total inhibition less osmotic inhibition. In the case of *Bromus rigidus* in the 4× extract, organic inhibition is 65% less 40%, or at least 38% of the total inhibition observed. Thus, it is believed that some water-soluble compounds are involved in inhibition of herbaceous species in bracken communities. Exposed to a series of leaching rains in the field, the toxins are

released from the fronds into the surrounding environment. Because of the depth of the rhizomes and roots of bracken, chemical interaction between the shallow rooted herbs and subterranean bracken parts is probably negligible. Therefore, leachates from the fronds would have to be the most ecologically effective.

To test this, four 3×3 m areas in bracken stands were cleared of fronds and litter for several consecutive years beginning in the autumn of 1968. At the end of the dry season before the rains commenced, the frond production of the previous growing season was removed without disturbing the soil surface. During the first subsequent growing season there was an increase in the mean number of herb individuals per 10-cm-diameter sample to 71.9 as compared to 35.3 in the undisturbed bracken. By the end of the third season (April 1971), it was very difficult to tell the difference between the species composition of the cleared areas and the undisturbed grassland. In the 1971 samples, the mean number of herb individuals per plot was 34.6 in the fern stand, 152.0 in the open grassland, and 120.7 in the clearings. The most significant species increases were *Bromus rigidus*, *B. mollis*, *Trifolium repens*, *T. ciliolatum*, and *Erodium cicutarium*. Total herb biomass in the 1971 grassland samples averaged 2.51 g/plot, 1.90 g in the clearings, and only 0.49 g in the interior of the undisturbed stand. The response was rather slow the first year, probably because of residual bracken litter in the soil as well as the exposed nature of the clearings, lacking any moisture-retaining litter above the soil surface from the herbs of the previous season. The probable factor keeping the clearings from achieving full development after three seasons is the grazing animals in the closely proximate fern stands.

Bioassays were performed to see if there was any correlation between the differential distribution of the herb species and variation in susceptibility to the phytotoxins being leached out of the fronds. Extracts were made of fronds collected before the rains of the 1970–1971 season by soaking the intact fronds in glass-distilled water for 2 hr (65 g in 1500 ml of water). The bioassays were done in triplicate, using the standard sponge method. As shown in Table 6, there is considerable variation among herb species in susceptibility to the toxins in the leachate. *Bromus mollis*, one of the most abundant herbs inside the stands, was the most tolerant, even in the concentrated extract. *Bromus rigidus*, abundant in the rodent free stands on the island, was also somewhat more tolerant. *Avena fatua* and *Festuca megalura* were both inhibited strongly. *Hypochoeris glabra* and *Clarkia purpurea*, both species almost never encountered inside the stands, were inhibited severely, *Hypochoeris* not even germinating in the concentrated extract.

To test further for the phytotoxic effects of bracken, but under more natural conditions, raindrip was collected in the field throughout the season. Liter bottles with funnels were placed both inside the stands under fronds

TABLE 6. INHIBITION OF SEVERAL GRASSLAND SPECIES BY FROND LEACHATES (30 SEEDS PER TREATMENT)

	Percent of distilled-water controls		
	Unconc. extract	4× Conc. extract	
Species	Growth	Growth	Germination[a]
Bromus rigidus	69.0[b]	45.7[c]	100
Bromus mollis	81.0	46.7[c]	100
Avena fatua	58.9[c]	29.6[c]	60.0[c]
Hypochoeris glabra	57.5[c]	0[c]	0[c]
Festuca megalura	40.0[c]	24.5[c]	57.5[c]
Clarkia purpurea	86.0	17.2[c]	23.0[c]

[a] Germination inhibited only in the extract concentrated 4×.
[b] *t* test significant at the 5% level.
[c] *t* test significant at the 1% level.

and in the open grassland as control. After the drip was filtered, bioassays were set up using the sponge method. As shown in Figure 4, the initial raindrip was the most toxic, all three of the species being inhibited significantly as tested by Student's *t* test at the 1% level of significance. The osmotic concentration of the drip from the first rain was 35 mosmol, and subsequent rains less than 10 mosmol; thus osmotic effects were minimal. Although the

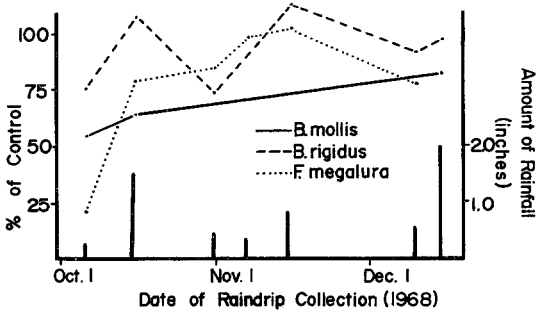

FIG. 4. Results of bioassays of raindrip from dead bracken fronds collected in the field with drip collected in the open grassland used as controls. Each point is the mean of three replicates, 10 seeds each.

TABLE 7. RADICLE GROWTH (mm) OF GRASSLAND SPECIES IN SOIL COLLECTED IN THE FIELD FOLLOWING THE FIRST RAIN OF THE SEASON[a]

Species	Control (grassland soil)	Test (fern soil)
Avena fatua	39.0 (100)	30.7 (78.9)[b]
Hypochoeris glabra	11.8 (100)	9.8 (83.6)[c]
Bromus rigidus	17.6 (100)	16.9 (96.1)
Bromus mollis	19.8 (100)	17.7 (89.5)[c]

[a] $N = 60$ for all means except $N = 90$ for *Hypochoeris*; numbers in parentheses are percent of control.
[b] t test significant at the 5% level.
[c] t test significant at the 1% level.

second and third rains were still toxic, the toxicity was not as pronounced. The second rain was not as toxic as expected because of the diluting effect from a 1.5-in. storm. It is hypothesized that the toxins are being concentrated in the upper layers of the soil, and thus can remain ecologically effective even with heavy rainfall. Although there was still slight inhibition produced in the drip in mid-December, most of the toxins had apparently been leached by then. Significantly, the pattern of inhibition became visible in the field by mid-November and was maintained for the rest of the season. So it is during the early rains that most of the initial inhibition takes place.

Because the raindrip proved to be inhibitory, it seemed likely that soil under bracken stands would acquire a significant level of toxicity. Following the first rain (0.20 in.) of the season on October 20, 1970, the wetted layer (upper 2–3 cm) was collected, screened, and 50 g placed in each of a series of 500-ml-capacity glass storage dishes. Soil collected from the open grassland was used as controls. Twenty seeds each of four different species (*Avena fatua*, *Hypochoeris glabra*, *Bromus rigidus*, and *B. mollis*) were planted in the soil in circular fashion, watered with 12 ml of glass-distilled water, and the dishes sealed with parafilm and stored for 72 hr in the dark at 26°C. As shown in Table 7, *Bromus rigidus* was not significantly inhibited but the other three species were. It is worth noting that toxicity was still evident despite the fact that the soil in each dish needed to be watered with distilled water, a treatment which would dilute the toxins and lessen their effectiveness.

In another set of experiments, soils were collected during the summer dry season from the interior of the stands and from adjacent grasslands, screened through a sieve with 2-mm openings, and placed in plastic greenhouse trays (20 × 40 × 10 cm) half filled with the soils. Four trays with each

TABLE 8. RADICLE LENGTH (mm) OF SEEDLINGS OF *Avena fatua* GROWN IN THE LABORATORY IN SOIL FROM GRASSLAND OR BRACKEN STANDS WHICH HAD BEEN EXPOSED ONLY TO THE FIRST RAIN OF THE SEASON[a]

Soil origin	Soil exposure	Mean growth	Percent control
Grassland	Grassland	48.5	100
Grassland	Bracken	42.7	88.0
Bracken	Grassland	40.8	84.0[b]
Bracken	Bracken	34.2	70.0[c]

[a] Grassland soil exposed to the rain in the open grassland used as control ($N = 60$ for all means).
[b] t test significant at the 5% level.
[c] t test significant at the 1% level.

type of soil were placed inside the stand under fronds and four in the open grassland. Immediately following the first rain of the season (October 20, 1970), the trays were brought into the laboratory and 100 g of each soil placed in separate storage dishes. Seeds were planted in the soil, the dishes sealed with parafilm, and incubated in the dark at 26°C for 72 hr. Moisture from the rain was sufficient so that no additional watering was needed. Results with seed of *Avena fatua* are presented in Table 8. Bracken soil exposed to rainfall in the bracken stand was the most inhibitory to seeds of *Avena*. Grassland soil exposed in the fern stand was also inhibitory, but to a lesser degree. Inhibition of *Avena* in the bracken soil exposed to rainfall in the grassland is an indication of residual toxicity in the soil for at least 1 year.

To test all of the above results, fronds from the stands were artificially transported to the grassland. Over a wooden frame 2.5 m square supported parallel to the ground on wooden legs 40 cm high, a grid of cotton twine was fashioned. Fully mature and dead fronds, collected at the end of the dry season before the rains began, were inserted into the string gridwork in the same upright position and same density as found naturally in the stands. The complete grid was then placed in the grassland, with another grid covered with shade fabric as an adjacent control. Light intensities under the grid averaged between 30 and 40% of full sunlight, intensities similar on the average to those of the bracken stands and under the shade frame. At the end of each growing season, to simulate the season's production, fronds were added to the grid in the density corresponding to this production. Thus, over a period of two consecutive growing seasons, extending from 1969 to 1971, leachates from the fronds were being added to the grassland environment. Since the fronds were not in physical contact with the herbs, any

TABLE 9. SPECIES COMPOSITION UNDER THE FERN GRID AND ADJACENT CONTROL ON APRIL 27, 1971, AFTER TWO GROWING SEASONS

	Shaded control		Fern grid	
Species	Number	Dry wt/indiv.	Number	Dry wt/indiv.
Bromus rigidus	3.4	14.8	7.0	9.1
Erodium cicutarium	0.3			
Lotus subpinnatus	1.3			
Micropus californicus	1.6			
Bromus mollis	10.7	2.7	94.4	0.9
Plantago hookeri	0.5			
Festuca megalura	0.3		0.2	
Lupinus bicolor	0.3			
Anagallis arvensis	0.3			
Clarkia purpurea	1.0		0.2	
Trifolium ciliolatum	1.8	6.6	0.2	2.5
Avena fatua	8.4	5.6	1.3	5.9
Trifolium repens	4.0	5.7	0.6	3.6
Silene gallica	1.7			
Hypochoeris glabra			0.1	
Galium aparine			0.2	
Mean total indiv./sample	35.6		104.2	
Mean total dry wt/sample		281.0		171.0

"smothering" effect of litter could be ruled out. Also, as evidenced by lack of such signs as runs or burrows, mammals did not concentrate their activities under the grid as they did in the fern stands.

Initially, there was not a very significant herb response, but at the end of the second season, herb growth under the grid had been severely altered. Ten 10-cm-diameter samples yielded numbers of each species (expressed as means per sample) and dry weights per individual for both the grid area and the shaded control, as given in Table 9. Species highly susceptible to the bracken toxins, such as *Avena fatua*, *Trifolium repens*, and *Lotus subpinnatus*, were greatly reduced in number as compared to adjacent grassland controls. But the tolerant species, *Bromus rigidus* and *B. mollis*, in the absence of grazing pressure of bracken-stand rodent populations and in reduced densities of other herbs, increased greatly in number. Total species diversity in general was low for this experiment because of low rainfall for the season and the resultant sparseness of herbaceous cover. Adjacent fern controls were very similar in density and yield to the grid, but those species subject to grazing, such as *Bromus rigidus*, were able to increase in numbers below the

fern grid where they were free of herbivore pressure. But the ability of the toxins to lower the average individual dry weight of both *B. rigidus* and *B. mollis* emphasizes the great differences there are in degree of susceptibility in the herb species. Although numbers of *B. mollis* were much higher under the fern grid, vigor was much reduced, many of the individuals not reaching reproductive maturity. Moist conditions under the fern grid may have favored the increase of this species.

Some Characteristics of the Toxins

A preliminary attempt has been made to isolate and identify the toxic compounds involved in the allelopathic interaction. Because the toxic principle is transported to the soil by rain or fog drip, attention was restricted to the water-soluble compounds in the frond extracts, predominantly phenolics. Fronds collected before the rains of the 1970 season were extracted with 10 parts glass-distilled water for 2 hr, filtered, and concentrated to a small volume in a flash evaporator at 45°C. In an attempt to isolate possible phenolic acids, the concentrate was then extracted with anhydrous diethyl ether 3 times, the ether fraction evaporated to dryness at room temperature, and then reconstituted in a small volume of 95% ethanol. Then 100 μl portions of the extract were spotted on 20 × 500 mm strips of washed Whatman #3 chromatogram paper. The chromatograms were developed in a descending

TABLE 10. R_f VALUES AND COLOR REACTIONS OF COMPOUNDS IN THE ETHER FRACTION OF WATER EXTRACTS OF BRACKEN FRONDS SEPARATED ON WHATMAN #3 CHROMATOGRAM PAPER STRIPS BY DESCENDING 2% ACETIC ACID[a]

Segment	Band centered on R_f	UV		DPN[b]
		Long	Short	
Origin	0			yel.
1	0.14			yel–br.
2	0.24	l.bl.	sky bl.	yel.
3	0.32	l.bl.		yel.
4	0.37	l.bl.	l. sky bl.	
5	0.49	vi.	bl.–vi.	
6	0.54	sky bl.	l.bl.	pu.
7	0.60	dk.vi.	dk.vi.	l.r–or.
8	0.64	l.vi.	l.vi.	l.vi.

[a] Key to colors: bl., blue; br., brown; dk., dark; l., light; or., orange; pu., purple; r., red; vi., violet; yel., yellow.
[b] DPN is diazotized *p*-nitroanaline.

chamber, using 2% acetic acid as the solvent. Results are presented in Table 10.

As can be seen, numerous bands separated quite well on the chromatogram strips. The bands were viewed under both long- and shortwave ultraviolet light, as well as sprayed with diazotized *p*-nitroaniline (DPN) followed by 10% Na_2CO_3 (after Hais and Macek, 1963). Several of these bands correspond to phenolic compounds identified from *Adenostoma fasciculatum* by McPherson et al. (1971), but further work is necessary before final identification can be made. Some of these may be the same compounds found in bracken by Bohm and Tryon (1967), such as caffeic acid, ferulic acid, and *p*-coumaric acid.

Bioassays were used to determine if the observed bands displayed toxicity. After development, unsprayed strips were marked and cut apart into segments centered on the R_f values in Table 10. The bioassays were performed by placing each of the paper segments in petri dishes, moistening them with distilled water, and planting 10 presoaked seeds of *Clarkia purpurea* or of lettuce (Great Lakes variety; Northrup, King and Co.) on each. These seeds are especially suited for this type of bioassay because of their small size and ease of germination. Several small cellulose sponges were

TABLE 11. INHIBITION OF LETTUCE AND *Clarkia purpurea* SOWN ON SEGMENTS OF CHROMATOGRAMS OF BRACKEN FROND EXTRACT[a]

R_f segment	Lettuce	*Clarkia*
Origin	5.96 (82.1)	6.00 (100)
0.14	6.20 (85.4)	6.39 (100)
0.24	6.12 (89.3)	6.24 (100)
0.32	5.82 (80.2)[b]	5.94 (100)
0.37	6.20 (85.4)	3.83 (74.0)
0.49	5.09 (70.1)[c]	6.84 (100)
0.54	6.29 (86.6)	3.60 (69.8)[b]
0.60	5.24 (72.1)[c]	3.47 (67.3)[b]
0.64	6.24 (85.9)	3.50 (67.8)[b]
0.73	5.00 (68.9)[c]	3.71 (71.8)
0.82	5.50 (75.7)[b]	1.72 (33.4)[c]

[a] Radical growth is expressed in millimeters. Numbers in parentheses are percentages of simultaneously run distilled water controls.
[b] *t* test significant at the 5% level.
[c] *t* test significant at the 1% level.

saturated with water and placed in the dish to keep the atmosphere moist. Care was taken to avoid contact between the sponges and the paper. The dishes were sealed with parafilm. Unspotted paper strips, developed but unsprayed and cut in the same way, were used as controls. There were three replicates of each test for *Clarkia* and two for lettuce. The lettuce was incubated for 48 hr in the dark at 26 °C, while *Clarkia* was incubated in the dark at 10 °C for 96 hr and then 26 °C for 24 hr. Seedling radicle lengths and percents of controls are summarized in Table 11. Some of the segments proved to be highly toxic, with others showing possible inhibition in these trials. The intermediate segments and those closest to the wetting front were the most toxic.

The water fraction of the ether extract has displayed similar diversity of spots and toxicity in bioassay as well, but more detailed work is still needed for characterization. Thus, the potential for the existence of an array of toxic compounds in the raindrip from bracken fronds is obvious. Perhaps the same compounds, or at least breakdown products of them, would also be found in the soil.

DISCUSSION

The role of phytotoxins in the interference of bracken with associated plants in the Mediterranean climate of southern California can be fully assessed in light of the data presented above.

The fronds of bracken, *Pteridium aquilinum*, contain chemical compounds that are toxic *in vitro* and *in vivo* to many of the herb species normally growing in grasslands adjacent to stands of the fern. The compounds are leached out of the fronds into the soil with the first series of rains during the winter season. At this time the fronds are dead but standing, the plants having died back as a result of low moisture levels in the soil or the first frosts of the winter. This is also the time of the initiation of the pattern of exclusion of herbs from bracken stands. Furthermore, reduction in growth and vigor of herb species able to establish inside the stands largely takes place in the winter and early spring, before the bracken fronds have emerged. Because of the dormant state of bracken during the time of active inhibition and other facts derived from this study, it is improbable that competition for light, water, or nutrients causes the pattern observed. In fact, the slightly higher level of available moisture inside the stands favours establishment of chemically restricted species unless they are susceptible to animal depredation.

The interaction between allelopathy and animals is very significant. Bartholomew (1970), working in a different context, questioned the role of animals in the interactions of plants, some of which have phytotoxic potential.

The present study suggests three types of plant response to the allelopathy–animal interaction. Some herb species, such as *Bromus rigidus*, are somewhat tolerant of the phytotoxins released from the bracken fronds, but are highly susceptible to grazing by the animals that inhabit the bracken stands. Other herbs are palatable to a certain extent, but are also mildly inhibited by the toxins. *Hypochoeris glabra* is an example of this type. Both factors working together help to restrict this species from bracken stands. The last group of species includes those severely inhibited by the phytotoxins and possibly not grazed appreciably, such as *Clarkia purpurea*. Because of their high susceptibility to the toxins, it is doubtful they would survive whether grazed or not. The absence of animals from the stands on Santa Cruz Island and the continued exclusion of many herb species is a good indication. Thus, it becomes apparent that allelopathy can strongly interact with other factors in the environment. This is true in most cases where plant–plant interactions are involved. Thus, it becomes necessary to assess fully the contribution of each factor of the environment to the maintenance of the total interaction.

The allelopathic potential of bracken may also explain the natural development of bracken when not disturbed for a long period of time. The interiors of old stands begin to degenerate (Watt, 1970, 1971). The grassland associates which are normally excluded from the mature stands are then able to reinvade. It is possible that there is an accumulation of phytotoxins in the interior of these old stands, and the fern may actually inhibit (autointoxicate) itself. With less phytotoxins after the reduction in density of the bracken fronds and possibly lower populations of herbivores associated with the opening up of the stand, the environment would be less hostile to the establishment and development of the grassland species. After a period of time, a time which could be called a "resting" phase, the bracken could begin to reinvade the areas it previously occupied. Then the cycle would be complete. Such a pattern has implications for the cyclic changes observed in plant populations in general, with allelopathy and possible autointoxication playing very important roles.

Bracken is dominant in situations covering a very wide range of habitats extending from the tropics to the edges of the boreal forests. This includes areas with climates ranging from semiarid to humid. Yet in all of these different ecological situations, the pattern of dominance is very similar. The mechanism establishing dominance has been demonstrated in this study for semiarid southern California. How this mechanism may vary in other habitats has been the subject of another study (Gliessman, 1976).

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RESPONSE OF THE ELM BARK BEETLE, *Scolytus multistriatus* (COLEOPTERA: SCOLYTIDAE), TO COMPONENT MIXTURES AND DOSES OF THE PHEROMONE, MULTILURE[1]

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Abstract—The response of the elm bark beetle, *Scolytus multistriatus* (Marsham), was measured to various doses and mixtures of the three components of its aggregation pheromone. The ratio of the components released, particularly heptanol to multistriatin, strongly influenced the number, but not the sex ratio, of beetles that responded. We concluded that a bait that released about 400: 100: 800 μg/day of heptanol–multistriatin–cubebene would be effective in mass-trapping beetles.

Key Words—aggregation pheromone, *Scolytus multistriatus*, elm bark beetles, *Ulmus*, attractant, Multilure.

INTRODUCTION

The European elm bark beetle, *Scolytus multistriatus* (Marsham), locates breeding sites by responding to host- and beetle-produced odors. Beetles respond initially to the odor of the elm itself (Martin, 1936; Meyer and Norris, 1967), but the mass attack is generated by a blend of attractants produced when virgin female beetles bore in the elm tissue (Peacock et al., 1971). This aggregation attractant consists of three synergistic components: (—)-4-methyl-3-heptanol (H), α-multistriatin (M), and α-cubebene (C) (Pearce et al., 1975).

[1] The use of a trade, firm, or corporation name 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 U.S. Department of Agriculture.

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H is produced exclusively by virgin females; M is produced by both mated and virgin females; C is released from elm tissue as beetles of either sex bore in the wood (Gore et al., 1977). After mating, female beetles boring in the elm wood continue to release M and C, but not H. The end of H production is apparently the basis for the cessation of attraction noted by Elliott et al. (1975), when males are allowed access to tunneling virgin females. A mixture of all three components is much more attractive than an individual component or a mixture of any two components (Lanier et al., 1977).

We tested the response of beetles to various doses and mixtures of the components of the synthetic pheromone, Multilure, to determine an effective and practical formulation for use in field tests to manipulate *S. multistriatus* populations.

METHODS AND MATERIALS

Tests were made during the summers of 1975 and 1976, in a 20-block area (200 × 3,000 m) of Detroit, Michigan. This area contained elms 50–70 cm in diameter that were about 12 m apart along the streets. The traps consisted of 46 × 66 cm sheets of paper (Bronstein Paper Co., Philadelphia, Pennsylvania) (solid, bleached white, 17-point board with a 3/4-mil double poly-coating) coated on one side with Stikem Special (Michel & Pelton, Emeryville, California). These traps were placed about 3 m high on the trunks of healthy elms that were between curbs and sidewalks. The traps were at least 50 m apart and 50 m from any diseased elm.

The three pheromone components (prepared by the Chemical Samples Co., Columbus, Ohio) were released from hollow fiber dispensers (Conrel Corp., Norwood, Massachusetts); each component was released from a separate dispenser. The nominal release rate from dispensers that contained H or M was 50 μg/day at 22°C. For dispensers containing C, the nominal rate was 100 μg/day. A bait consisting of one dispenser for each component was used as a standard.

The standard bait [H, M, and C at a 1: 1: 2 ratio (50: 50: 100 μg/day)] was designed to approximate the daily output of 5×10^3 virgin female beetles boring in an elm tree (Pearce et al., 1975). However, recent studies indicated that the synthetic H used was only about 1/4 as attractive as H produced naturally—presumably because the synthetic material consisted of equal parts of four enantiomers, and only one of these enantiomers [(—)-4-methyl-3-heptanol] is biologically active (Lanier et al., 1977). Therefore the standard bait more closely mimicked the output from a tree infested with 1.25×10^3 virgin and 3.75×10^3 mated female beetles.

During the first test period, in June 1975, we ran seven series of tests

with four different combinations of components per test, and one test of the combination of components in the standard bait (29 combinations total). There were four replications per combination. In test series 1–3, the dose of one of the three components was varied geometrically and the other two were held constant. In series 4–6, the doses of two components were varied and the third was constant. In series 7, the doses of all three components were varied. The different combinations of components were formed by varying the number of dispensers placed on a trap, and were assigned at random to 116 trap sites in the test area. The traps were removed after two weeks, and the beetles on each trap were counted. Samples of 50 beetles, picked from each of the traps during the counting process, were washed with kerosene and acetone and were examined to determine their sex.

In August 1976, we evaluated the response of beetles to various doses of two mixtures of components. We evaluated these two mixtures because the 1975 test indicated they might be more attractive than the 1: 1: 2 mixture tested in 1975. Procedures were identical to those used in 1975 except that trapped beetles were not sexed.

RESULTS AND DISCUSSION

The beetles were clearly affected by the ratio of pheromone components. Increases in the amount of any single component relative to the other components generally caused large changes in the number of beetles trapped. The largest changes were in response to M. When amounts of H and C were held constant at the standard dose (one dispenser of each per trap = 50 μg/day H, 100 μg/day C), the number of beetles responding was reduced as the amount of M was increased (Figure 1). For example, when the amount of M was doubled, the beetle catch decreased by more than half. Increasing the output of M to four times that of H virtually terminated attraction.

On the other hand, increases in the amount of H or C had a positive but smaller effect on catch. Catches were higher when H was increased by two and four times that of the standard dose; but catches were lower or were unchanged by further increases in H (Figure 2). The effect of C was more consistent. When H and M were held constant, each increase in C resulted in a larger catch, although a 16-fold increase in amount caused only a threefold increase in catch (Figure 3).

The importance of the H-to-M ratio is illustrated by the number of beetles trapped when the amounts of two components were increased simultaneously and the amount of the third component was held constant. When M and C were increased and H was held constant (a smaller ratio of H to M), the catch was lower (Figure 4). Each increase in M and C resulted in an even

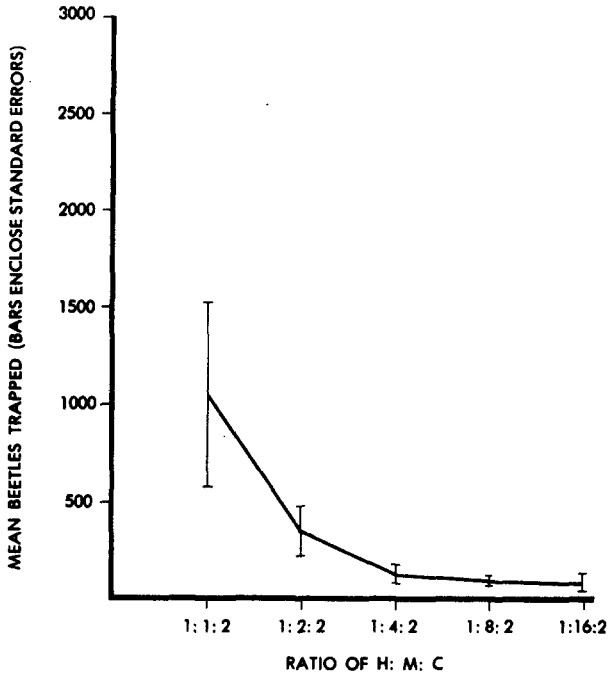

FIG. 1. *Scolytus multistriatus* trapped when M was varied and H and C were held constant. Detroit, Michigan, 1975.

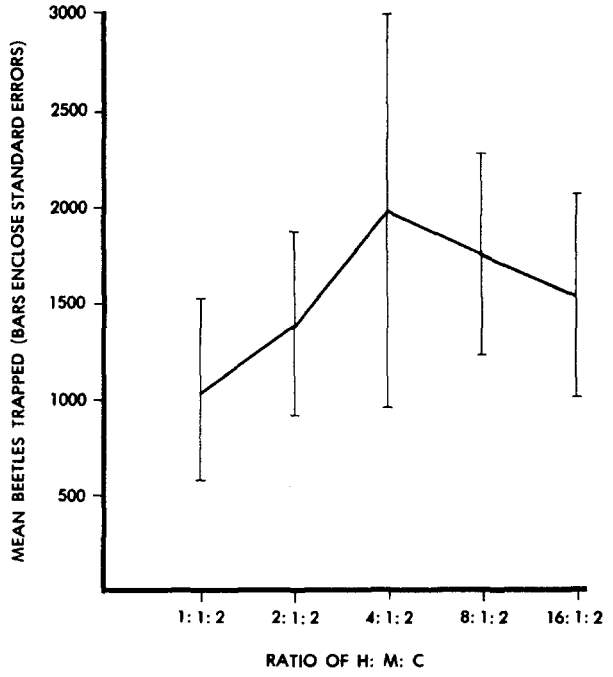

FIG. 2. *Scolytus multistriatus* trapped when H was varied and M and C were held constant. Detroit, Michigan, 1975.

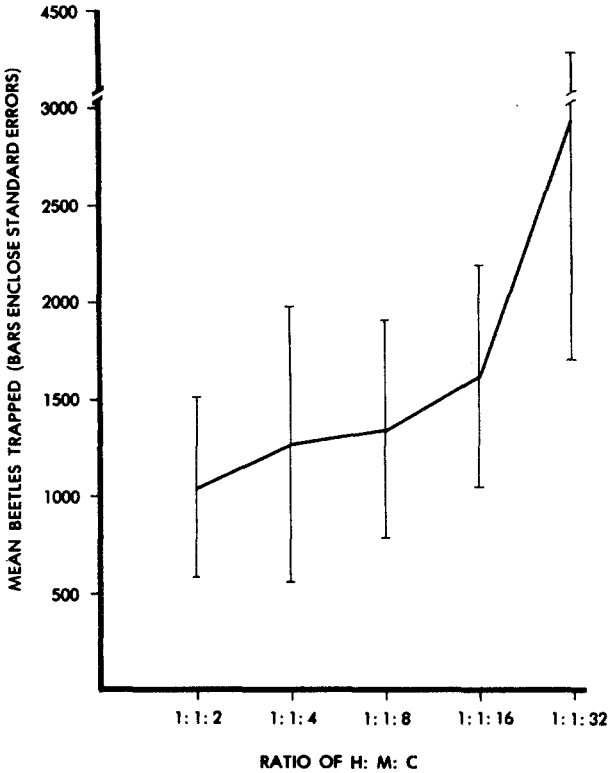

FIG. 3. *Scolytus multistriatus* trapped when C was varied and H and M were held constant. Detroit, Michigan, 1975.

lower catch, despite the expected positive effect of C. However, when H and M were increased simultaneously (the ratio of H to M was unchanged), the catches were nearly the same (Figure 5). The strong negative effect of M was partially offset by H.

When H and C were increased and M was held constant (a greater ratio of H to M), the catches were higher (Figure 6). However, in view of the results from varying H alone or C alone (Figures 2 and 3), these increases in catch probably resulted more from the greater dose of C (with H in excess of M) than from the greater ratio of H to M.

Compared to the large differences in the number of beetles trapped at the various combinations of components, the sex ratio of trapped beetles varied little. The percentage of males per treatment (sets of four traps with identical combinations of components) ranged from 33 to 53%, but the differences among these treatments were not significant (χ^2, $P < 0.05$). This

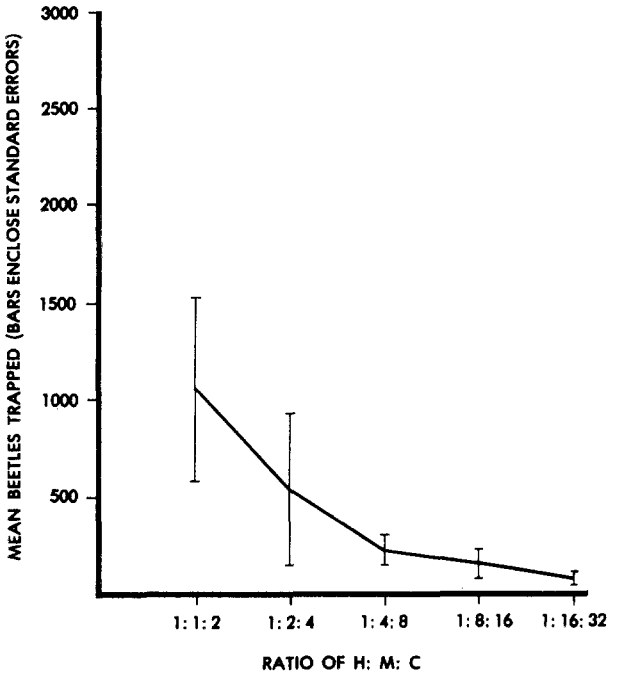

FIG. 4. *Scolytus multistriatus* trapped when M and C were varied and H was held constant. Detroit, Michigan, 1975.

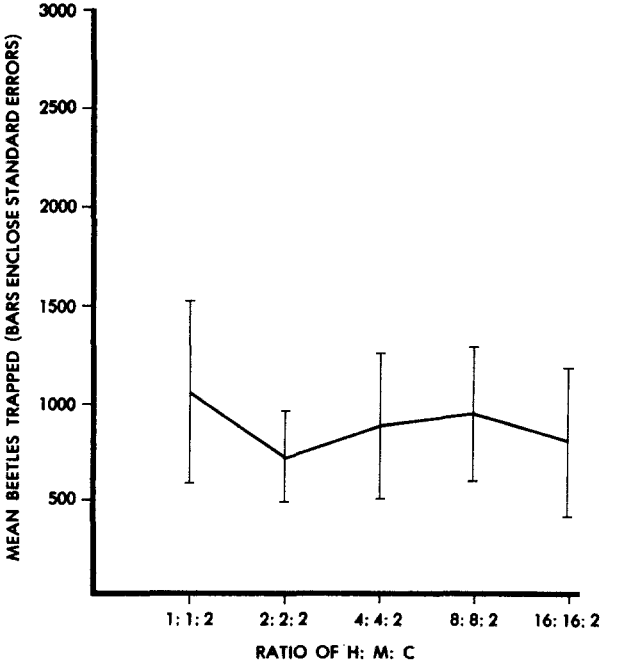

FIG. 5. *Scolytus multistriatus* trapped when H and M were varied and C was held constant. Detroit, Michigan, 1975.

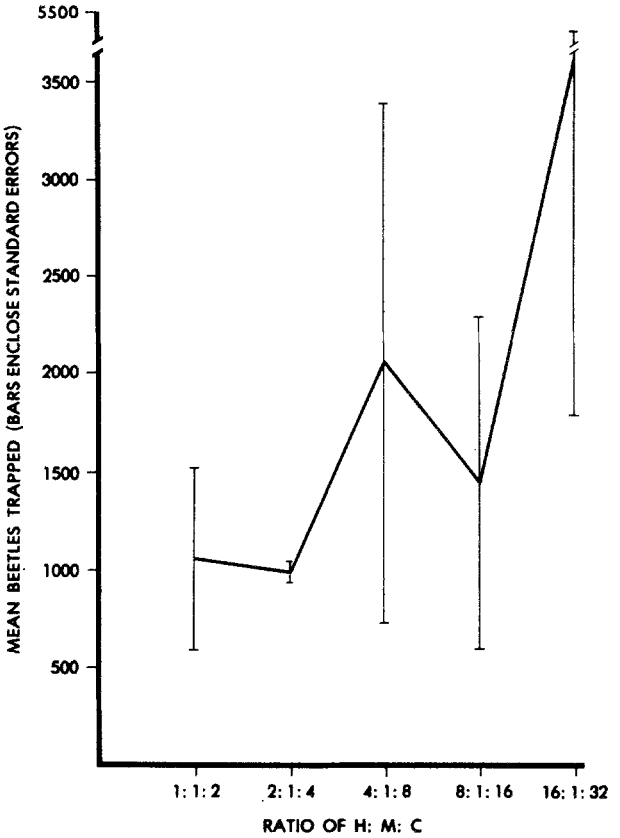

FIG. 6. *Scolytus multistriatus* trapped when H and C were varied and M was held constant. Detroit, Michigan, 1975.

constant sex ratio is unusual in scolytids; in most species the sex ratio and the number of responding beetles depend on the ratio of components released (summarized by Borden, 1974). With the data from all treatments pooled, the sex ratio of trapped beetles (44♂ : 56♀) differed from the nearly equal sex ratio of emerging beetles (Wallace, 1940; Bartels and Lanier, 1974), but this small difference probably resulted from a systematic error in our sexing procedure. Stikem obscured the setae on the heads of some males (the basis for sex determination), and therefore biased the results.

Changes in the total pheromone dose, with the three components at the standard ratio (H: M: C, 1: 1: 2), had an erratic effect on the number of beetles trapped (Figure 7). Because of the variation, which we assumed could be due to the suboptimal ratio of H to M, we ran two series of tests in 1976 at two higher H to M ratios. Catches were lower in 1976 than in 1975 because the

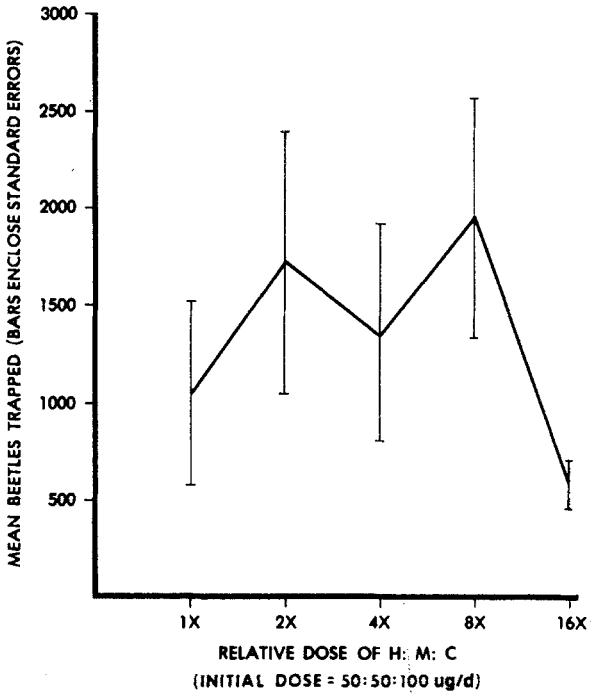

FIG. 7. *Scolytus multistriatus* trapped when doses of all three pheromone components were varied proportionally. Detroit, Michigan, 1975.

general beetle population was low at the time of the test. Again, there was no clear dose–response relationship. As expected, in tests with the higher H-to-M ratio (Figure 8), more beetles were trapped than at the lower ratio (Figure 9). But, if anything, high doses lowered the catch, regardless of the H-to-M ratio.

The variation in catches within a treatment combination in all of our tests was large—the standard error was about 40% of the mean for each treatment. Thus, statistically significant differences (ANOVA at 5% level) between treatments were detected only in those series of tests in which M exceeded H, e.g., Figures 1 and 4. However, the differences between each of the series of tests usually were so large and consistent that trends were obvious despite the variation within treatments. This variation in catches, which has occurred commonly in other field experiments with this insect, may be caused by different beetle densities between trap sites.

We concluded that the ratio of H to M is the most important factor in the attractiveness of the pheromone. Increases in the total dose of these two

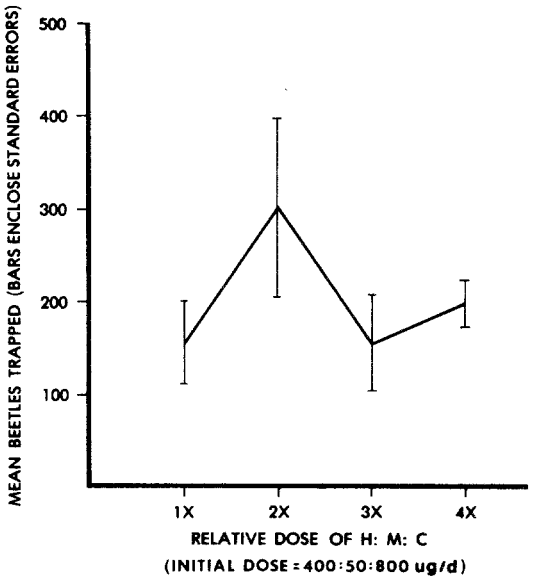

FIG. 8. *Scolytus multistriatus* trapped when doses of all three pheromone components were varied proportionally. Detroit, Michigan, 1976.

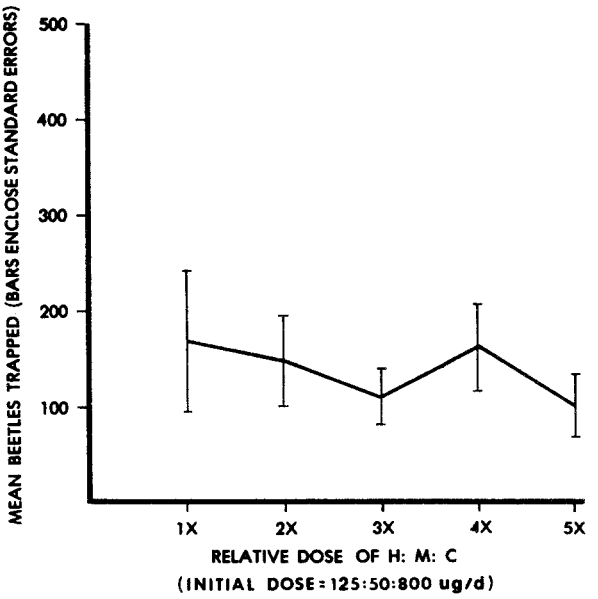

FIG. 9. *Scolytus multistriatus* trapped when doses of all three pheromone components were varied proportionally. Detroit, Michigan, 1976.

components, as long as H exceeds M, is relatively unimportant. In contrast, the attractiveness appears to be enchanced by increasing the dose of C. On the basis of these trends in beetle response alone, we would formulate baits to release no more than 50 μg/day of M (minimum dose tested), 50–100 μg/day of H (equal to or greater than M), and at least 1600 μg/day of C (maximum dose tested).

But in designing dispensers for field use we had to consider factors such as the cost of materials, the decline in release rates from dispensers over time, the variability in release rates within a dispenser (i.e., the differences between the nominal rates and the actual rates of each component relative to the others),[2] in addition to the beetle response. Therefore, we suggest that dispensers be designed to release more than the minimum dose of M to account for the decline in release rate over time. The release rate of C should be less than the maximum dose tested because the small increases in catch resulting from high doses do not appear to repay the cost of materials and formulation. The H-to-M ratio should be greater than that indicated by the beetle responses. The excess H would not affect the catch significantly, but it would minimize the chance of a suboptimal ratio, which would strongly decrease the catch. A high H-to-M ratio provides a safety factor to offset the variability in dispensers, and since H is far cheaper than M and C, this safety factor is inexpensive. In view of all these factors, we now formulate baits that release H, M, and C at the nominal rates of 400: 100: 800 μg/day.

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[2] J.W. Peacock, 1976. Preliminary studies of release rates in similar dispensers indicated that the actual release rates vary as much as 100% from the nominal rates. Since the components are released independently, the actual ratio of components released would differ considerably from the nominal ratio in some of the dispensers. Unpublished report on file at Northeastern Forest Experiment Station, Delaware, Ohio 43015.

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PHENOLS AS DEFENSIVE SECRETION IN A MALAYAN COCKROACH, *Archiblatta hoeveni* VOLLENHOVEN

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Abstract—A sternal abdominal defensive gland in adults of the Malayan cockroach *Archiblatta hoeveni* is described. It produces *p*-cresol and as a minor compound, phenol. When the animals are attacked, they eject the secretion as a spray that can be aimed in the direction of the stimulus. The position, morphology, and chemistry of the gland support the taxonomic position of *Archiblatta* within the Blattoidea.

Key Words—*Archiblatta hoeveni*, cockroach, abdominal defensive gland, *p*-cresol, phenol, taxonomic position.

INTRODUCTION

In the secondary rain forests near Kuala Lumpur, the cockroach *Archiblatta hoeveni* was found feeding nocturnally on fruits lying on the ground. These large animals defended themselves in an almost frightening way when attempts were made to capture them. They produced sharp chirping noises and repeatedly sprayed us with large quantities of a stinking fluid. In the following article, the origin and the chemical composition of their defensive secretion are reported.

METHODS AND MATERIALS

The animals were found in Gombak valley near the field station of the University of Kuala Lumpur. To avoid a release of the secretion, we cautiously

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let them run into plastic vessels. The secretion was taken directly from the gland opening with a capillary tube and then stored at −20 °C. The analyses were carried out using spot tests, GLC, and TLC.

GLC was done on a Perkin-Elmer gas chromatograph model 900, using stainless-steel columns (1.8 m × 2.7 mm) packed with Chromosorb W AW-DMCS 80–100, coated with 15% Celanese ester 9 (30 ml/N_2/min, FID). For TLC, the phenols were coupled with fast red salt AL (Knappe and Rohdewald, 1964) and separated on modified silica gel G plates.

RESULTS

Origin and Release of the Secretion

When irritated, the brachypterous females (5 cm in length, 2.5 in width) of *Archiblatta hoeveni*, which were more often caught, ejected a milky- to brownish-colored fluid secretion. It is produced in a large bilobed gland (Figure 1), which opens in the intersegmental membrane between the 6th and 7th abdominal sternites. The release of the secretion was also observed in the rarely collected normally winged males. Larvae were not obtainable. The spraying response, which was studied more thoroughly in the females, could be induced by touching the animals. In some cases, even the approach of a hand released spraying. When touched on their bodies, legs, or antennae, females exhibited an aiming reaction of turning and lifting their bodies in a

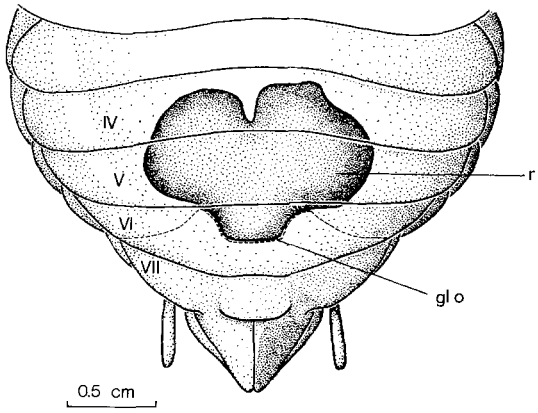

FIG. 1. Semidiagrammatic drawing of the ventral gland of an adult female of *Archiblatta hoeveni* (ventral view) in relation to the sternites (IV–VII). r, reservoir; gl o, gland orifice.

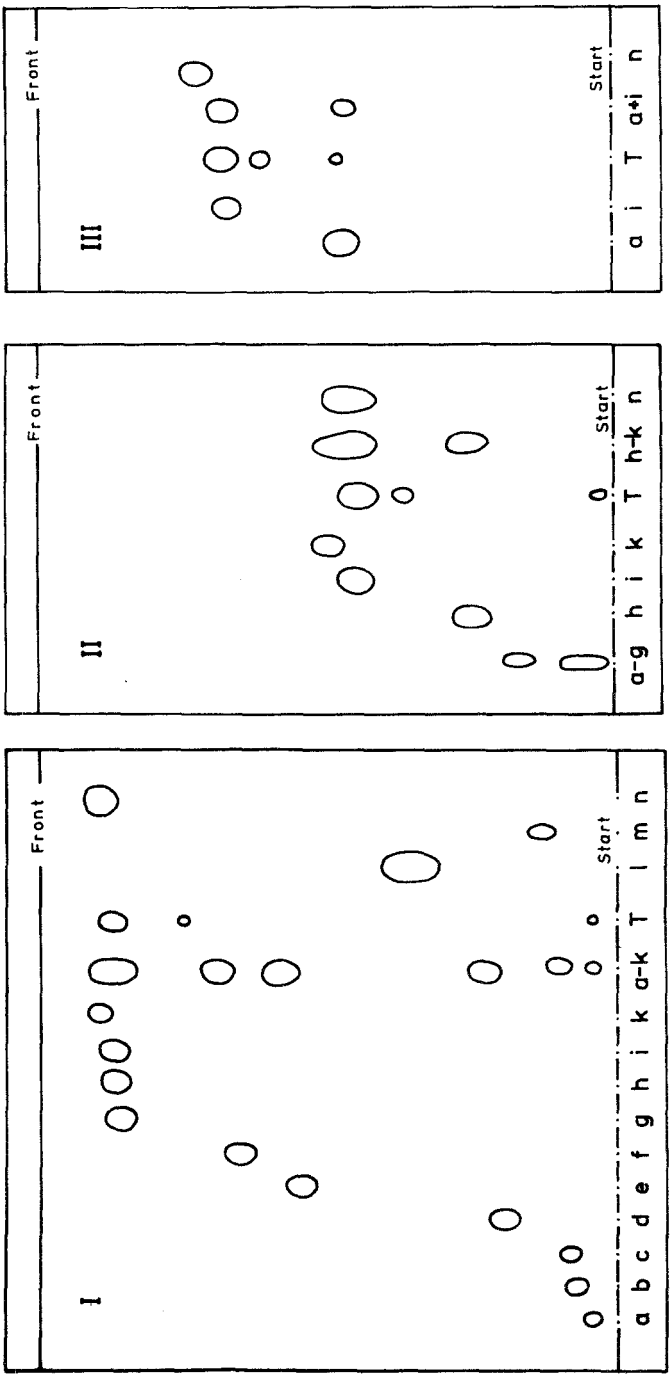

FIG. 2. Thin-layer chromatograms of the coupling products of fast red salt AL with phenol, all isomeric cresols, xylenols, and ethylphenols, and with the defensive secretion of *Archiblatta hoeveni*, on modified silica gel G plates. Coupling products with (a) phenol, (b) 3,5-xylenol, (c) *m*-cresol, (d) *o*-cresol, (e) 2,3-xylenol, (f) 2,5-xylenol, (g) 2,6-xylenol, (h) 3,4-xylenol, (i) *p*-cresol, (k) 2,4-xylenol, (l) *o*-ethylphenol, (m) *m*-ethylphenol, (n) *p*-ethylphenol, (T) defensive secretion. I. Silica gel G modified with potassium carbonate, solvent methylene chloride–acetic acid ethylester–diethylamine 92:5:3. II. Silica gel G modified with oxalic acid, solvent benzene; 2 runs. III. Plate as in I, solvent chloroform–acetic acid ethyl ester–ethanol 93:5:2.

jerking movement toward the stimulus. With their aiming movement, the animals achieved fairly accurate delivery of the secretion. In 30 tests with three animals, the direction sprayed never deviated more than 45° from the stimulus direction regardless of which part of the body had been touched. The mode of the spray was variable. The animals could eject either a few far-reaching drops or a spray of many tiny droplets. One animal tested was able to spray repeatedly 20 times, reaching a distance up to 60 cm. Simultaneously with the spraying reaction, the females often stridulated by moving a rough spot underneath the atrophied hindwings against a granulated field on the first abdominal tergite. The resultant loud noise therefore seems to be a defense reaction. It was formerly interpreted by Karny (1924) as a brood-caring signal. According to his observations, only females are able to stridulate, and females and their young stay together. The lack of observed stridulation in males, in our opinion, may rather be understood in connection with their well developed ability to fly and flee.

Chemical Composition of the Secretion

The secretion is a fluid which smells like phenol, as already pointed out by Varley (cited in Wallbank and Waterhouse, 1970). The spot test with Millons reagent was positive, as was the Liebermann reaction (Feigl, 1960), indicating the presence of simple phenols. For their identification, chroma-

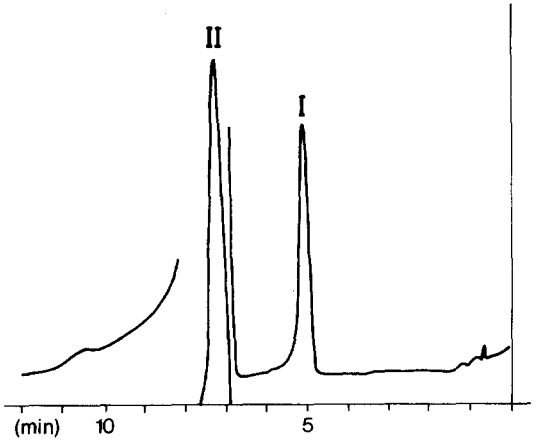

FIG. 3. Gas chromatogram of the defensive secretion of *Archiblatta hoeveni* (column temperature 200°C). Peak I and peak II are identical in their retention times with phenol and *p*-cresol, respectively.

tographic methods were used. Thin-layer chromatography allows a definite separation and identification of phenol and all cresol, xylenol, and ethylphenol isomers, using three different solvents on modified silica gel plates. As Figure 2 shows, two secretion compounds were identical with *p*-cresol and phenol in all systems, *p*-cresol being the main compound. A third spot was not identical with any of the reference substances. In GLC analysis we found only two main compounds, a major identical in its retention time with *p*-cresol, and a minor with phenol.

A quantitative analysis gave a ratio of 274: 1 (*p*-cresol to 1 phenol). Further compounds were found only in traces (Figure 3).

DISCUSSION

Several types of defensive glands have been found in Blattaria (Alsop, 1970; Beier, 1974): single ventral intersternal glands, paired eversible pleural glands, paired dorsal intertergal glands, paired tracheal glands, and hypodermal tergal glands on the terminal abdominal segments. The latter can be found in females and nymphs of many species of oviparous cockroaches. They have been investigated thoroughly in *Blatta orientalis* (Roth and Stahl, 1956; Plattner et al., 1972), where they produce a sticky proteinous secretion. Unlike the other gland types, the hypodermal defensive glands possess no reservoir. Additionally, *Blatta* possesses a ventral intersternal gland and a paired dorsal intertergal gland (Liang, 1956; Beier, 1974).

In the tracheal glands associated with the second abdominal spiracles, odoriferous repellent secretions are produced, e.g., in *Leucophaea* and *Diploptera*. In *D. punctata*, the secretion contains quinones. In the system of Princis (1960), these cockroaches are placed in two different families of the Blaberoidea; in the system of McKittrick (1964) in the Blaberidae.

The first gland type, the single ventral glands, which are situated between the 6th and the 7th abdominal sternites (Liang, 1956; Stay, 1957; Waterhouse and Wallbank, 1967), mainly produce odoriferous repellents, too. Mostly unsaturated or saturated, straight-chained or branched aldehydes have been found (Roth et al., 1956, Chadha et al., 1961; Blum, 1964; Wallbank and Waterhouse, 1970). Takahashi and Kitamura (1972) found *p*-cresol and *p*-ethylphenol in very low concentrations in the small ventral gland of adult *Periplaneta americana*, whereas odoriferous substances consisting of shorter-chain fatty acids are found in this cockroach according to Gilby and Cox (1963). The typical odor of *P. americana* suggests that there are additional compounds present.[3]

[3] For further compounds in the secretion of the ventral gland of *Periplaneta americana* see Brossut, R., Sreng, L., Rigaud, J., and Dubois, P. 1977. Glands and defensive secretions in Blattidae. Proc. VIII Int. Congr. IUSSI: 291. Wageningen.

Based on its ventral position and its structure, the large abdominal gland of *Archiblatta hoeveni* appears to be homologous to the first gland type. The systematic position of *Archiblatta* is not clear. According to Kirby (1904), it belongs to the family Blattidae. Princis (1960, cit. in Beier, 1974) regarded the monogeneric family Archiblattidae with the single genus *Archiblatta* as belonging to the suborder Blaberoidea.[4] In all other blaberoid species investigated, however, single ventral glands are lacking (Brossut, 1973). The Blattidae are placed in the suborder Blattoidea by Princis. All other cockroaches which are presently known to possess single ventral glands (Beier, 1974) belong to this family. The presence of the apomorph character, "single ventral gland," in *Archiblatta hoeveni* therefore supports, in our opinion, a systematic position of this genus in the Blattidae/Blattoidea rather than the Blaberoidea sensu Princis. Its phenolic compounds may indicate closer relationships to the Blattinae. Thus ventral defensive glands as well as other glands (Roth, 1969; Brossut, 1973) seem to be reliable characters for the classification of cockroaches.

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[4] McKittrick (1964), who revised the order Blattaria, did not investigate the systematic position of *Archiblatta*.

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STUDIES ON THE NATURE OF CHEMICAL SIGNALS IN SCENT MARKS AND URINE OF *Saguinus fuscicollis* (CALLITRICIDAE, PRIMATES)

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Abstract—The circumgenital scent marks of *Saguinus fuscicollis* are mixtures of skin gland secretions, urine, and perhaps vaginal discharge. One of their ingredients, urine, is readily investigated by conspecifics. Female tamarins are able to utilize urine to discriminate between the sexes, but it appears that urine, unlike the scent marks, offers no clues for individual discrimination. Moreover, urine is less attractive to conspecifics than complete scent marks, which signal sex and individuality. The sexual identity of marks is recognized even if the tamarins are prevented from contacting the material directly and seems to be retained in a mixture of scent from both sexes.

Key Words—Primates, *Saguinus fuscicollis*, tamarin, marmosets, scent marking, urine, chemical communication.

INTRODUCTION

A large number of recent reviews have illustrated the importance of chemical communicants in the social and reproductive life of various mammalian species. Examples are the monographs edited by Birch (1974) and by Doty (1976) and the books of Shorey (1976), Stoddart (1976), and Thiessen (1976). Much of the knowledge, however, relates to rodents while chemical communication in primates is still a largely unexplored field. Nevertheless, observational and experimental studies recently reviewed by Epple (1974a–c, 1976) show that some primates make use of chemical signals in urine, feces, genital discharge, saliva, and the secretions of skin glands to communicate the identity of the species and the individual, its gender, reproductive condition, social status and emotional state.

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We are presently involved in investigating the biological role and chemical nature of scent communication in the South American tamarin *Saguinus fuscicollis* in some detail. In captivity these highly arboreal primates form extended family groups consisting of a permanently mated adult pair and its offspring. Within the family the parents dominate, and only one female, the dominant mother, breeds. So far we know almost nothing about the social behavior of wild *Saguinus fuscicollis*, but two field studies on related tamarin species suggest that it does not differ dramatically from that of laboratory animals (Dawson, 1978; Neyman, 1978).

Adult males and females possess specialized scent glands in the circumgenital–suprapubic regions and above the sternum (Perkins, 1966). Both sexes scent mark by rubbing the glands against objects in the environment and against conspecifics. While sternal marking is relatively rare, marking with the circumgenital–suprapubic scent gland is a regular component of daily activities (Epple, 1975). It results in the deposition of the secretions from the scent gland, containing sebaceous and apocrine units (Perkins, 1966), as well as the regular addition of a few drops of urine. Female marks possibly also contain some vaginal discharge (Epple, 1975).

Scent marking occurs in a number of behavioral contexts and appears to be involved in controlling a variety of sexual and social behaviors such as courtship and pair bonding as well as intragroup and intergroup aggressive interactions (Epple, 1975, 1976, 1978). We have found that the complex circumgenital marks of males and females communicate gender, individual identity, and social status (Epple, 1971, 1973). However, since the scent marks are complex mixtures of sebaceous and apocrine secretions, urine, and possibly other ingredients, it is necessary to learn more about their biological nature and the relative importance of their ingredients. The present paper reports studies on the relative attractiveness and communicatory content of urine and on some characteristics of the sex-specific signals in circumgenital scent marks. Since sternal marking is such a rare behavior, sternal scent marks, although they might have been occasionally present in the stimuli, are not considered in this study.

METHODS AND MATERIALS

A total of 40 adult males and 35 adult females were used in the experiments reported here. Ten males and 5 females served as scent donors only. All other animals served as subjects in some experiments and as scent donors in others, but no animal was used as donor and subject in the same experiment. Table 1 lists the number of subjects and donors for each experiment.

The monkeys were permanently maintained in male–female pairs or

TABLE 1. EXPERIMENTAL PROCEDURES

Exp. no.	Stimuli tested	Subjects	Donors	Stimulus objects	No. of tests on each subject
I	1 ml of urine v. scent marks from same donor	12 ♂♂ 11 ♀♀	3 ♂♂ 4 ♀♀	Perches of white pine	2 with ♂ donors 2 with ♀ donors
II	0.4 ml ♂ urine v. 0.4 ml ♀ urine	14 ♂♂ 14 ♀♀	5 ♂♂ 5 ♀♀	Perches of white pine	4
III	♂ scent marks v. ♀ scent marks	11 ♂♂ 12 ♀♀	4 ♂♂ 4 ♀♀	Glass plates presented in screened Plexiglas cases	4
IV	Mixture of 2 ♂ marks and 2 ♀ marks v. 4 ♂ marks	16 ♀♀	19 ♂♂ 22 ♀♀	Aluminum plates	4
V	0.4 ml of urine from 2 individuals of same sex	6 ♂♂ 6 ♀♀	18 ♂♂ 21 ♀♀	Aluminum plates	4–6

small family groups. Only the breeding members of each family (highest ranking male and female) were used. The animals lived in wire mesh cages of $2 \times 4 \times 4$ ft (pair) and $4 \times 6 \times 4$ ft (groups) or small rooms (10×10 feet) containing a $2 \times 4 \times 4$-ft cage. All cages were divided by wire mesh into two equal-sized compartments accessible through a sliding door. The monkeys were maintained on a mixed diet of various fruits, cereals, meat, and commercial preparations (Hill's Science Marmoset Diet, Purina Monkey Chow 25). Females were tested during all stages of the reproductive cycle, including pregnancy. However, these primates show no external sign of cycling. Therefore, the exact stage of the females' cycles was unknown at the time of testing.

General Testing Procedures

The ability of the tamarins to discriminate between different kinds of

scent marks or urine samples was investigated by choice tests in which the subjects were simultaneously presented with two samples. The occurrence of a statistically significant discriminatory response was interpreted as proof that the animals discriminated between the stimuli. Each subject was only tested once a day. During each choice test the tamarins were given two stimulus objects which carried the scent material. Fresh pieces of white pine ($24 \times 3/4 \times 1\ 1/3$ in.), aluminum plates ($24 \times 2 \times 1/4$ in.), or Plexiglas boxes ($25 \times 2 \times 2$ in.) served as stimulus objects. Scent material was applied to the stimulus objects either by allowing donor monkeys to mark them or by applying a predetermined amount of stimulus fluid (e.g., urine) to their full lengths. Wooden perches were discarded after one use, aluminum plates were cleaned with a detergent (Alconox) and rinsed with water followed by 96% ethanol. The Plexiglas cases used in experiment III were washed with detergent and rinsed with tap water followed by distilled water. Their aluminum tops and glass inserts were rinsed with 96% ethanol following the detergent wash and water rinse.

The subjects were tested in one unit of their double-unit home cage while their group mates were confined to the other half of the cage, within sight and hearing of the subjects. Both stimulus objects were introduced simultaneously into the testing cage and were placed on the cage floor, parallel to each other, about 1 ft apart. Their left–right position was counterbalanced across the subjects of each experiment, and across all tests on each subject. The stimuli were coded and the observer was unaware of their identity. Each test lasted 5 min. The total testing time was divided into 60 intervals of 5 sec each. For every interval the subjects received a score of 1 for contacting, sniffing, and marking either stimulus object with the circumgenital glands, regardless of the actual frequencies of these behaviors. In experiment II, the time spent in contact with either perch was also recorded on stopwatches. The observer sat in full view of the monkeys, about 6 ft from the cage. Table 1 shows the specific procedures of each experiment. More details are given, whenever necessary, under the appropriate experiment. Mean scores for each subject were analyzed by nonparametric tests following Siegel (1956).

RESULTS AND DISCUSSION

Experiment I

As pointed out above, urine is one of the components of the complex scent marks. In itself, it is an attractive stimulus which is readily investigated by the tamarins, eliciting sniffing and scent marking. However, Epple

(1974b) found that scent marks applied by a tamarin to a wooden perch are preferred over a 0.4-ml urine sample from the same individual, demonstrating that the complex mark is more attractive than one of its components. Since the scent glands are situated in the circumgenital area, it is possible that the relative attractiveness of urine depends on material washed off the glandular surface during elimination and accumulating in the urine. If this were true, larger amounts of urine should be more attractive relative to scent marks than small amounts and the tamarins should show no preference when presented with a choice between scent marks and large urine samples or reverse their preference in favor of large urine samples. Experiment I presents the first step in testing this possibility by giving the subjects a choice between a scent mark and 1 ml of urine from the same donor.

One milliliter of urine was chosen because this amount is considerably more than the few drops of urine mixed into a mark but not enough to be difficult to apply to the stimulus perch without prior concentration. Uncontaminated urine was collected in glass beakers by aspirating it from clean plastic sheets spread under the donors' cages. The subjects were presented with a choice between scent marks of an adult individual and 1 ml of urine from the same donor. Each subject received two tests using two male donors and two tests using two female donors.

Male and female tamarins preferred the scent marks of males and females over the urine samples, despite the relatively large amount of urine being presented. Figure 1 shows that the subjects obtained higher scores for

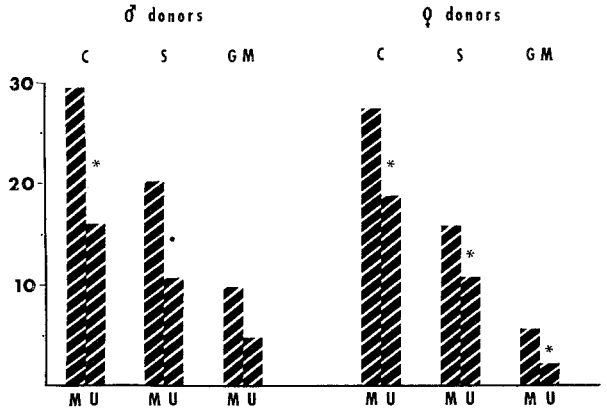

FIG. 1. Mean contact (C), sniffing (S), and genital marking (GM) scores on wooden perches carrying scent marks (M) and urine (U). Walsh test, 1-tailed (*P = 0.005, •P = 0.048).

contacting, sniffing, and scent marking the perch carrying scent marks. This result shows that urine, as one of the components of a complex mark, is not as attractive as the mark and that the attractive properties contained in the scent mark do not readily accumulate in urine. Future experiments will show whether the present results can be altered by presenting concentrates of even larger amounts of urine as an alternative to scent marks. Moreover, it would be of interest to test the relative attractiveness of bladder urine. Unfortunately, we have not found it possible to catheterize these small and fragile primates.

Experiment II

Previous studies have shown that *Saguinus fuscicollis* discriminates between scent marks of males and females and that the tamarins express discrimination in a choice test mainly by scent marking more frequently on top of male odor (Epple, 1971, 1974b). Female subjects are particularly motivated to show this response and have done so in numerous experiments in our laboratory. Males, on the other hand, are much less motivated to discriminate between male and female scent marks, although occasional significant preferences for male odor demonstrate that they are able to do so (Epple, 1974b). Experiment II tests whether the tamarins are able to discriminate between the sexes on the basis of urine alone.

Uncontaminated urine from five adult males and five adult females was collected from clean plastic sheets placed under the donors' cages and pooled by sex. The subjects received four replications of a choice between a 0.4-ml sample of male urine and a 0.4-ml sample of female urine.

Male subjects did not prefer either of the urine samples, although they showed a tendency to sniff and mark female urine preferably. Female subjects showed their usual preference for male odor over female odor. As Figure 2 demonstrates, females tended to spend more time in contact with the perch carrying male urine and sniffed and scent marked it significantly more frequently. This suggests that small quantities of naturally voided urine alone enable the tamarins to discriminate between the sexes but that males are not motivated to show a preference. This is not surprising since males do not always respond differentially to male and female scent marks either, although they are capable of discriminating between them (Epple, 1974b). Moreover, it is possible that their tendency to sniff and mark the female urine, offered in this experiment more frequently than the male samples, reflects an attraction to some urinary or vaginal compounds which are specific for certain stages of the females cycle. Since the donor females were at random stages of the cycle, including pregnancy, not enough of a possible attractant to cause a clearcut preference for female urine might have been present.

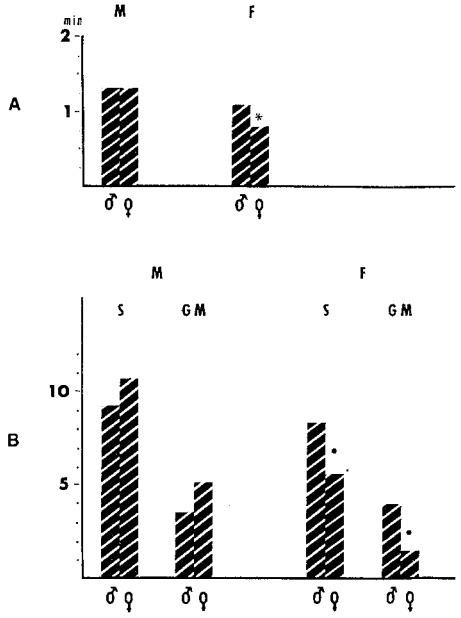

FIG. 2. Discrimination between male and female urine. (A) Mean time (min) spent in contact with stimulus perches. (B) Mean sniffing (S) and genital marking (GM) scores on perches carrying male (♂) and female (♀) urine. M = male subjects, F = female subjects. Walsh test, 1-tailed (*P = 0.047; •P = 0.005).

The question of the production site of the sexual identifiers in urine, of course, remains unsolved. Although the urine samples tested here are small, they could still have been contaminated by scent gland material during elimination. It is possible, on the other hand, that sexual identifiers of the same or different chemical nature are produced in the skin as well as in one or more additional organs (e.g., the kidney). Further experimentation is needed to solve this problem.

Experiment III

Tamarins, investigating conspecific scent marks and urine, frequently contact the stimuli directly with their noses. Moreover, in addition to sniffing, the stimuli are frequently and sometimes extensively licked. This observation

suggests that, in addition to olfaction, other chemical senses such as taste or the vomeronasal organ, which is well developed in these primates (Stephan, 1965), are involved. The present experiment tests whether the discrimination between male and female scent marks can be made when the stimuli are presented in such a way that the subjects cannot contact them while investigating.

Donor males and females scent marked $24 \times 1\frac{3}{4} \times \frac{1}{4}$-in. plates of frosted glass. The scent-marked glass plates were inserted into clean cases ($25 \times 2 \times 2$ in.) made of clear Plexiglas. The open top of each case was covered by a sliding panel of aluminum screening. There was approximately 1 in. between the screened top of the Plexiglas case contacted by the monkeys and the surface of the scent marked plate. The screening prevented any kind of direct contact between subjects and stimuli, allowing the free exchange of air. The subjects were each tested once with the scent marks of each of four donor pairs.

As Figure 3 shows, the subjects obtained a significantly higher score for contacting, sniffing, and scent marking the Plexiglas case containing male scent marks, showing that they could discriminate gender on the basis of volatiles alone.

Experiment IV

Previous experiments have suggested that sexual discrimination between scent marks is made on the basis of qualitative differences between male and female marks rather than of quantitative ones (Epple, 1971). If this were true, the tamarins should be able to recognize either sex in a mixture of scent marks from both sexes. Experiment IV tests this assumption by offering the subjects a choice between a mixture of male and female marks

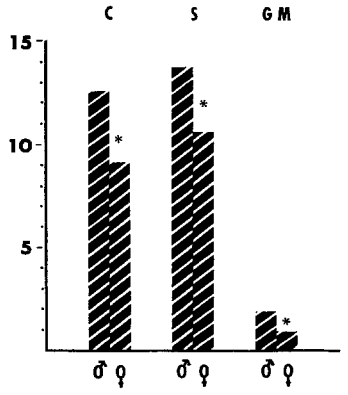

FIG. 3. Discrimination between male and female scent marks on the basis of volatiles alone. Mean contact (C), sniffing (S), and genital marking (GM) scores on Plexiglas cases containing male (♂) and female (♀) scent marks. Wilcoxon matched-pairs signed-ranks test, 1-tailed (*$P = 0.005$).

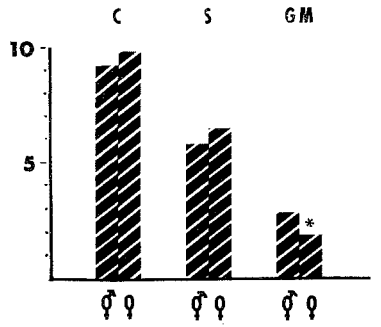

FIG. 4. Discrimination between a mixture of male and female scent marks (♂) and female marks (♀). Mean contact (C), sniffing (S), and genital marking (GM) scores on metal stimulus plates. Wilcoxon matched-pairs signed-ranks test, 2-tailed (*$P = 0.01$).

and female marks only. It was expected that the subjects would display their usual preference for male odor by preferring the male–female mixture over female marks if they were able to recognize the presence of male material in the mixture. Scent marks were collected by allowing the donors to mark clean, frosted-glass plates ($24 \times 1\frac{3}{4} \times \frac{1}{4}$ in.), recording the number of marks obtained. The material was pooled by placing the plates in chromatography columns ($24 \times 2\frac{1}{2}$ in.) and successively washing them with 100 ml of dichloromethane and 100 ml of methanol. For each series of eight choice tests, 32 female marks were pooled into one column and 16 male marks plus 16 female marks were pooled into the second column. Not all of the donors were represented in each collection but care was taken to avoid overrepresentation of any single individual and to represent the same females in both pools. The washings were concentrated to dryness by distillation under reduced pressure, dissolved in methanol, adjusted to equal volumes, and stored at −60 °C no longer than 3 days.[1] The volume of 1 "scent mark equivalent" was calculated by dividing the total volume by the number of marks. All subjects received four replications of a choice between an aluminum plate carrying a sample of the male–female mixture (2 male marks and 2 female marks) and a bar carrying four female marks.[2] The stimuli were presented after the solvent had completely evaporated. Only eight subjects were tested on any given day and a fresh collection was made for every day of testing.

The subjects contacted and sniffed both samples with about equal frequency. Their scent marking score on top of the mixture containing male material, however, was significantly higher than that on the female samples (Figure 4). Although the difference is small, it is significant at the 0.01 level,

[1] Control tests have established that fresh concentrates are not preferred over concentrates stored in this manner for several months.

[2] Previous experiments (Epple, 1974a) have established that the messages communicating gender are contained in these washings and withstand the chemical manipulations used in this experiment.

reflecting the fact that only 3 of 16 subjects did not show more marking on the plate carrying the mixture. We interpret this result as another indication that gender is communicated chemically by a qualitative difference between the sexes.

One possibility contradicting this interpretation is that males might produce a significantly higher amount of the same compounds as females and that the subjects were preferentially attracted to a higher concentration in the mixture. Several findings, however, make this explanation unlikely and support the notion of qualitative differences between the sexes. In behavioral experiments, perches marked by one donor male are preferred over those marked by two females, and over those marked by one female, as are perches carrying the odor of two males (Epple, 1971).

Thus, a preference for male marks is shown regardless of the number of donors who marked the perch, i.e., of the amount of odor present on them. This result could either be caused by qualitative differences between the two samples or, in their absence, by a rather large quantitative difference which overrides the experimental variations created by the different number of donors. Gas chromatographic profiles of male and female marks, however, reveal no dramatic quantitative differences (Yarger er al., 1977, and unpublished data) and therefore also support the idea that sex is encoded by different compounds. However, additional careful chemical studies, presently conducted in our laboratory, are needed to confirm this suggestion.

Experiment V

Epple (1973) reported that scent marks communicate individual identity and that the tamarins can remember the scent of a particular individual for several days. Since urine, which is one of the ingredients of a mark, serves as individual identification in some other mammals (e.g., Dagg and Windsor, 1971; Halpin, 1974; Seitz, 1969), experiment V investigates this possibility in the tamarins by means of choice tests.

In order to motivate the tamarins to prefer the urine of one individual over that of another, we replicated an earlier method in which we made use of their spontaneously displayed interest in the odor of a conspecific with whom they had recently interacted aggressively (Epple, 1973). In the course of a study on aggressive behavior (Epple, 1978) permanently cohabiting male–female pairs, living in a 10×10-ft room, underwent a series of social encounters, during which either one adult male or one adult female, the opponent, was introduced into their room for 10 min (for details see Epple, 1978). The resident males and females served as subjects for the present experiment, and the social encounters were used as a means to motivate their interest in the odor of the recent opponent.

One hour following an encounter the subjects received a choice between 0.4 ml urine from the "opponent" and 0.4 ml urine from a "neutral" donor of the same sex who had been an opponent during an encounter several days before the choice test. Urine samples from both donors were collected prior to the social encounters to avoid possible stress-related changes in the opponent's urine.

The subjects contacted, sniffed, and scent marked both stimulus objects with equal frequency, showing no indication that they discriminated between them. Since the tamarins showed a very clear and significant preference for the scent marks of recent opponents over the scent marks of single neutral donors when tested under almost identical conditions (Epple, 1973), the results of Experiment V suggest that individual discrimination is not, at least not easily, made on the basis of urine alone.

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PATTERNS OF SEX PHEROMONE RELEASE FROM ADULT FEMALES, AND EFFECTS OF AIR VELOCITY AND PHEROMONE RELEASE RATES ON THEORETICAL COMMUNICATION DISTANCES IN *Trogoderma glabrum*[1]

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Abstract—By means of olfactory communication models, theoretical maximum communication distances for dispensers releasing synthetic (*E*)-14-methyl-8-hexadecenal (trogodermal) were confirmed in wind-tunnel tests with *Trogoderma glabrum* males. Pheromone release rates of *T. glabrum* females are at least 10^4 greater during than before calling, and are gradually reduced after calling. The models predict that, even under ideal conditions, females may attract males from only several meters. The models are used to estimate active space lengths for female and synthetic pheromone sources, as a function of realistic release rates and wind velocities.

Key Words—Coleoptera, chemical communication distances, Dermestidae, 14-methyl-8-hexadecenal, pheromone, release rates, *Trogoderma glabrum*, trogodermal.

INTRODUCTION

Olfactory communication theory allows modeling of approximate communication distances in insect pheromone systems when the parameters of pheromone release rates, receiver thresholds, and wind velocities can be estimated (Wright 1958; Bossert and Wilson, 1963; Sower et al., 1973; Nakamura and Kawasaki, 1977). Such models can provide useful ecological information

[1] Coleoptera: Dermestidae.

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regarding the reproductive strategy of the pheromone system users, and in the case of pest species, may represent an important step in design and spacing of pheromone-releasing devices for manipulation of the pest populations.

With the recent characterization of 14-methyl-8-hexadecenal (named trogodermal by Levinson et al., 1977) as the most important long-range component in the airborne pheromone systems of several *Trogoderma* species (Cross et al., 1976), we felt that olfactory communication theory could help answer many questions regarding this compound's role in the reproductive biology of *Trogoderma* species and its potential for use in population monitoring (Barak and Burkholder, 1976) or suppression (Shapas et al., 1977). We report here on the comparison between predicted and measured communication distances of synthetic sources releasing *T. glabrum* (Herbst) pheromone in a wind tunnel, and on release rates of live females. Via models, we explore the limits placed on such communication when the constraints imposed by practical release rates and air velocities are considered.

METHODS AND MATERIALS

Experimental Insects and Environmental Conditions

All insects were laboratory-reared and prepared for testing according to the methods of Hammack et al. (1976). To summarize, males and females were sieved from media as pupae, and segregated by sex in 9.5-cm plastic petri dishes in separate incubators. At daily intervals, adults were removed and transferred to petri dishes lined with filter paper at a density of approx. 100/dish. Before testing, males had no prior exposure to female-released volatiles or to synthetic sex pheromone. Standard environmental conditions of 27 $\pm$ 2°C, 40 $\pm$ 10% relative humidity and a 16:8 light/dark photoperiod with lights on at 0700 hours, central standard time (CST) were maintained throughout rearing and testing. During bioassays light intensity was approx. 70 foot-candles provided by overhead cool-white fluorescent lamps.

Pheromone and Dispensers

Synthetic pheromone used during tests was 14-methyl-8-hexadecenal supplied by Farchan Chemical Company, Willoughby, Ohio.[2] Assays against *T. glabrum* males were run using the (*E*) isomer, which was >99% aldehyde, >98% (*E*) isomer. Release-rate studies were run with (*Z*)-trogodermal, purity >99% aldehyde, >98% (*Z*) isomer. Dispensers were 12.7-mm-

[2] Mention of a commercial product does not constitute an endorsement by the USDA.

diameter antibiotic assay filter disks (Schleicher and Schuell, Keene, New Hampshire).

Wind-Tunnel Bioassay

A small wind tunnel was used for assays, which tested for upwind attraction of males and permitted comparison of communication distances (or release rates) of pheromone dispensers. The wind tunnel will be described in detail elsewhere.[3] Briefly, the tunnel consisted of a $28 \times 28 \times 270$-cm, glass-walled enclosure, provided with temperature- and humidity-controlled and activated charcoal-filtered air. Horizontal air velocity was 48 cm/sec, measured in the center of the tunnel, 8 cm above the floor. Prior to assays, the floor of the tunnel was lined with white butcher paper to provide a suitable substrate on which the test insects could walk. Longitudinal edges of the paper were curled upward for 4 cm and were rimmed at the top with cellulose tape to prevent insect escape. The pheromone dispenser was positioned on the center of the tunnel floor approx. 25 cm downwind from its far upwind edge.

At least 60 min before each assay, groups of 20 each 6- to 12-day-old, virgin males were placed on 8×8-cm flat, white cardboard squares and covered with 2.7×5.5-cm shell vials. At assay time, individual cardboard squares with males were placed on the central axis of the tunnel, either 25 or 110 cm downwind from the pheromone dispenser. At 15 cm upwind from these male release points, a cellulose tape strip was run across the tunnel floor (perpendicular to the direction of air flow) to serve as a boundary for attracted males. A live female or pheromone-treated filter disk was then placed at the pheromone dispenser position. Treated disks were placed on clean glass slides to prevent contamination of the tunnel floor and were exposed 60 sec after treatment. The shell vial was then removed (after a few seconds for pheromone plume development) and the males were exposed to the test source. Males were removed by aspiration as they arrived 15 cm upwind at the cellulose tape boundary. The percentage of the 20 released males arriving at the tape boundary during each 60-sec assay was recorded. A new butcher paper tunnel liner was installed when control assays with no pheromone source detected contamination.

Olfactory Communication Models

To predict maximum distances over which synthetic pheromone dispensers or live females are capable of attracting *T. glabrum* males, we used

[3] C. Gorsuch and M. Karandinos, Department of Entomology, University of Wisconsin. Unpublished data.

the olfactory communication models developed by Wright (1958) and Bossert and Wilson (1963), based on mathematical descriptions of gas diffusion in the lower atmosphere (Sutton, 1949, 1953). For a stationary source releasing pheromone into the wind, the maximum downwind distance (X_{max}) at which the pheromone density is sufficient to cause a response in intended receivers is described in equation (1):

$$X_{max} = \left(\frac{2Q}{\pi C_y C_z v K} \right)^{\frac{1}{2-n}} \tag{1}$$

where Q = pheromone source release rate in μg/sec; C_y = a diffusion coefficient in $cm^{1/8}$; C_z = a diffusion coefficient in $cm^{1/8}$; v = average air velocity in cm/sec; K = pheromone density in $\mu g/cm^3$, sufficient for eliciting a response in intended receiver; n = a non-dimensional constant, = 0.25.

Values for C_y and C_z probably depend on surface roughness but are not readily calculated or easily measured, according to Sellers (1965). For diffusion over level surfaces with little temperature gradient and moderate wind, however, Sutton (1953) suggests values of $C_y = 0.4$ $cm^{1/8}$, $C_z = 0.2$ $cm^{1/8}$. With $n = 0.25$, equation (1) reduces to equation (2) (Sower et al., 1973; after Bossert and Wilson, 1963):

$$X_{max} = \left(\frac{8Q}{vK} \right)^{0.571} \tag{2}$$

Release Rates of Pheromone Dispensers, Q

Release rates of pheromone from filter-disk dispensers were determined by measuring with GLC the loss per unit time of (*Z*)-trogodermal from disks aged under assay conditions. Disks were first treated with approx. 100 μg of (*Z*)-trogodermal applied as a 10-μl aliquot in hexane. After aging disks in both a small glass chamber under conditions similar to those of the wind tunnel and in 1.5 × 5-cm shell vials under vial bioassay conditions, sets of three disks were removed from aging after each 0 (for extraction efficiency determination), 1.5, 3, 6, 12, and 24 hr. Each removed disk was immediately placed in a preweighed 1.5 × 5-cm stoppered shell vial and stored at −30 °C. When all disks had been aged, each was extracted for 24 hr in 1.0 ml hexane at 20 °C on a shaking apparatus. Each disk was removed, subjected to a second 24-hr extraction, and its extracts combined (minus the disk) in the preweighed vials. At least three 2-μl samples of each extract were chromatographed (1.5% OV-17 on Chromosorb-G, high purity, 100/120 mesh;

1.3 m × 3 mm OD stainless-steel column, 40 ml/min N_2 flow rate at 190°C; trogodermal retention time approx. 5 min), and their average peak areas were compared with those of standard pheromone solutions. Pheromone recoveries for each disk were calculated as the quantity of trogodermal in 2 μl of extract times the total extracted volume (determined by weight of the extract times 1/density of hexane), divided by the extraction efficiency. Release rates were then calculated as the average amount of pheromone lost, divided by the time interval during which the loss took place.

Effects of Time of Day on Female Pheromone Release Rates

Release rates of sex pheromones from live females were determined by comparing the average wind-tunnel bioassay response to individual 6- to 10-day-old females with that produced by synthetic pheromone sources releasing known amounts of (*E*)-trogodermal. Females were held (5/petri dish) under normal photoperiod (16 hr photophase beginning at 0700 CST) or in separate incubators which were phase-shifted (while the females were still pupae) so that photophase began at 1300, 1900, or 0100 CST. [Hammack and Burkholder (1976) have shown that temporal placement of calling behavior of *T. glabrum* females is "corrected" to a new photophase 3–4 days after such shifts.] For approx. 5 min prior to assays, females were left in uncovered dishes at the downwind end of the wind tunnel to reduce possible cuticular surface contamination. Strips of white bond typing paper (5 × 10 mm) were bent in an L shape and attached by double-stick tape to the centers of 4 × 4-cm squares of flat, white cardboard to serve as calling platforms for females. The cardboard squares were edged with a 1.5-cm-wide band of cellulose tape to discourage female escape. Just prior to assay time, females were transferred to the centers of cardboard squares and covered with a 1.5 × 5-cm shell vial. Tunnel bioassays at 1100–1200 and 1400–1500 CST (during the period of maximum male sensitivity to (*E*)-trogodermal, Shapas and Burkholder, 1978) were used to measure the male response to 3–5 females from each of the four photoperiods, thus comprising samples of females from eight consecutive 3-hr periods. We estimated release rates of pheromone from females by comparing their bioassay response with that of filter disks releasing known amounts of (*E*)-trogodermal, assuming that (*E*)-trogodermal alone is responsible for long-range attraction of males by females.

The release-rate values determined by wind-tunnel bioassay were also integrated for one day and compared with calculations based on Porapak-Q recovery of (*E*)-trogodermal from 2000 females releasing volatiles into a small glass collection chamber (Cross et al., 1976). The raw Porapak value was increased by dividing by the recovery efficiency, determined by releasing

approx. 1600 μg of (*Z*)-trogodermal from filter disks into the Porapak aeration chamber over a 4-day period, recovering the trapped pheromone with the standard techniques used for female-released pheromone (Cross et al., 1976), and calculating the fraction recovered.

Male Arousal Threshold, K

The concentration of (*E*)-trogodermal in air necessary to release arousal behavior in *T. glabrum* males was estimated as follows. A modification of the small-vial bioassay technique described by Vick et al. (1970) was used to determine the 50% arousal thresholds of individually tested males when filter disks were used as dispensers. Briefly, the bioassay involved pipetting 10-μl samples of serially diluted pheromone in hexane onto 12.7-mm-diameter filter disks and suspending each disk 0.5 cm above virgin 6- to 12-day-old males which had no prior exposure to pheromone and had been kept for at least several hours prior to assay in individual 1.5 × 5-cm shell vials lined with untreated filter disks. The percentage of males which responded by raising their antennae and taking at least 2 steps during a 60-sec exposure to the pheromone sources was recorded. For each active pheromone concentration, at least four groups of 10 males were assayed. Thresholds for 50% response were then calculated by best linear fit of the probits of percent responses plotted against the logs of applied pheromone concentrations.

The pheromone release rate of the filter disk at the dose producing a 50% arousal response was determined using GLC as described in a previous section. The average pheromone density in air necessary to elicit arousal was then calculated as the release rate of filter disks at 50% threshold times the average bioassay time (approx. 30 sec), divided by the volume of air beneath the filter disk in the assay vial (1.43 cm^3). The threshold determined in this manner differed slightly from that measured in the wind-tunnel bioassay. In the tunnel assay, males must not only become aroused, but must also travel 15 cm upwind.

To be certain that wild *T. glabrum* males had response thresholds for trogodermal similar to those of laboratory-reared males, vial assays were also run on a population of 120 males which had been live-trapped in the field. The live-trap used a filter disk pheromone dispenser with 100 μg of applied (*E*)-trogodermal, suspended with string across the 15-cm-wide mouth of a glass funnel. The funnel was positioned so that the narrow end emptied into a 100-ml glass jar, which was covered with aluminum foil to reflect solar radiation. The trap, placed 1 m above the ground on a grassy knoll on the University of Wisconsin campus, captured a total of approx. 200 flying males during 3-hr exposure periods on each of three successive days during July 1976. Males were removed from the trap, distri-

buted in two filter-paper-lined, 9.5-cm-diam petri dishes, and stored in pheromone-free incubators alongside laboratory-reared males. To minimize possible contaminating effects from pheromone in the trap, males were transferred to clean petri dishes each day after capture. After 4 days for adjustment to laboratory conditions, males were tested for 50% arousal threshold using the small-vial technique.

Air Velocity, v

The air velocity 8 cm above the tunnel floor was estimated by measuring the speed of TiO_2 vapor puffs, generated from a 12.7-mm filter disk on which several drops of $TiCl_4$ had been placed. It is known that the vertical wind profile decreases as one approaches a horizontal surface, with the amount of decrease proportional to surface roughness. The actual wind velocity v_z at the height of the pheromone dispenser above the tunnel floor (0.2 cm) was estimated by equation (3) (Porter et al., 1973; after Sellers, 1965):

$$v_z = v_r \frac{\ln\left(\frac{z}{z_0}+1\right)}{\ln\left(\frac{z_r}{z_0}+1\right)} \tag{3}$$

where v_z = wind velocity in cm/sec at any height z; v_r = wind velocity in cm/sec at reference height; z = height in cm above horizontal surface; z_0 = surface roughness length in cm.

Sutton (1953) lists a range of z_0 values, from 0.001 cm for smooth surfaces such as mudflats, to 5.0 cm for 50-cm-high grass. We assume our tunnel floor is similar to a mudflat in smoothness ($z_0 = 0.001$), which yields an air velocity at the pheromone source of 28.3 cm/sec.

Real Communication Distances, X_{max}

Pheromone-treated filter disks of known release rate were tested in the standard wind-tunnel bioassay to compare real communication distances with theoretical values generated by equation (2). The percent response of males to pheromone sources of various release-rates was first determined at downwind ranges of 25 and 110 cm. The release rates which would yield a 50% response at 25 and 110 cm were then determined by best linear fit of the probits of the percent responses vs. logs of the pheromone release rates. These release rates were then substituted for Q in equation (2) to generate

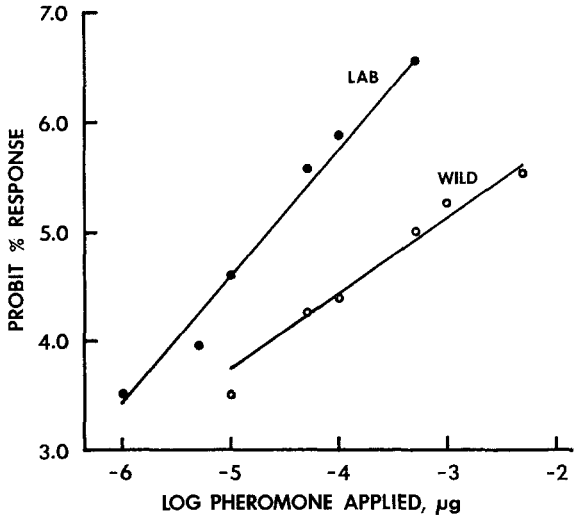

FIG. 1. Standard curves relating pheromone applied to 12.7-mm filter disks to percent male responses in the small-vial bioassays. The vial assay response of 6- to 12-day-old laboratory males is compared with wild *T. glabrum* males of unknown age distribution.

the theoretical values for X_{max}, or the distances at which 50%of males would be expected to respond.

RESULTS AND DISCUSSION

Trogodermal release rates (Q for filter disks) were approx. 10%/hr ($= 2.8 \times 10^{-3}$%/sec) from 12.7-mm filter disks in the wind tunnel, and approx. 0.8%/hr ($= 2.2 \times 10^{-4}$%/sec) from disks in the shell vials. The dose–response curves for the vial bioassays are shown in Figure 1. With the vial assay 50% response threshold of laboratory-reared males at a release rate of 4.8×10^{-11} μg/sec, the 50% response threshold density of (*E*)-trogodermal in air was calculated as

$$K = \frac{(4.8 \times 10^{-11}\ \mu g/sec)\ (30\ sec\ average\ assay\ time)}{(1.43\ cm^3\ assay\ vial\ volume)}$$

$$= 1.0 \times 10^{-9}\ \mu g/cm^3$$

This estimate assumes that all pheromone released from the filter disk is airborne, e.g., none is deposited on the vial walls, insect, or filter disks.

Also shown in Figure 1, the sensitivity of our sample of wild *T. glabrum* males to (*E*)-trogodermal was less than that of 6- to 12-day-old males raised in the laboratory. We attribute the sensitivity difference, and the difference in slopes of the two dose-response curves, to the unknown age distribution of the wild males. If the wild males were considerably older than 12 days (which we assume to be the case, since the wild males lived only a few days after capture), the approximate 1 decade range difference in sensitivity at the 50% response level may partly reflect slightly decreased pheromone sensitivity with age (Shapas and Burkholder, 1978). Since our sample of the wild population was selected on the ability of its members to respond to a pheromone trap, we consider the average sensitivity of the entire local population in the trap range to be no greater than those trapped. We conclude that the sensitivities of wild and laboratory-reared males to (*E*)-trogodermal are not vastly different.

Comparison of wind-tunnel bioassay responses to 6- to 10-day-old females with the standard curves in Figure 2 revealed diel changes in female pheromone release rates, as shown in Figure 3. Female pheromone release increased abruptly during the 1300–1600 sampling period, and trailed off after calling. This period of maximum release of pheromone corresponds

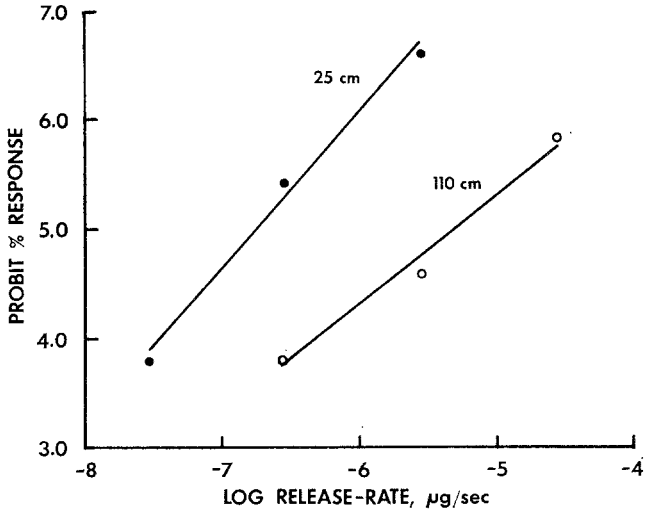

FIG. 2. Standard curves relating release rates of filter-disk trogodermal sources in the wind tunnel to response of males 25 and 110 cm downwind.

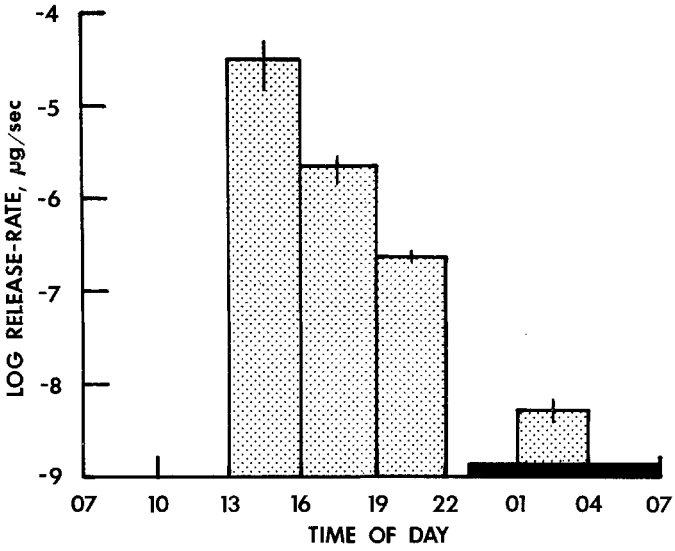

FIG. 3. Release rates of pheromone from live *T. glabrum* females with respect to time of day. Values were determined by wind-tunnel bioassay, comparing the response to females with the response to filter disks releasing known amounts of (*E*)-trogodermal. The dark bar (2300–0700 hr) denotes scotophase, and the short, vertical lines denote $s_{\bar{x}}$.

with the times during which maximum frequency of calling (Hammack et al., 1976) and male and female exposure behavior (Shapas and Burkholder, 1978) have been reported. The peak release rate for females was calculated as 3.2×10^{-5} ($\pm 1.7 \times 10^{-5}$ SE) µg/sec. Since pheromone release is probably through cuticular pores over an epidermal glandular area (Hammack et al., 1973), and not via an eversible gland as in some Lepidoptera, we expected the gradual decrease in pheromone released after the calling period.

Collection on Porapak-Q of volatiles from 2000 females (Cross et al., 1976) revealed that 28.3 ng/female/day of (*E*)-trogodermal could be recovered. This was divided by 0.23, the recovery efficiency of trogodermal from Porapak-Q, to yield 122.8 ng/female/day, a more accurate estimate of the release of (*E*)-trogodermal from live females. Integrating our release-rate data from tunnel assays over an entire day (= 372.9 ng/female/day) compares somewhat favourably with the value determined from Porapak-Q. The wind-tunnel data may be inflated since we have considered females to be releasing pheromone maximally for an entire 3-hr period (1300–1600). However, the Porapak data may be an underestimate, due to low air velocities and therefore reduced release rates in the aeration chamber.

TABLE 1. ESTIMATES OF OLFACTORY COMMUNICATION THEORY PARAMETERS FOR *Trogoderma glabrum*, UNDER WIND-TUNNEL CONDITIONS[a]

Q, pheromone release rate for filter disks (μg/sec)	K, 50% response threshold density (μg/cm^3)	v, air velocity Z=0.2 cm (cm/sec)	Theoretical x_{max} (cm)	Measured x_{max} (cm)
1.27×10^{-7}	1.0×10^{-9}	28.3	7.7	25
4.94×10^{-6}	1.0×10^{-9}	28.3	62.9	110

[a] Based on equation (2), theoretical x_{max} is the distance at which 50% of males would be expected to respond.

Our estimates for the parameters Q, K, and v, are summarized in Table 1, along with a comparison of the predicted and measured X_{max}. Since we had hoped the predicted and measured values would differ by no more than one order of magnitude, the model accurately predicts communication distances under our wind tunnel conditions. A better fit at 110 cm is seen when equation (2) is modified to equation (4)

$$x_{max} = \left(\frac{21Q}{vK}\right)^{0.571} \tag{4}$$

This change results from changing C_y and C_z from equation (1) to values whose product equals 0.03, instead of 0.08 which was used to generate equation (2). Such a modification is not unreasonable since accurate values for C_y and C_z remain unavailable. Under our wind-tunnel conditions, diffusivity in the y and z planes is probably less than for the "average" conditions described by Sutton (1953).

Using equation (4), we have produced a series of curves (Figure 4) representing theoretical maximum communication distances between pheromone sources and *T. glabrum* males, as a function of pheromone release rates and wind velocities. The predictions may bear only small resemblance to values measured in the field, since effects of many other factors must be considered there. Air convection and turbulence, for example, may play important roles in limiting the length of the pheromone-active space in field situations, but were probably minimized in the wind tunnel. The possible dependency of pheromone release-rates and receiver thresholds on temperature has not been considered in this discussion.

Figure 4 also predicts theoretical communication distances for calling females. We have plotted female communication distances as a band, limited by females with peak release rates of twice (6.4×10^{-5} μg/sec) and

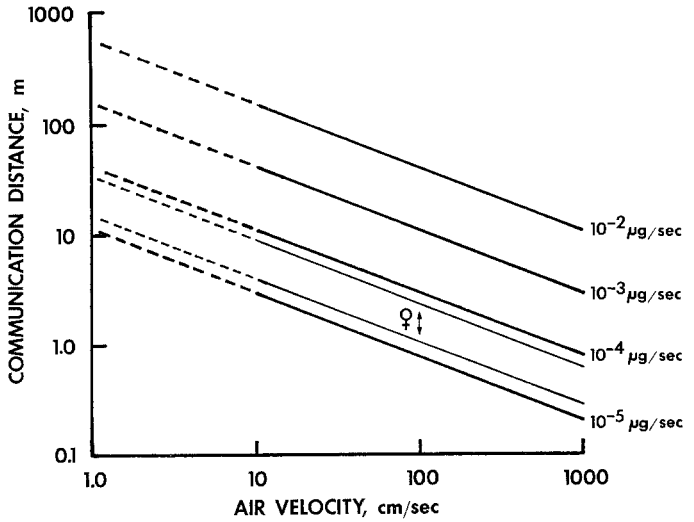

FIG. 4. Predicted communication distances of (*E*)-trogodermal pheromone sources [equation (4)], as a function of release rates and wind velocities. Communication distance is defined as that range downwind from a pheromone source at which 50% of males would be expected to respond. Communication distances of females are represented by a band, limited by females which release half (1.6×10^{-5} μg/sec) and twice (6.4×10^{-5} μg/sec) as much pheromone as the average. The model may become increasingly inaccurate below air velocities of 10 cm/sec, due to air velocity-dependent pheromone release rates.

half (1.6×10^{-5} μg/sec) the average value. Based on such predictions, laboratory-reared females are severely limited in range of attraction for males. Release rates of wild females remain unknown, however.

Equation (1) implies an increasing communication distance with decreasing air velocity. To take into account effects at low air velocities as a result of deposition of the released pheromone on ground surfaces (Chamberlain, 1953), Nakamura and Kawasaki (1977) utilized a modified release-rate.

$$Q_x = Q^{-\left(\frac{4v_g x^{n/2}}{nv\pi^{\frac{1}{2}}Cz}\right)}$$

where v_g = velocity of deposition in cm/sec and is equal to v_f^2/v, where v_f is the friction velocity.

According to equation (5), length of the active space is expected to decrease rapidly at wind velocities below 10–25 cm/sec, due to a reduction in airborne pheromone resulting from pheromone deposition. We have con-

sidered this possibility in Figure 4 by plotting theoretical communication distances from air velocities below 10 cm/sec as dotted lines. If this effect is real, it would help to explain observations (Hammack et al., 1976) that *T. glabrum* females seek high places prior to calling and release of sex pheromone, and assume "headstand" postures during calling. Air velocities can easily approach zero in the boundary layer a few millimeters above rough surfaces, as can be calculated from equation (3). Perhaps females exhibit calling behavior and seek high places not simply to reach increased wind velocities (which above a certain point will actually decrease olfactory communication distances), but primarily to escape the boundary layer of greatly reduced air movement. The boundary layer may present a formidable obstacle in olfactory communication among some insects by virtue of significantly reducing release of chemical messages.

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DIEL AND AGE-DEPENDENT BEHAVIORAL PATTERNS OF EXPOSURE–CONCEALMENT IN THREE SPECIES OF *Trogoderma*[1]

Simple Mechanisms for Enhancing Reproductive Isolation in Chemically Mediated Mating Systems

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Abstract—Adult *Trogoderma glabrum*, *T. inclusum*, and *T. variabile* exhibit diel periods of exposure–concealment behavior, the frequency and duration of which are age-dependent, and the temporal placement of which is species-dependent. Exposure periods correspond with daily maxima in male sensitivity to the most active female-released sex pheromone component, 14-methyl-8-hexadecenal, and with respective periods of sex pheromone release in females. Exposure–concealment behavior may be a factor in reproductive isolation between these species, since potential communication distances are greatly reduced when either males or females are concealed.

Key Words—Coleoptera, Dermestidae, diel behavioral patterns, 14-methyl-8-hexadecenal, pheromone, reproductive isolation, *Trogoderma glabrum*, *T. inclusum*, *T. variabile*, trogodermal.

INTRODUCTION

Recent reports (Cross et al., 1976; Greenblatt et al., 1977) have shown that 14-methyl-8-hexadecenal is responsible for interspecific responses among several *Trogoderma* species under laboratory conditions. While laboratory matings between all combinations of seven North American species of *Trogoderma* produce no viable hybrids (Strong and Arndt, 1962), questions

[1] Coleoptera: Dermestidae.

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remain regarding the identity of isolating mechanisms which may prevent attempted matings in the field between species which utilize similar pheromone systems. Diel changes in both male pheromone sensitivity (Vick et al., 1973), and female pheromone release (Hammack et al., 1976) have been described as contributing to such isolation. There has been, however, little quantitative assessment of the relative importance of these or other factors in preventing presumably nonadaptive cross-attraction between sympatric field populations of *Trogoderma* species.

Trogoderma glabrum (Herbst), *T. inclusum* LeConte, and *T. variabile* Ballion may be the most economically important Dermestidae in North America today. For these three species, we report on changes in male sensitivity to the common sex pheromone component 14-methyl-8-hexadecenal with respect to age and time of day, and on previously unreported diel behavioral patterns of exposure–concealment which are exhibited by both males and females. We also attempt to assess the relative importance of such simple behavioral patterns in the reproductive isolation of these *Trogoderma* species, as measured by effects on theoretical chemical communication distances.

METHODS AND MATERIALS

Experimental Insects and Environmental Conditions

All insects were laboratory-reared and prepared for testing according to the methods of Hammack et al. (1973, 1976). To summarize, males and females were sieved from media as pupae, segregated by sex in 9.5-cm plastic petri dishes, and held in separate incubators. At daily intervals, adults were removed and transferred to petri dishes lined with filter paper, with each dish containing approx. 100 insects. Before testing, males had no prior exposure to female-released volatiles or to synthetic sex pheromone. Standard environmental conditions were maintained throughout rearing and testing, consisting of $27\pm2°C$, $40\pm10\%$ relative humidity and a 16:8 light/dark photoperiod with lights on at 0700 hours, central standard time (CST). During bioassays and observations of male behavior, light intensity was approx. 70 foot-candles provided by overhead cool-white fluorescent lamps. For wind-tunnel bioassays, air velocity was 48 ± 4 cm/sec, measured in the center of the tunnel, 8 cm above the floor.

Pheromones and Dispensers

Synthetic pheromone used during these tests was 14-methyl-8-hexadecenal (trogodermal), supplied by Farchan Chemical Company, Willoughby,

Ohio.[2] The (*E*) isomer [purity >99% aldehyde, >98% (*E*)] was used in assays for *T. glabrum*, and the (*Z*) isomer [purity >99% aldehyde, >98% (*Z*)] for assays of *T. inclusum* and *T. variabile*. Dispensers were 12.7-mm antibiotic assay filter disks (Schleicher and Schuell, Keene, New Hampshire) which, as determined by GLC recovery from disks aged under bioassay conditions, released pheromone at approximately 10%/hr in the wind tunnel and 0.8%/hr in the small-vial assay.

Bioassays

Synthetic pheromone sources were bioassayed against males using either a modification of the small vial technique of Vick et al. (1970) or the wind tunnel assay described in detail elsewhere (Shapas and Burkholder, 1978). Briefly, both assays involved measuring the response of 6- to 12-day-old virgin males to 12.7-mm filter disks which had been treated with 10 μl of synthetic pheromone of various dilutions in hexane. For the vial assays, disks were suspended 0.5 cm above males which had been kept for at least several hours prior to assay on untreated filter disks in individual 1.5 × 5-cm shell vials. A positive response was recorded if a male raised its antennae and took at least 2 steps during a 60-sec exposure to a pheromone source. In the tunnel assays, treated disks were placed on the floor of a 28 × 28 × 270-cm glass-walled wind tunnel. At 110 cm downwind from the disks, groups of 20 males were released from under 5-cm-diameter petri dish halves. A positive response was recorded if a male traveled 15 cm upwind from its release point during a 60-sec exposure to a pheromone source. Males were tested only once in both types of assays. Hexane controls did not yield positive responses in either bioassay.

Male Pheromone Sensitivity as a Function of Time of Day and Age

To determine the variations in male sensitivity to pheromone with respect to time of day and age, males were vial-bioassayed with synthetic (*E*)- or (*Z*)-trogodermal. All assays were run between 1200 and 1600 CST for the age tests, and as described below for the time-of-day tests. At each time or age point, four sets of 10 males were tested at each of 3–6 pheromone concentrations spanning at least 3 log-unit concentration ranges. Thresholds for 50% responses were calculated by best linear fit of the probits of percent response average, plotted against the logs of respective pheromone concentrations.

For determinations of pheromone sensitivity with respect to age, males

[2] Mention of a commercial product does not constitute an endorsement by the USDA.

were vial-bioassayed at 3- to 6-day intervals posteclosion, until 25% mortality had occurred.

For time-of-day tests, 6- to 12-day-old males were vial-assayed at 2-hr intervals (assay duration approx. 40 min) after lights on. Scotophase assays were run using a 10-W red light for illumination. For convenience, most scotophase assays were run on males which were subjected to a standard photocycle, but phase-shifted (while still in the pupal stage) so that the 8-hr scotophase began at 0900 CST.

Exposure–Concealment Behavior

Exposure–concealment behavior was quantified in the following manner: Adults of each species were maintained in 5-cm-diameter, filter paper-lined petri dishes with 10 males or females per dish. Each dish was provided with a 2 × 2-cm single-faced, A-flute, corrugated fiberboard square as a concealment substrate. White butcher-paper cylinders, 5 cm diameter × 20 cm high, were fitted around each dish to provide even and equal illumination and to minimize effects of observer presence on insect behavior.

After beetles had acclimated in dishes for at least 24 hr, observations were made from above at 2-hr intervals throughout the standard photocycle. Most scotophase observations were made using a 10-W red light on insects which were photocycle phase-shifted by 10 hr. Recordings were made of numbers exposed (observation of at least 50% of an entire beetle was necessary to qualify as "exposed") per dish at each time or age point. Observations were made at 1-day age intervals until 25% mortality was observed. Data is expressed as average percent of total beetles exposed in each of 5 dishes at various time or age observation points.

Determination of Exposure–Concealment Effects on Communication Distances

We believe that the relative importance of some presumed reproductive isolating mechanisms in chemically-mediated mating systems can be measured by their effects on communication distances between sender and receiver. In Bossert and Wilson's (1963) model, communication distance is proportional to sender release rate and inversely proportional to receiver threshold. Parameters of this model have been estimated for *Trogoderma* by Shapas and Burkholder (1978). An important isolating factor would be expected to minimize communication distances between closely related species, either by effectively reducing the pheromone release rate of the sender or by effectively increasing the response threshold of the receiver. One advantage of using communication distances to measure these effects is that it creates a continuum of comparable values.

To test the effects of concealment behavior on effective male response thresholds, we compared the response of exposed vs. concealed *T. glabrum* males in a standard wind-tunnel assay. Six- to eight-day-old males were assayed during late morning, a period during which such males normally would be concealed. Half the sets of 20 males were provided with a standard 2×2-cm corrugated fiberboard square (with open flutes perpendicular to the air flow) for concealment, while the other half were left exposed.

To test the effects of concealment behavior on effective pheromone release-rates, we compared the tunnel assay response of *T. glabrum* males to exposed vs. concealed pheromone sources. Standard bioassay filter disks were used as pheromone sources, and were tested in the concealed condition by placement inside standard 2×2-cm corrugated fiberboard squares, after detaching the center fiberboard flute from its backing. Again, open flutes were positioned perpendicular to the airflow.

RESULTS AND DISCUSSION

Diel biological rhythms, especially when they facilitate sexual union, are generally presumed to provide the users certain, obvious adaptive advantages. Coincident male–female activity rhythms in a chemically mediated mating system, for example, might tend to minimize mate-seeking energy expenditure in terms of locomotion and biosynthesis of sex pheromones, which could ultimately maximize reproductive success. Such patterns might also tend to minimize the chances of mortality from predation or the physical environment.

What we see in certain aspects of the adult behavior of these three *Trogoderma* species, at least under one set of laboratory conditions, is an apparent tendency to optimize chemical communication conditions for mating. A probable scenario for premating activities of these species, based on current knowledge of behavior and reproductive biology, can be developed as follows. Males remain inactive, concealed, and nonresponsive to sex pheromone stimulation until approx. 3–4 days postpupal eclosion (Figures 1A, 2A, 3A). From 2 to 6 days of age, sex pheromone sensitivity increases, then remains relatively constant for the remainder of the males' lives (Figure 4). Sensitivity also has a broad diel peak, which is about 2 log units greater than the daily minimum (Figure 5). This agrees roughly with the findings of Vick et al. (1973) for male responses to female extracts of unknown aldehyde or alcohol content. Peak sensitivity occurs during the age- and species-dependent period in which males are most likely to leave concealment (peaking at lights-on for *T. variabile*, 7 hr after lights-on for *T. inclusum*, and 8 hr after lights-on for *T. glabrum*), presumably to create more favorable

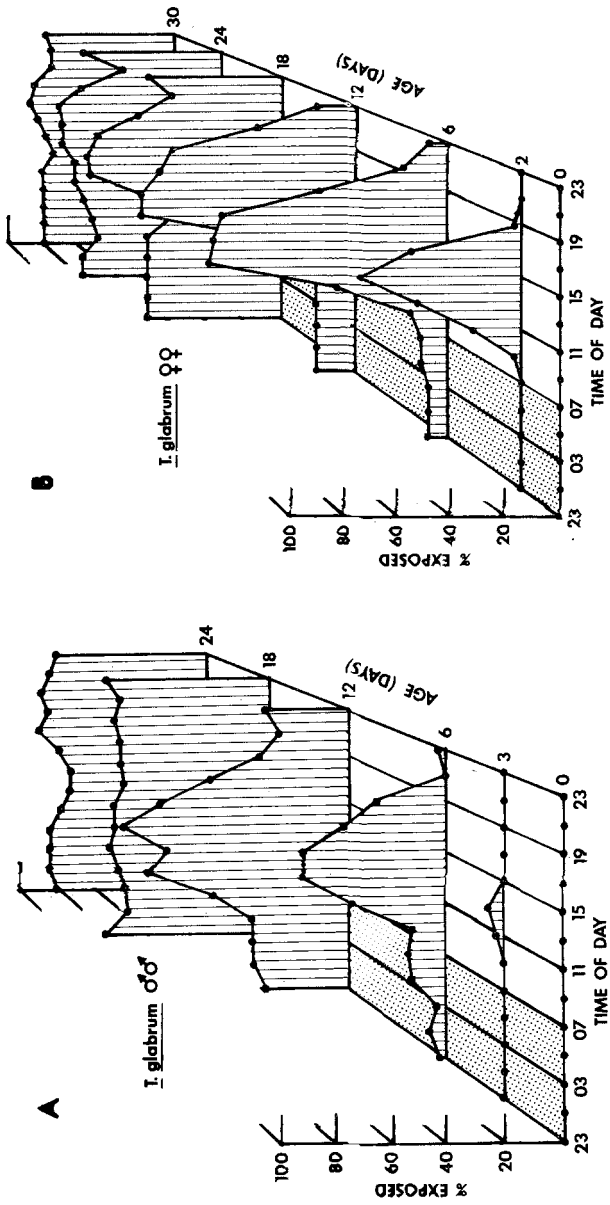

FIG. 1. Exposure–concealment behavior in virgin *Trogoderma* males (A) and females (B), with respect to age and time of day. Points represent average percentages of males or females exposed (from populations of 50 for each sex and species, held 10 per petri dish), when observed under standard environmental conditions. Shaded areas (2300–0700 hr) denote scotophase.

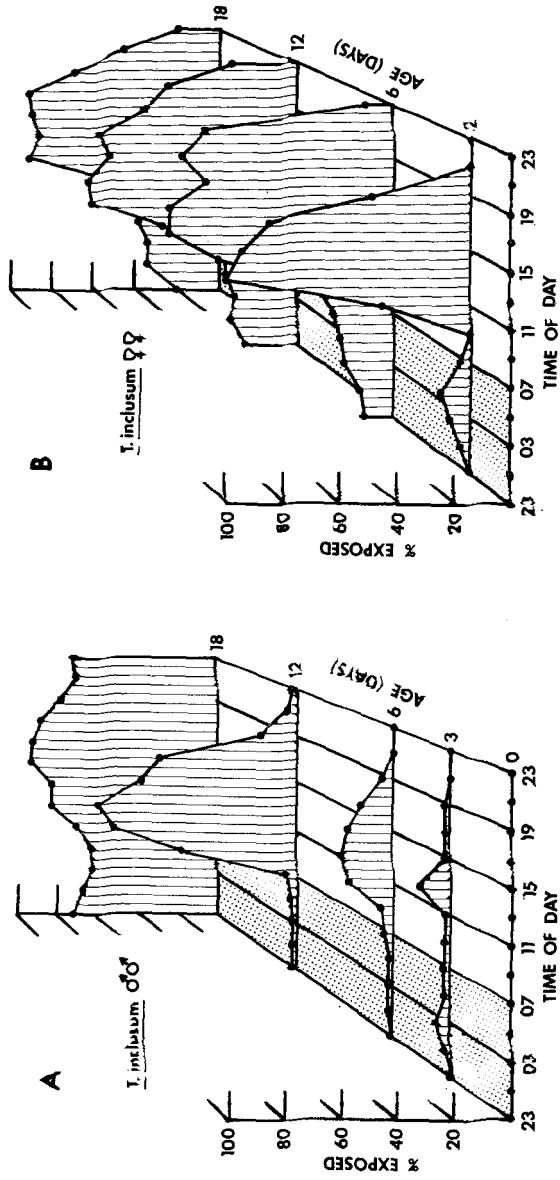

FIG. 2. Exposure–concealment behavior in virgin *Trogoderma* males (A) and females (B), with respect to age and time of day. Points represent average percentages of males or females exposed (from populations of 50 for each sex and species, held 10 per petri dish), when observed under standard environmental conditions. Shaded areas (2300–0700 hr) denote scotophase.

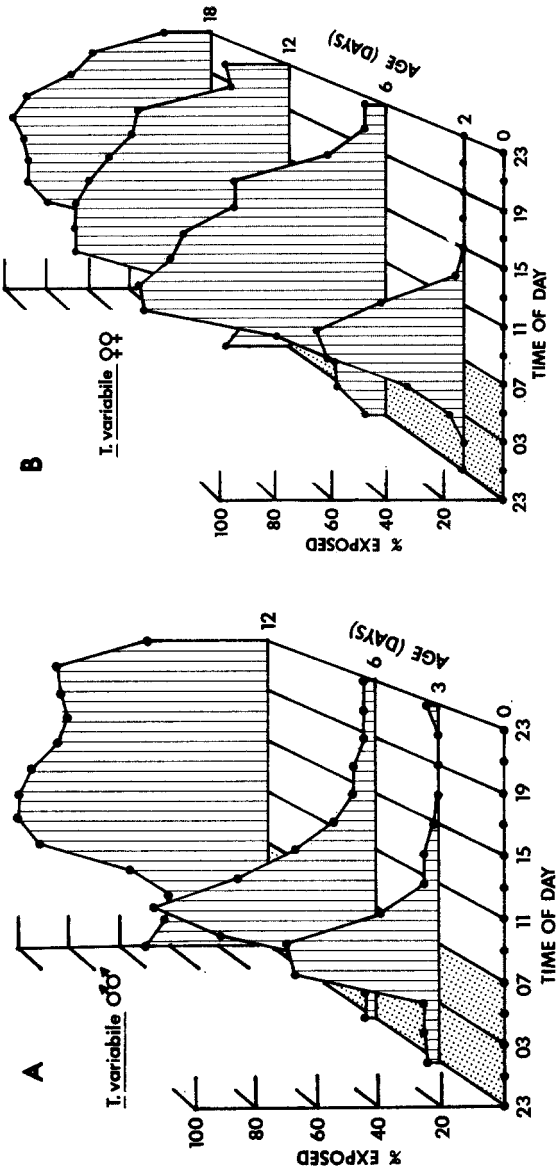

FIG. 3. Exposure–concealment behavior in virgin *Trogoderma* males (A) and females (B), with respect to age and time of day. Points represent average percentages of males or females exposed (from populations of 50 for each sex and species, held 10 per petri dish), when observed under standard environmental conditions. Shaded areas (2300–0700 hr) denote scotophase.

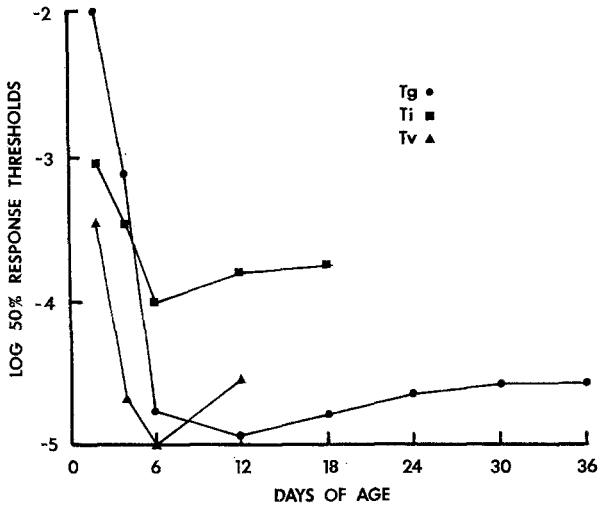

FIG. 4. Sensitivity of *Trogoderma* males to sex pheomone with respect to age. Each point represents the 50% arousal threshold as determined by probit analysis of vial bioassay re sponses to (*E*)- or (*Z*)-trogodermal.

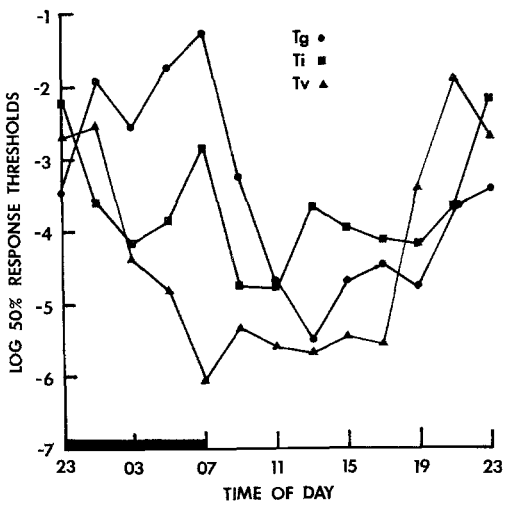

FIG. 5. Sensitivity of *Trogoderma* males to sex pheromone with respect to time of day. Each point represents the 50% arousal threshold as determined by probit analysis of vial bioassay responses to (*E*)- or (*Z*)-trogodermal.

conditions for receiving female sex pheromone signals. Such exposure may also increase energy expenditure and risks from predation and the physical environment. Although male pheromone sensitivity is maintained until death, locomotory abilities deteriorate rapidly during the last quarter of life.

Females remain inactive and concealed until 2–3 days of age, after which occur daily exposure periods coinciding with those of conspecific males (Figures 1B, 2B, 3B). The percentage of females exposed at any hour increases slightly with age, but is not as age-dependent as male exposure behavior. During exposure periods, females seek elevated locations and assume characteristic "calling" postures during which sex pheromone is released (Hammack et al., 1976). For *T. glabrum*, sex pheromone release rates are at least 10^4 greater during than before calling, and are gradually reduced after calling (Shapas and Burkholder, 1978).

Under our laboratory conditions (27°C, 50% relative humidity, 16:8 light/dark photoperiod), the three species we have studied have daily behavioral patterns which would tend to maximize male–female encounters at mid-afternoon (7–8 hr after lights-on) for *T. glabrum* and *T. inclusum*, and at dawn (lights-on) for *T. variabile*.

While *T. glabrum* and *T. inclusum* have nearly coincident exposure periods, they utilize opposite isomers of trogodermal as their long-distance sex attractants. This feature may prevent cross-attraction, even though both species may be sexually active at the same time of day.

An interesting aspect of the exposure–concealment behavioral patterns observed in both sexes is its age dependency. If mating does not take place, duration of exposure periods increases with age, implying a possible trade-off of greater risk-taking and/or energy expenditure vs. an increased probability of successfully sending or receiving sex pheromone signals.

We have not attempted to explore the nature of the observed daily patterns of exposure–concealment, except to report their occurrence and possible significance regarding pheromone-mediated communication distances. Possibly we are observing activity rhythms which in some way use the photoperiod to provide *Zeitgebers* for temporal placement. Because temporal placement of sex pheromone-releasing behavior in *T. glabrum* females is circadian in nature and is at least partially regulated by photoperiod (Hammack and Burkholder, 1976), it would be surprising not to find similar controls for exposure–concealment behavior. The presence of light at some time during exposure periods may also provide direction for dispersal, which as shown for other Dermestidae (Butler and Hunter, 1969), may also be an age-dependent phenomenon.

Using airborne chemical communication models (Bossert and Wilson, 1963; Sower et al., 1973), we have estimated the relative effects of rhythmic changes in male pheromone sensitivity and exposure–concealment behavior

on theoretical communication distances. At moderate air velocities, the communication distance x for the *Trogoderma* sex pheromone trogodermal can be predicted fairly accurately in a wind-tunnel environment by equation (1) (Shapas and Burkholder, 1978; after Bossert and Wilson, 1963),

$$x = \left(\frac{21Q}{vK}\right)^{0.571} \tag{1}$$

where Q is the pheromone release rate, v is the air velocity, and K is the pheromone concentration in air necessary for male arousal.

As shown in Figure 5, male pheromone sensitivities reach daily minima which increase their arousal threshold K by a factor of approximately 100. Concealment of males effectively raises K also by a factor of approximately 100 as shown in Figure 6. Concealment of the pheromone source, which we here consider to accurately represent a pheromone-releasing female, effectively reduces the pheromone release rate Q by a factor of approximately 10 as shown in Figure 7. Periods of female concealment would also be accompanied

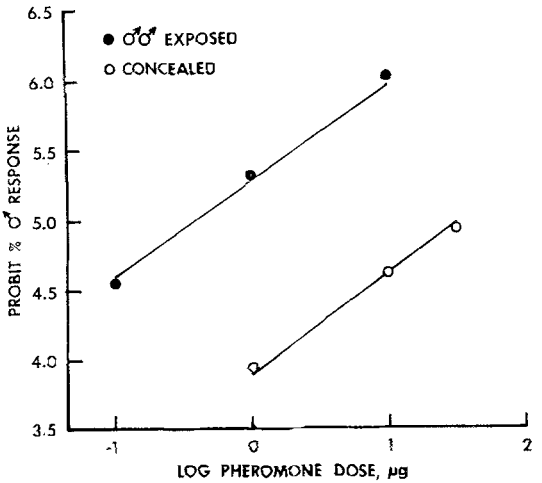

FIG. 6. Effects of exposure–concealment behavior of males on effective response thresholds. Filter-disk pheromone sources were used to compare the wind-tunnel bioassay responses of exposed vs. concealed 6- to 7-day-old *T. glabrum* males. A 2×2-cm corrugated fiberboard substrate was used for male concealment. Assays were run during late morning, a period during which males were normally concealed.

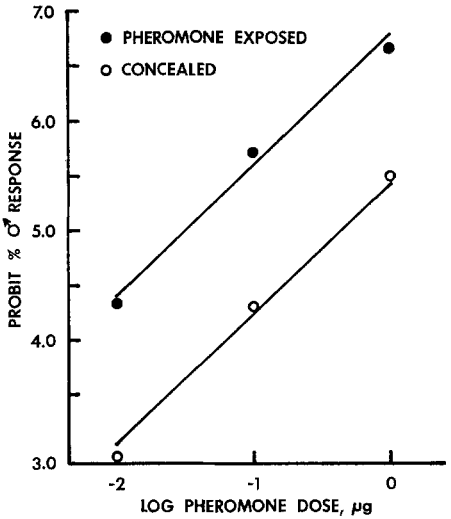

FIG. 7. Effects of exposure–concealment of the pheromone source on effective release rates. The wind-tunnel bioassay response of 6- to 8-day-old *T. glabrum* males to filter disks releasing (*E*)-trogodermal was compared with the response to similarly treated disks which were "concealed" inside 2 × 2-cm. corrugated fiberboard substrates.

by a real 10^4 decrease in pheromone release, (Shapas and Burkholder, 1978), which is probably the key factor in delimiting attraction distances. If we consider a sex pheromone communication distance x between a female pheromone source and male responder, concealment of either male or female has an effect on the model of decreasing x by 93 or 73%, respectively. If both are concealed, x has been reduced by 98%, and if male sensitivity is also at its daily minimum, x is further reduced to 0.14% of its maximum value. Further, if the female is at minimum emission rate, x is reduced to 0.0007%. Even though *T. inclusum* and *variabile* utilize the same long-distance pheromone component [(*Z*)-trogodermal], for example, calling females would be unlikely to attract other than conspecific males, since when one species is most likely to be releasing sex pheromone, males of the other species are most likely to be concealed and with significantly raised thresholds for arousal. These relationships are illustrated in Table 1, and suggest that exposure–concealment behavior, especially when accompanied by daily changes in olfactory sensitivity, can act as an effective mechanism to limit

TABLE 1. THEORETICAL EFFECTS OF MALE OR FEMALE EXPOSURE OR CONCEALMENT, AND EFFECTS OF MAXIMAL CHANGES IN MALE PHEROMONE SENSITIVITY AND FEMALE PHEROMONE RELEASE ON *Trogoderma* COMMUNICATION DISTANCES[a]

Communicating conditions	Maximal theoretical communication distances (%)
Female (or synthetic source) exposed and releasing pheromone maximally; male exposed and at maximum sensitivity	100
Female (or synthetic source) exposed and releasing pheromone maximally; male concealed and at minimum sensitivity	0.52
Female concealed and releasing pheromone minimally; male exposed and at maximum sensitivity	0.14
Female concealed and releasing pheromone minimally; male concealed and at minimum sensitivity	0.0007

[a] Using communication theory models which have been verified in wind tunnel tests (Shapas and Burkholder, 1978), the relative effects of these factors are compared.

attraction distances. Such temporal isolation would be expected to decrease when densities of either species become high, since distances between individuals would decrease and concealment would no longer provide a barrier to attraction. Presumably age would also effect such isolation, since older populations of *Trogoderma* remain exposed for longer periods than younger populations.

Concealment effects on male arousal thresholds and pheromone-source release rates are probably a result of microclimate modifications in and around the concealment substrates. In the wind tunnel, the concealment substrates probably create a boundary-layer extension or area inside of which air velocities and air exchange with upper layers are reduced (Rosenberg, 1974). If this is true, the effect on concealed males would be to dilute the amount of pheromone reaching them. Similarly, the effect on concealed pheromone sources would be a reduction in the amount of pheromone reaching the main airstream. This may be a result of a real decrease in pheromone released, caused by reduced airflow over the pheromone source (Nakamura and Kawasaki, 1977). The selection of corrugated fiberboard squares as concealment substrates was somewhat arbitrary; however, they approximate the effects of structural cracks and crevasses which *Trogoderma* are known to inhabit.

From the standpoint of using synthetic pheromone sources for monitoring or controlling *Trogoderma* populations, this information implies that attraction of males will be most successful during the exposure period of the

target species. It also suggests that the youngest males, and perhaps those with the greatest reproductive potential, may be difficult to lure due to their relatively brief daily periods of exposure.

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SYNERGISTIC INHIBITORY EFFECTS OF VANILLIC AND *p*-HYDROXYBENZOIC ACIDS ON RADISH AND GRAIN SORGHUM

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Abstract—Radish and grain sorghum germination and sorghum growth were inhibited in a synergistic manner by combinations of vanillic and *p*-hydroxybenzoic acids. At threshold inhibition levels, 2.5×10^{-3} M vanillic acid-treated radish seeds had 71% of control germination after 24 hr and 2.5×10^{-3} M *p*-hydroxybenzoic acid-treated radish yielded 95% germination. A mixture of 2.5×10^{-3} M of each of these two phytotoxins showed 52% germination after 24 hr. Equimolar mixtures of 5×10^{-3} M vanillic and *p*-hydroxybenzoic acids allowed sorghum germination of 60% of untreated seeds after 24 hr, whereas separate treatments of individual phenols had 93% and 96% of control seed germination. Sorghum root and shoot elongation and total seedling growth were more sensitive than germination to vanillic and *p*-hydroxybenzoic acid treatments, and synergistic effects also were apparent. A combination of 5×10^{-3} M vanillic with 5×10^{-3} M *p*-hydroxybenzoic reduced root length more than either did individually, and a mixture of 5×10^{-4} M vanillic with 5×10^{-4} M *p*-hydroxybenzoic acid reduced sorghum seedling growth to approximately that resulting from a 10^{-3} M concentration of either phenol alone. Phytotoxin levels inhibitory to sorghum growth caused small increases in lower leaf surface diffusive resistance, but did not close stomates, and this effect was not judged to be the cause of reduced sorghum growth.

Key Words—synergism, vanillic acid, *p*-hydroxybenzoic acid, allelopathy, inhibition, sorghum, radish.

INTRODUCTION

Vanillic acid (4-hydroxy-3-methoxybenzoic) and parahydroxybenzoic acid rank high among those compounds most frequently reported as inhibitory

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in soils and as allelopathic agents found in plants. In an early report of soil phenols, *p*-hydroxybenzoic acid was identified (Walters, 1917). Recently, investigators have extracted *p*-hydroxybenzoic and vanillic acids from a variety of soil types and from soils under several cultivation practices (Guenzi and McCalla, 1966b; Hennequin and Juste, 1967; Wang et al., 1967, 1968; McCalla, 1971; Whitehead, 1974). Agronomic crop residues often contain these two phenols as well as several additional phytotoxins (Guenzi and McCalla, 1966a; McCalla, 1971; Patrick, 1971; Chou and Patrick, 1976). Naturally occurring plant species have also been found to leach or release, as part of the decomposition process, both of these phenols (Chou and Muller, 1972; Tinnin and Muller, 1972; Chou and Young, 1975). In tests on radical growth of lettuce, Chou and Patrick (1976) showed the phytotoxicity of *p*-hydroxybenzoic and vanillic acids in concentrations the authors stated to be within the range found naturally.

Plant materials containing other phenolic acids may also contribute to the presence of vanillic and *p*-hydroxybenzoic acids in the rhizosphere due to the decomposition process. Henderson (1963) proposed vanillic acid as a breakdown product of ferulic acid. Turner and Rice (1975) verified this decomposition sequence in the metabolism of *Rhodotorula rubra*, a major soil microorganism they found active in ferulic acid decomposition. Several investigations indicate ferulic acid is a common inhibitory component of allelopathic species or the soil (del Moral and Muller, 1970; Rasmussen and Rice, 1971; Lodhi, 1975; Rice and Pancholy, 1974).

Since vanillic and *p*-hydroxybenzoic acids are of extensive distribution and are often found together in plant and soil, the possibility of their combined inhibitory effects is important. If small amounts of each of these phenols may work together in cooperative inhibitory action, this would increase their potential hazard to plant growth. Evidence of cooperative inhibition from several phytotoxins has been demonstrated by Asplund (1969), Lodhi (1975), and Rasmussen and Einhellig (1977).

Experiments of this project were designed to test the hypothesis that vanillic and *p*-hydroxybenzoic acids in combination may act in additive or synergistic ways in inhibiting germination and seedling growth.

METHODS AND MATERIALS

Radish (Cherry Belle, Gurney's Nursery, Yankton, South Dakota) was chosen for initial germination experiments because of its sensitivity to phytotoxic compounds. All subsequent experiments utilized grain sorghum (Hybrid 701-Gurney's). Sorghum also has been shown to be sensitive to

phenolic compounds (Einhellig and Rasmussen, 1973; Rasmussen and Einhellig, 1975, 1977), and it is an agronomic crop which may be affected by interference from both allelopathic weeds and phenols released from decomposition of crop residue.

Radish seed germination experiments were done in 10-cm petri dishes containing a disk of Whatman No. 1 paper. Five milliliters of test solution were added to each dish and 200 radish seeds were placed in each dish, two dishes per treatment. These experiments monitored seed germination in aqueous solutions of 10^{-2} M, 5×10^{-3} M, and 2.5×10^{-3} M *p*-hydroxybenzoic and vanillic acids (Sigma Chemical Co.). Two combinations of these benzoic acid derivatives were also tested: (1) 5×10^{-3} M *p*-hydroxybenzoic with 5×10^{-3} M vanillic and (2) 2.5×10^{-3} M *p*-hydroxybenzoic with 2.5×10^{-3} M vanillic. The first combination was made by mixing equal amounts of 10^{-2} M *p*-hydroxybenzoic acid and 10^{-2} M vanillic acid. The second combination was made in a similar manner using equal amounts of 5×10^{-3} M of both phenols. Control dishes contained deionized water since prior work had established there was no difference in germination between deionized water and a phosphate buffer medium. Tests were carried out in darkness at room temperature, 26 °C $\pm$ 3 °C. Germination counts were taken at 12, 18, 24, 36, 48, and 60 hr with a 2-mm extrusion of the radical taken as evidence of germination. Germinated seeds were removed from the dishes when counted. This experiment was replicated three times, making a total of 1200 seeds per treatment.

Sorghum seed germination bioassays were similar to that used for radish, except that 15-cm petri dishes were used with 100 seeds and 10 ml test solution per dish, 2 dishes per experimental group. Test solutions in the sorghum bioassay were 10^{-2} M and 5×10^{-3} M vanillic, *p*-hydroxybenzoic, or a combination of these two phenols. The combination dishes were made by adding 5 ml 10^{-2} M vanillic and 5 ml 10^{-2} M *p*-hydroxybenzoic to a petri dish, making an effective concentration of 5×10^{-3} M of each of these two compounds. The sorghum germination experiments were conducted five times.

In two additional replicas of the sorghum germination sequence, but with 50 seeds per petri dish, sorghum shoot and root lengths were measured for all seeds that had germinated after 6 days.

Experiments designed to test the effect of vanillic and *p*-hydroxybenzoic acids on sorghum seedling growth utilized these phenols dissolved in nutrient culture (Floyd and Rice, 1967; Rasmussen and Einhellig, 1977). Sorghum was germinated in vermiculite for 7 days, and then seedlings were individually transplanted to 40-ml light-free vials containing a 2:5 aqueous dilution of Hoagland's nutrient solution (Hoagland and Arnon, 1950). After several days stabilization in the aqueous culture, plants were selected for uniformity and

transferred to nutrient solution containing the appropriate phenols dissolved in the diluted Hoagland's solution. Experimental solutions for sorghum growth were: 10^{-3} M vanillic, 10^{-3} M *p*-hydroxybenzoic, 5×10^{-3} M vanillic, 5×10^{-3} M *p*-hydroxybenzoic, a combination of the two phenols, and a control (diluted Hoaglands). The combination vials were made by mixing equal amounts of 10^{-3} M vanillic and *p*-hydroxybenzoic, making a combination of 5×10^{-4} M vanillic with 5×10^{-4} M *p*-hydroxybenzoic. Sorghum seedlings were grown in a Percival growth chamber at 20,000 lux and 30/18°C day/night conditions. Additional nutrient and test solution generally had to be added on the fourth day. After 7 days, plants were oven dried (48 hr, 105°C) and data analyzed with a *t* test. This experiment was replicated four times.

Einhellig and Kaun (1971) and Kadlec (1973) found several phytotoxic phenols interfered with stomatal function at concentrations that caused stunting of growth. This background indicated leaf diffusive resistance might be a sensitive index of inhibitor effects. Therefore, two additional growth experiments were carried out under summer greenhouse conditions and with diffusive resistance monitored each day for 6 plants per group. Resistance was measured daily between 1 and 3 PM with a diffusive resistance meter, Lambda Instrument Co., model L1-60 and L1-15 sensor fitted with a narrow plate. Because previous work in our laboratory had shown that sorghum seedlings of this size had more stomates on the abaxial surface than the adaxial, only the lower leaf surface was measured as an index of effects.

RESULTS

All three radish germination experiments showed similar trends, and only the combined data are shown in Table 1. For brevity, 12- and 60-hr counts are not shown. Both benzoic acid derivatives strongly reduced radish germination at concentrations of 10^{-2} M (Table 1). Although this inhibition was most pronounced during early germination, there was still considerable depression of germination after 60 hr, whereas over 92% of the control seeds were germinated by 48 hr. Vanillic and *p*-hydroxybenzoic acids at concentrations of 5×10^{-3} M also inhibited germination, with vanillic acid inhibition being the greater as in the 10^{-2} M treatments (Table 1). The experimental group containing a combination of 5×10^{-3} M of each phenol consistently depressed radish germination more than either of these alone, demonstrating the cooperative effect of the two. Germination reduction in this combination approximated the germination rate of 10^{-2} M vanillic acid. Counts in the combination dishes after 36 and 48 hr showed the combination to depress germination more than the added effects of 5×10^{-3} M vanillic

TABLE 1. EFFECTS OF VANILLIC ACID (V-A) and *p*-HYDROXYBENZOIC ACID (PHB-A) ON RADISH SEED GERMINATION[a]

Treatment	Seed Germination											
	18 hr			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	No. germ.	Dif. from control	% of control
Control	614			834			1028			1112		
10^{-2} M V-A	157	(−457)	25.6	294	(−540)	35.3	484	(−544)	47.1	593	(−519)	53.3
10^{-2} M PHB-A	290	(−324)	47.2	452	(−382)	54.2	673	(−355)	65.5	868	(−244)	78.1
5×10^{-3} M V-A	254	(−360)	41.4	397	(−437)	47.6	564	(−464)	54.9	696	(−416)	62.6
5×10^{-3} M PHB-A	436	(−178)	71.0	679	(−155)	81.4	958	(−70)	93.2	1036	(−76)	93.2
Combination: 5×10^{-3} M V-A with 5×10^{-3} M PHB-A	192	(−422)	31.3	271	(−563)	32.5	440	(−588)[b]	42.8	576	(−536)[b]	51.8
2.5×10^{-3} M V-A	458	(−156)	74.6	589	(−245)	70.6	807	(−221)	78.5	958	(−154)	86.2
2.5×10^{-3} M PHB-A	535	(−79)	87.1	795	(−39)	95.3	997	(−31)	97.0	1051	(−61)	94.5
Combination: 2.5×10^{-3} M V-A with 2.5×10^{-3} M PHB-A	251	(−363)[b]	40.9	432	(−402)[b]	51.8	633	(−395)[b]	61.6	763	(−349)[b]	68.6
Reduction of germ. from: 2.5×10^{-3} M V-A and 2.5×10^{-3} M PHB-A (added—not in combination)		(−235)			(−284)			(−252)			(−215)	

[a] Total data of three experiments, 400 seeds/treatment/experiment.
[b] Synergistic effects shown.

TABLE 2. EFFECTS OF VANILLIC and *p*-HYDROXYBENZOIC ACIDS ON SORGHUM SEED GERMINATION

		Seed Germination								
		24 hr			36 hr			48 hr		
Treatment	Exp.	No. germ.	Dif. from control	% of control	No. germ.	Dif. from control	% of control	No. germ.	Dif. from control	% of control
Control	1	97			160			172		
	Total[a]	455			790			853		
10^{-2} M	1	48	(−49)	49.5	99	(−61)	61.9	148	(−24)	86.0
vanillic acid	Total[a]	265	(−190)	58.2	570	(−220)	72.2	745	(−108)	87.3
10^{-2} M	1	68	(−29)	70.1	129	(−31)	80.6	162	(−10)	94.2
p-hydroxybenzoic	Total[a]	337	(−116)	74.4	657	(−133)	83.2	790	(−63)	92.6
5×10^{-3} M	1	88	(−9)	90.7	144	(−16)	90.0	168	(−4)	97.7
vanillic acid	Total[a]	425	(−30)	93.4	770	(−20)	97.5	855	(+2)	100.2
5×10^{-3} M	1	89	(−8)	91.8	160	(0)	100.0	184	(+12)	107.0
p-hydroxybenzoic	Total[a]	435	(−20)	95.6	785	(−5)	99.4	883	(+30)	103.5
Combination:										
5×10^{-3} M vanillic with	1	56	(−41)[b]	57.7	116	(−44)[b]	72.5	146	(−26)[b]	84.9
5×10^{-3} M *p*-hydroxybenzoic	Total[a]	270	(−183)[b]	59.6	636	(−154)[b]	80.5	770	(−83)[b]	90.3
Reduction of Germ. from:	1		(−17)			(−16)			(+8)	
5×10^{-3} M vanillic and 5×10^{-3} M PHB-A (added—not in combination)	Total[a]		(−50)			(−25)			(+32)	

[a] Total data of 5 experiments, 200 seeds each.
[b] Synergistic effects shown.

plus 5×10^{-3} M *p*-hydroxybenzoic (synergism). Vanillic acid at concentrations of 2.5×10^{-3} M caused more inhibition of radish than did 2.5×10^{-3} M *p*-hdroxybenzoic, which approached the rate of controls. However, the combination of these two together consistently caused marked synergistic effects (Table 1).

Sorghum seeds were less sensitive than radish but did show reduction in germination to be caused by either 10^{-2} M vanillic or *p*-hydroxybenzoic acids (Table 2). The depression of germination was greatest in early counts. Both 5×10^{-3} M vanillic and *p*-hydroxybenzoic acids also caused some depression of sorghum germination after 24 hr, but later germination rates were similar to controls with slight stimulation observed in 5×10^{-3} M *p*-hydoxybenzoic-treated seeds after 48 hr. A combination of 5×10^{-3} M treatment of both phenolic acids showed synergistic effects when compared to effects of 5×10^{-3} M vanillic plus 5×10^{-3} M *p*-hydroxybenzoic. The sorghum germination depression in the combination treatment was comparable to that seen in either 10^{-2} M vanillic or *p*-hydroxybenzoic treatments (Table 2).

Whereas the 5×10^{-3} M vanillic and *p*-hydroxybenzoic acid treatments had only small effects on initial germination of sorghum seeds, these phenols

TABLE 3. EFFECTS OF VANILLIC AND *p*-HYDROXYBENZOIC ACIDS ON SHOOT AND ROOT ELONGATION IN GERMINATING SORGHUM

Treatment	Experiment	Mean length[c] (mm±SE)	
		Shoot	Root
Control	1	47.6±2.0	42.1±2.8
	2[d]	50.7±2.4	52.3±3.7
10^{-2} M vanillic	1	22.7±1.4[a]	3.3±0.4[a]
	2	19.2±1.5[a]	1.8±0.3[a]
10^{-2} M *p*-hydroxybenzoic	1	28.5±1.6[a,b]	2.9±0.3[a]
	2	24.2±1.8[a]	2.2±0.3[a]
5×10^{-3} M vanillic	1	26.9±1.7[a]	4.3±0.7[a]
	2	34.0±2.2[a]	6.1±1.0[a,b]
5×10^{-3} M *p*-hydroxybenzoic	1	36.5±2.0[a,b]	14.8±1.5[a,b]
	2	31.2±2.5[a]	11.3±1.8[a,b]
Combination:			
5×10^{-3} M vanillic with	1	23.5±1.5[a]	3.7±0.4[a]
5×10^{-3} M *p*-hydroxybenzoic	2	30.5±1.8[a]	2.8±0.4[a]

[a] Significant deviation from the control at $P < 0.001$.
[b] Difference from combination mean significant at $P < 0.05$, or better.
[c] Measurements based on all seeds germinated out of 100, after 6 days.
[d] Duplicate experiment.

TABLE 4. EFFECTS OF VANILLIC AND *p*-HYDROXYBENZOIC ACIDS ON SORGHUM GROWTH

Treatment	Experiment	Mean[c] oven-dry weights (mg±SE)
Control	1	312.4±18.1
	2[d]	183.1±11.9
10^{-3} M vanillic	1	174.1±15.3[a]
	2	88.0± 6.3[a]
10^{-3} M *p*-hydroxybenzoic	1	212.7±15.9[a]
	2	96.0±10.3[a]
5×10^{-4} M vanillic	1	236.2±17.4[a,b]
	2	135.5± 8.6[a,b]
5×10^{-4} M *p*-hydroxybenzoic	1	240.2±16.0[a,b]
	2	176.3±11.6[b]
Combination:		
5×10^{-4} M vanillic with	1	159.2±15.9[a]
5×10^{-4} M *p*-hydroxybenzoic	2	101.0±10.5[a]

[a] Difference from control mean significant, $P < 0.001$.
[b] Difference from combinations mean significant, $P < 0.05$.
[c] Each mean represents 10 plants, 7 days treatment.
[d] Duplicate experiment.

both caused significant depression of shoot and root elongation (Table 3). Root elongation was more severely depressed than was shoot elongation, and roots generally showed a brownish discoloration. In both the vanillic and *p*-hydroxybenzoic acid treatments there was considerable variation among the roots. Most of the roots were very short, but those which had elongated considerably were threadlike in the segment between tip and base. In both replicas of this experiment, a combination of 5×10^{-3} M vanillic with 5×10^{-3} M *p*-hydroxybenzoic acids reduced root elongation significantly more than either phenolic alone (Table 3). The effect of the combination of phenols can be observed to be similar to separate treatments with either 10^{-2} M vanillic or 10^{-2} M *p*-hydroxybenzoic. Among shoot elongation comparisons, the combination mean was below that of separate 5×10^{-3} M treatments, with this deviation statistically significant in one comparison to 5×10^{-3} M *p*-hydroxybenzoic (Table 3).

Short-duration experiments with the growth of sorghum showed effects on total growth at much lower concentrations of vanillic and *p*-hydroxybenzoic acids than for germination, since both 10^{-3} M vanillic and *p*-hydroxybenzoic acids significantly inhibited seedling growth (Table 4). As in germination experiments, vanillic acid was slightly more inhibitory than

TABLE 5. EFFECTS OF VANILLIC ACID (V-A) AND *p*-HYDROXYBENZOIC ACID (PHB-A) ON DIFFUSIVE RESISTANCE OF SORGHUM LEAVES

Treatment	Resistance[c] (sec/cm±SE)					
	Days after treatment					
	1	2	3	4	5	6
Control	2.8±0.3	2.6±0.3	3.3±0.2	2.0±0.4	2.1±0.3	2.0±0.2
10^{-3} M V-A	4.0±0.4[a]	4.7±0.6[a]	6.5±1.1[a]	5.4±0.6[a]	3.5±0.2[a]	3.1±0.2[a]
10^{-3} M PHB-A	3.0±0.4	4.0±0.5[a]	4.1±0.3[a]	3.2±0.2[a]	3.8±0.1[a]	3.7±0.2[a]
5×10^{-4} M V-A	2.5±0.4[b]	3.0±0.3[b]	3.7±0.2[b]	2.9±0.2[a]	2.9±0.2[a,b]	3.3±0.2[a]
5×10^{-4} M PHB-A	2.5±0.2[b]	3.2±0.3	2.9±0.2[b]	2.8±0.2	3.2±0.3[a]	3.1±0.2[a]
Combination: 5×10^{-4} M V-A with 5×10^{-4} M PHB-A	4.2±0.6[a]	4.5±0.7[a]	5.7±0.9[a]	5.6±1.7[a]	3.6±0.3[a]	5.0±1.4[a]

[a] Significantly different from the control, $P < 0.05$ or better.
[b] Difference from combination mean significant, $P < 0.05$ or better.
[c] Each is the mean of 6 plants, abaxial resistance only.

p-hydroxybenzoic. Growth inhibition was caused by concentrations as low as 5×10^{-4} M of both compounds, but a combination of these two together showed growth depression significantly below either of them separately, an effect that can be considered synergistic (Table 4). While only two replicas are shown in Table 4, similar data were collected in four separate experiments and two experiments where plants were greenhouse grown.

Diffusive-resistance information obtained from plants grown in the greenhouse showed that in most cases both 10^{-3} M vanillic and *p*-hydroxybenzoic acid-treated plants had greater leaf resistance than controls (Table 5). On several days this was also apparent in more dilute treatments. Although each of the concentrations of these phenols was inhibitory to growth, the deviation of diffusive resistance from control values does not suggest much interference with stomatal function and certainly not stomatal closure. Pertinent to our experiments is the fact that a concentration of 5×10^{-4} M of both benzoic acid derivatives caused an effect that was greater than separate treatments with these phenols (Table 5). The duplicate experiment showed similar trends in all respects, with only mild interference of stomatal diffusive resistance by vanillic and *p*-hydroxybenzoic acids that stunted growth. Variations in temperature and light conditions account for the day-to-day variations seen in control plants.

DISCUSSION

When the seed germination data of Tables 1 and 2 are evaluated by comparing the germination inhibition created by a mixture of vanillic and *p*-hydroxybenzoic acids with the action of either phenol alone, it is evident that cooperative inhibitory effects occurred. This synergistic action is most apparent at concentrations on the threshold of inhibition, which was 5×10^{-3} M for vanillic and *p*-hydroxybenzoic acids in the case of sorghum (Table 2) and half of this concentration for radish (Table 1). At these threshold levels, combinations of the two inhibitors produced a much greater effect than either phenolic acid did independently. The synergistic effects of the two benzoic acid derivatives, vanillic and *p*-hydroxybenzoic, are similar to the cooperative action between *p*-coumaric and ferulic acids reported by Rasmussen and Einhellig (1977).

When data of Table 3 are compared with data from Table 2, it is evident that sorghum root elongation is much more sensitive to vanillic and *p*-hydroxybenzoic acids than is seed germination. This is suggested by the fact that 5×10^{-3} M concentrations of these phenols had mild and temporary effects on germination, but caused severe stunting of roots. Additional differences in sensitivity are noted when comparisons are made to growth experiments (Table 4). Sorghum seedling growth is affected by about one tenth (5×10^{-4} M), or less, of the quantities of vanillic and *p*-hydroxybenzoic acids required to inhibit seed germination. Rasmussen and Einhellig (1977) reported also that seedling growth was more sensitive than germination to phytotoxic effects from phenols. As seen in germination, elongation of shoots and roots and seedling growth (Tables 3 and 4) showed synergistic effects when combinations of vanillic and *p*-hydroxybenzoic were utilized together.

We initially hypothesized that concentrations of these phytotoxins that reduced growth would cause stomatal closure. Einhellig and Kuan (1971) and Kadlec (1973) found scopoletin, chlorogenic acid, caffeic acid, and *p*-coumaric acids all to cause stomatal closure at levels inhibitory to growth. Although both 10^{-3} M vanillic and *p*-hydroxybenzoic acids did cause an increase in lower-surface leaf resistance (Table 5), the higher resistances did not approach values obtained when sorghum stomates are closed, as observed both in our laboratory and reported by Beardsell and Cohen (1974). We doubt that the observed increase in resistance has biological significance in limiting gas exchange to the extent of interfering with growth. It does give some evidence of stress, and the cooperative action of 5×10^{-4} M vanillic and *p*-hydroxybenzoic acids together is of note.

While our experiments suggest stomatal closure is not the cause of vanillic and *p*-hydroxybenzoic acid-induced growth inhibition in sorghum, they do not indicate the mechanism(s) involved. Glass (1973) found even

5×10^{-5} M vanillic acid reduced uptake of phosphate ions by barley roots by 24%, with stronger concentrations further reducing uptake. Glass (1973) found 5×10^{-4} M *p*-hydroxybenzoic acid to be even more inhibitory to phosphate ion uptake than 5×10^{-4} M vanillic. Both of these phenols caused loss of membrane potential and allowed nonselective loss of both anions and cations (Glass, 1974). He hypothesized this general increase in permeability could account for the allelopathic nature of these compounds. Demos et al. (1975) reported neither vanillic nor *p*-hydroxybenzoic affected mitochondrial respiration, although they did reduce hypocotyl growth. Vanillic acid inhibited mitochondrial Ca^{2+} uptake, but *p*-hydroxybenzoic acid did not. Demos et al. (1975) concluded that *p*-hydroxybenzoic growth inhibition was not due to adverse effects on mitochondria, but vanillic did influence metabolic processes of the mitochondria.

Exact mechanisms of inhibitory action for both vanillic and *p*-hydroxybenzoic acids on germination and growth are not certain. These two phenolic acids need not even act in the same ways. Further, their inhibitory roles in processes of germination could be quite different from their phytotoxic activity on growth. In any case, the synergistic action of vanillic and *p*-hydroxybenzoic acids on germination in radish and germination and growth in sorghum is important to practical considerations in allelopathy. It suggests that even if small concentrations of individual phytotoxins are present in the soil, they may act cooperatively in natural communities and agronomic fields.

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LINALOOL IN MANDIBULAR GLAND SECRETION OF *Colletes* BEES (HYMENOPTERA: APOIDEA)[1]

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Abstract—Linalool was identified as the main component of the mandibular gland secretion in females and males of four species of *Colletes* bees, viz., *C. cunicularius*, *C. daviesanus*, *C. impunctatus* and *C. succinctus*. It was also shown to be the dominant volatile compound in the same gland in male *C. floralis* (the female of this species has not yet been investigated). Further, in another species, *C. similis*, linalool is present in the mandibular gland secretions of females and males, but the dominant volatile component in these secretions is geranial, together with neral. Females of the species *C. fodiens* seem to lack monoterpenes altogether; nonadecane is the dominant volatile compound of the cephalic secretion (based on analysis of a single individual). When linalool is put out in the area of nest aggregation of *C. cunicularius*, where the males are "patrolling", a distinct increase in flight activity is noted. The function of linalool is discussed on the basis of field observations.

Key Words—*Colletes* spp., bees, mandibular gland secretion, linalool.

INTRODUCTION

The genus *Colletes* Latr. belongs to the family Colletidae. This family is considered to contain the most primitive bees in the superfamily Apoidea. *Colletes* is found in the Holarctic and the Ethiopian regions. We have analyzed the volatile cephalic secretion from seven of the eight species occurring in Sweden.

[1] This report forms part XXI in the series "Studies on Natural Odoriferous Compounds."

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METHODS AND MATERIALS

Collection and Taxonomy of the Bees

All bees used in this study, except *C. impunctatus* Nyl., were collected on Öland, an island near the Swedish mainland in the southern Baltic. The specimens of *C. impunctatus* were collected in Abisko, Torne Lappmark, in the northernmost part of Sweden.

Preparation of Glands and Techniques of Chemical Analyses

Analyses were made on whole heads. The mandibles were pulled out of their attachments to expose the mandibular glands. In 1971 analyses of *C. cunicularius* L. were made from hexane extracts of heads. In order to localize the emanation of the secretion, the mandibular glands were first dissected under the microscope and analyzed together with the mandibles. In the next analyses, we tested the rest of the head, which had been kept in a freezer during the interval. The chemical techniques and equipment used in this study have been described earlier (Ställberg-Stenhagen, 1972; Bergström, 1973; Bergström and Tengö, 1974). A combined capillary gas chromatograph/mass spectrometer (LKB-2091) served as the main instrument.

Deposition of Material

Specimens, gas chromatograms, and mass spectra from this study are deposited at the Ecological Station on Öland.

RESULTS

The volatile secretion emanating from the mandibular glands has been analyzed from seven species of *Colletes* bees (Table 1). In five of these, both females and males were studied, whereas in the other two, only one sex has been analyzed so far. Five of the investigated species contained the acyclic monoterpene alcohol linalool (3, 7-dimethyl-1, 6-octadien-3-ol) as the dominating volatile compound. In *C. similis*, geranial, neral, and linalool were present in about equal amounts, the main component being geranial. This cooccurrence is interesting since the two aldehydes may be obtained, biosynthetically, from linalool by oxidation. Only one female *C. fodiens* specimen has been studied, but this species appears to lack monoterpenes altogether. Nonadecane was found by this analysis to be the dominating compound.

Table 2 summarizes the chemical findings. Volatile secretions of both

TABLE 1. ANALYSES OF VOLATILE COMPOUNDS FROM MANDIBULAR GLANDS OF *Colletes* 1968–1977

Colletes species	Sex	Combined capillary gas chromatography/mass spectrometry[a]
cunicularius	♀	1968:1
		1974:3[b]
	♂	1968:1
		1969:2
		1974:5[c]
daviesanus	♀	1975:1
	♂	1974:4
floralis	♂	1974:3[d]
fodiens	♀	1975:1
impunctatus	♀	1975:1
	♂	1975:3
similis	♀	1974:1
		1975:2
	♂	1974:3
succinctus	♀	1977:2
	♂	1975:3

[a] All refer to preparations of single individuals. Whole heads were used except in 3 cases—see [b] and [c]. Figure after the year gives the number of separate analyses.
[b] In two analyses only mandibular glands.
[c] In one analysis only mandibular glands.
[d] In one analysis 2 males were used.

TABLE 2. MAIN COMPOUNDS IDENTIFIED IN THE MANDIBULAR GLAND SECRETION OF *Colletes*[a]

	C.c.		C.d.		C.fl.	C.fo.	C.i.		C.si.		C.su.	
	♀	♂	♀	♂	♂	♀	♀	♂	♀	♂	♀	♂
Monoterpenes												
Linalool	*x*[b]	*x*	*x*	*x*	*x*		*x*	*x*	x	x	*x*	*x*
Neral									x	x		
Geranial									*x*	*x*		
Straight-chain hydrocarbons												
Nonadecane						*x*						
Heneicosene	x	x	x	x								
Heneicosane	x	x	x	x		x						
Tricosene			x	x	x			x			x	x
Tricosane		x	x	x		x	x				x	x

[a] C.c. = *Colletes cunicularius* (L.); C.d. = *C. daviesanus* Sm.; C.fl. = *C. floralis* Ev.; C.fo. = *C. fodiens* (Fourcr.); C.i. = *C. impunctatus* Nyl.; C.si. = *C. similis* Schck.; C.su. = *C. succinctus* (L.).
[b] Dominating components are in italics.

male and female *C. cunicularius* heads were analyzed previously in 1968 and 1969, but the results have not been reported earlier. The 1974 and 1975 analyses agreed with the earlier results. This indicates the constancy of the secretional composition. In addition to combined GC-MS, three separate capillary gas chromatographic analyses were made of *C. daviesanus* in 1973. These supported the GC-MS results fully.

Together with the monoterpenes, straight-chain saturated and mono-unsaturated hydrocarbons are present in the mandibular gland secretion. The hydrocarbon pattern differs slightly from species to species. The odd numbered hydrocarbons from C_{15} to C_{25} have been identified in some of the species; C_{21} and C_{23} are the most common ones. The presence of small amounts of some sesquiterpenes is indicated in *C. cunicularius* and *C. floralis*. They may emanate from flowers visited by the bees but also showed up in a preparation of mandibular glands from a *C. cunicularius* male. *C. impunctatus* and *C. succinctus* seem to contain smaller amounts of linalool than *C. cunicularius*, *C. daviesanus*, and *C. floralis*. This may be due to the condition of the bees, at least for *C. impunctatus*, which was transported from Abisko in northern Sweden. For *C. cunicularius*, the amount of linalool per individual is around 10 μg.

The gas chromatograms presented here (Figures 1–6), were all made with a glass capillary column with OV-101 as stationary phase. Sharp vertical lines in the gas chromatograms indicate mass spectral recordings. The

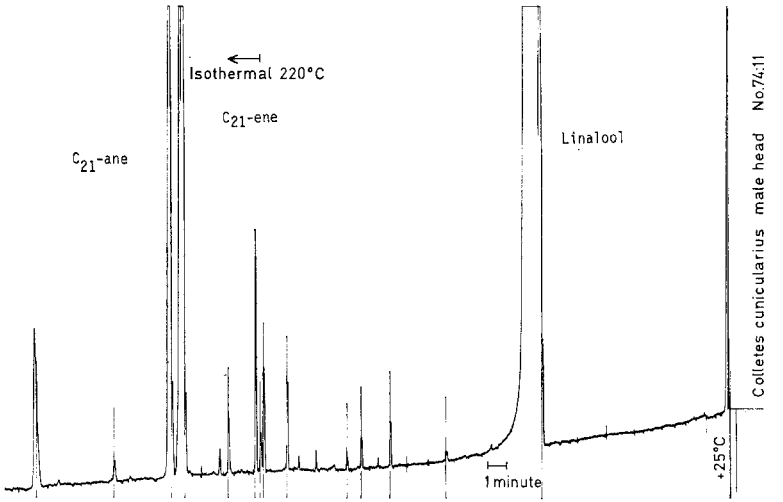

FIG. 1. Capillary gas chromatogram of volatile compounds from the head of one female *C. cunicularius*.

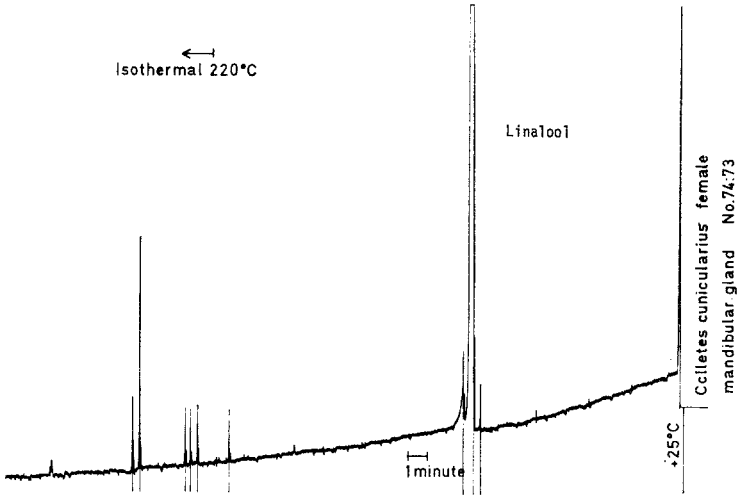

FIG. 2. Capillary gas chromatogram of volatile compounds from the mandibular gland of one female *C. cunicularius*.

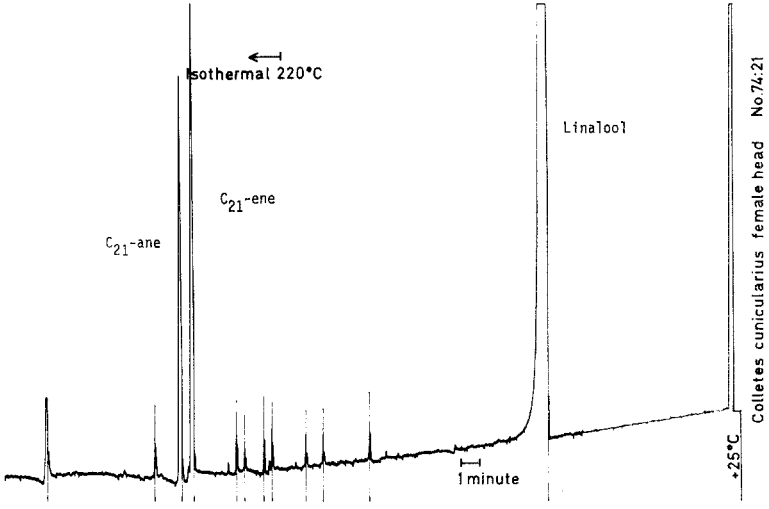

FIG. 3. Capillary gas chromatogram of volatile compounds from the head of one male *C. cunicularius*.

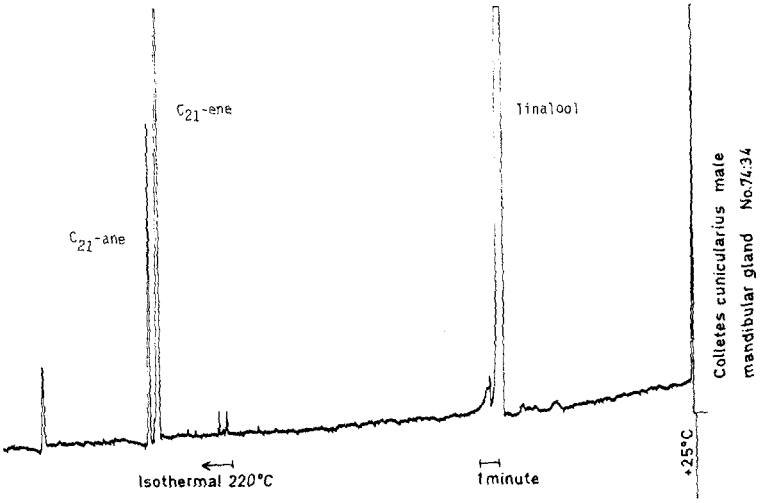

FIG. 4. Capillary gas chromatogram of volatile compounds from the mandibular gland of one male *C. cunicularius*.

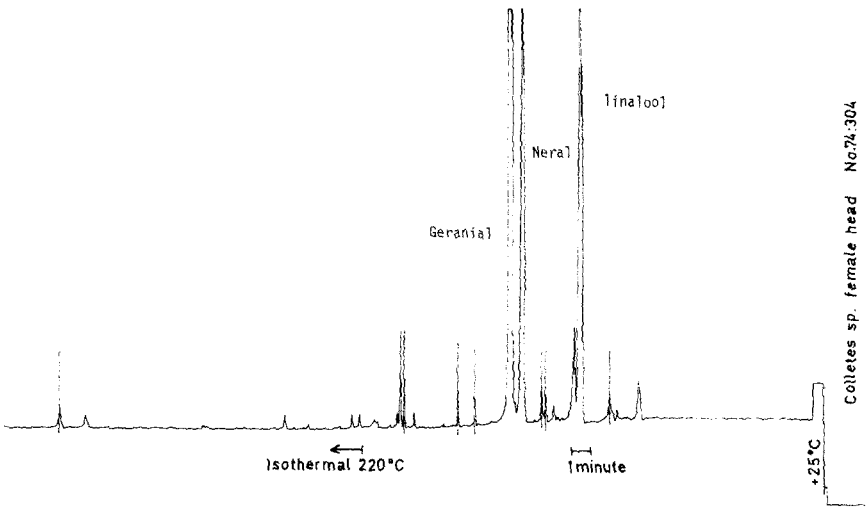

FIG. 5. Capillary gas chromatogram of volatile compounds from the head of one female *C. similis*.

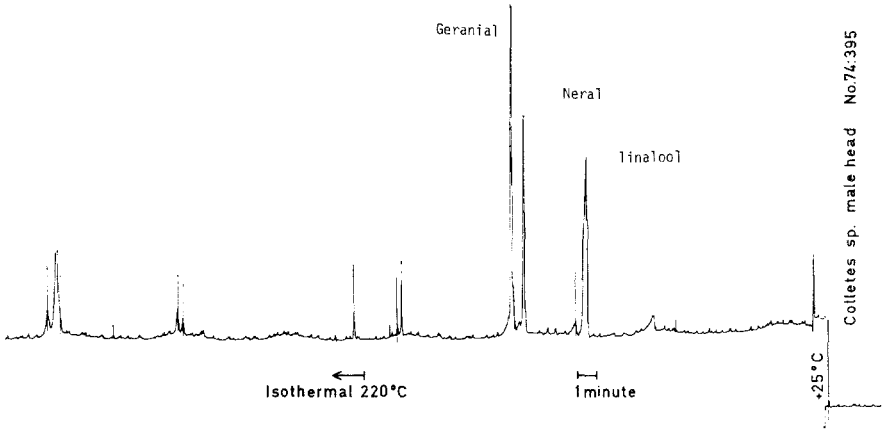

FIG. 6. Capillary gas chromatogram of volatile compounds from the head of one male *C. similis*.

temperature was programed from 25 to 220°C, 8°/min. Linalool (M = 154) was identified through its mass spectrum, with the fragment $m/e = 71$ as a particularly indicative character, and by its capillary gas chromatographic retention time. Both the mass spectrum and the retention time of the natural compound were compared with those of a sample of synthetic linalool and

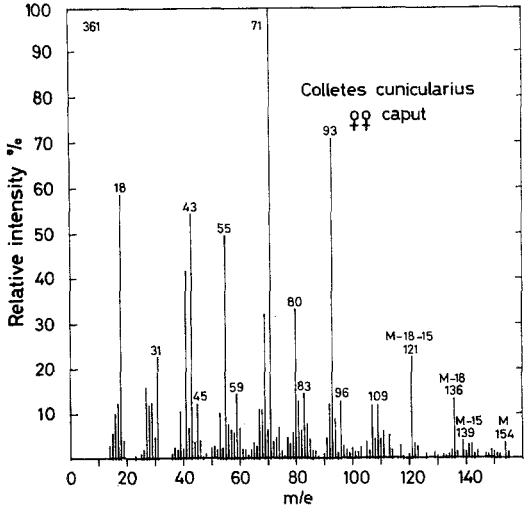

FIG. 7. Mass spectrum of natural linalool from *Colletes cunicularius*.

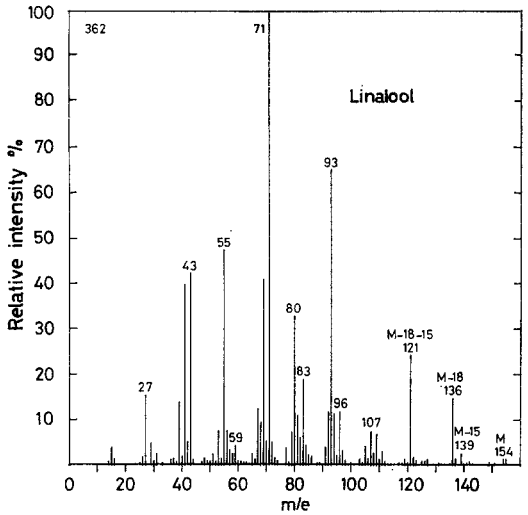

FIG. 8. Mass spectrum of synthetic linalool.

found to be identical. Mass spectra of natural (*Colletes cunicularius*) and synthetic linalool are given in Figs. 7 and 8.

By comparison of Figures 1 and 2, and 3 and 4, it is demonstrated that the volatile cephalic secretion emanates from the mandibular glands. For the analyses shown in Figures 1 and 3, whole heads were put on a precolumn tube of the GC, whereas in the analyses shown in Figures 2 and 4, prepared whole mandibular glands were used in the same way. From these figures it can be seen that not only linalool but probably also the hydrocarbons come from the mandibular gland.

Figures 5 and 6 show typical capillary gas chromatograms from the species *C. similis*, which has about equal amounts of linalool, neral, and geranial.

The males of *C. cunicularius* "patrol" the area where the nests are aggregated. A velvet dummy, 11 times 6 mm, was dipped into a 1% solution of linalool in hexane and concealed within a piece of green tulle. When such a dummy was placed in the male-patrolled area, a distinct and increased male behavioral activity was released. Very few males were resting on the ground. All flying ones cruise more rapidly than normally to and fro in a seemingly undirected and excited manner. The experiment was repeated on several occasions giving the same result. Kullenberg (personal communication) observed the same phenomenon when exposing hexane extracts of flower labella of early-flowering *Ophrys* (Orchidaceae) forms of the "form-spectrum arachnitiformis-sphecodes (s. l.)" (cf. Sundermann, 1975, pp. 87–95).

DISCUSSION

In Colletidae, (salivary) secretions from glands debouching from the head are considered to be used in the coating or lining of the nest cells (Malyshev, 1927), in which the larvae of the bee are bred on a stored amount of a pollen and nectar mixture. It is not known from which gland this secretion originates. Some workers have observed a strong odor when nest cells are opened (Enslin, 1922; Scheloske, 1974). This odor may be emitted from the larva or the adult bee in the nest cell, from the stored food, or from glands of the nest-building female. Nest odors are of significance in solitary bee behavior (Butler, 1965). In bee genera where the female has a large Dufour gland, the secretion of this gland is exploited for nest cell lining (de Lello, 1971; Batra, 1972; May, 1974). Females of *Colletes* bees have large Dufour glands, which are well filled with secretion during the nest building phase of the bee. It has been shown to contain macrocyclic lactones (Bergström, 1973) as main components. The corresponding hydroxy acids could

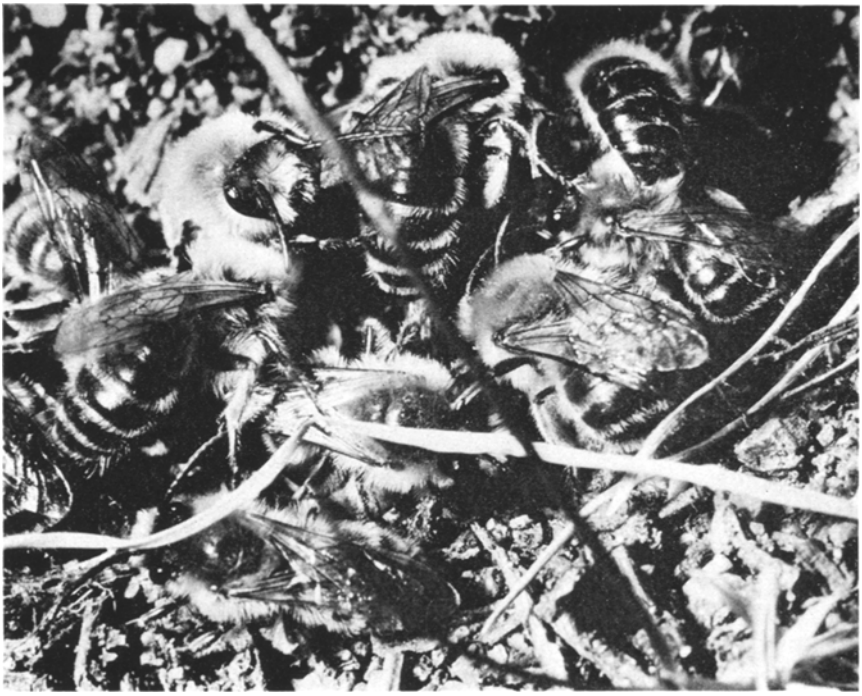

FIG. 9. Aggregation of *C. cunicularius* males digging for an emerging female. (Photo B. Kullenberg.)

FIG. 10. *C. cunicularius* in copula. (Photo B. Kullenberg.)

form a hydrophobic cell lining by polymerization (Bergström and Tengö, 1974).

Bees of the genus *Colletes* very often build their nests in aggregation. Males fly and hover over such nesting places (Rau and Rau, 1916; Rau, 1923; Stephen, 1954). In *C. cunicularius* (Malyshev, 1927, and our own observations), *C. succinctus* (Jones, 1930), *C. stepheni* (Hurd and Powell, 1958), and *C. halophila* (Guichard, 1974), the males, which emerge some weeks or days before the females, congregate in clusters on spots in the nest area, often eagerly digging (Figure 9). They "help" females to emerge from ground and immediately copulate with them. Observations suggest that odor is the signal that attracts the males as they assemble by flight without any contact with the ground, which might transmit vibrations from the female. Visual stimuli are also involved and resting males as well as other insects on the ground are pounced upon by the males. We have observed fist-sized balls of up to 50 males of *C. cunicularius* around one emerging female. This behavior has also been seen by Kullenberg (personal communication) and is reported for *Centris pallida* by Alcock et al. (1976). A typical aggregation of digging males

is shown in Figure 9. This behavior leads to one male succeeding in copulating with the female (Figure 10). Even if small groups of males can build up around a resting male, they disperse after a few seconds. In connection with sexual excitation, the bees emit the odor of the cephalic secretion, and thus the cluster of males around a female gives off a strong smell. Observations regarding odors emitted by *Colletes* bees when copulating were reported by Kullenberg (1956). Of the species studied here, in addition to the two mentioned above, *C. daviesanus* was also observed to nest in aggregation.

Odor involved in mating behaviour has significance in species separation. When two or more species have, for instance, different phenology or different mating habitat, the species specificity of the odor makes no difference.

Concerning the species studied here, our knowledge of the choice of habitat for different activities in the different species does not provide an explanation of species separation. *C. succinactus* and *C. fodiens* prefer to build their nests in sand, while *C. daviesanus* chooses harder substrates such as sandstone, claywalls or hard clayey soil. *C. succinctus* is only found on heath land with *Calluna vulgaris*, but *C. fodiens*, *C. similis* and *C. daviesanus* also occur at the edge of the heath (Richards, 1937). *Chrysanthemum vulgare* and *Achillea millefolium* seem to be of great importance as food flowers, especially to *C. floralis*, *C. daviesanus*, and *C. similis*, but are also visited by *C. impunctatus*. On the basis of morphological studies, Noskiewics (1936) considers that there are close relationships between *C. fodiens*, *C. similis*, and *C. daviesanus* and between *C. impunctatus* and *C. floralis*. In some localities on Öland, *C. similis* is found together with *C. daviesanus*, in others together with *C. fodiens* or *C. floralis*. Species which have been found to have the same composition of their mandibular gland secretion may be separated by minor constituents, too small to be identified with the GC-MS technique. Further studies of their behavior may reveal other species-isolating mechanisms.

Linalool is a widespread compound in plants. Both optical isomers occur in nature. The R form is, for instance, found in large amounts in oil of *Coriander* and the S form in the oil of rosewood. The absolute configuration of linalool was determined by Prelog and Watanabe (1957). They concluded that (—) -linalool has the S configuration. It has recently been shown (Parliment and Scarpellino, 1977) that linalool, together with *cis*-3-hexenol, is essential to the characteristic flavor of blueberry. In the present work we have not had sufficient biological material for determination of the absolute configuration. It is not surprising that linalool has also been found in insects. Silverstein et al. (1973) identified it in volatiles produced by *Ips paraconfusus* females and from frass produced by *I. pini* males. Linalool, together with some other monoterpenes, has earlier (Hamamura, 1965) been reported to attract the silkworm, *Bombyx mori*.

Linalool has a relatively high vapor pressure and therefore it ought to

evaporate rather quickly. The mandibular glands of one individual are estimated to contain around 10 μg in the female and less in the male. If this is given off all at once, quite high concentrations could be obtained for a short time, either in the air after a flying bee, deposited on an object like a leaf or a flower, or secreted by an emerging bee in the ground. Linalool per se is a fairly common compound and therefore not ideal as a highly specific volatile signal. The context of the secretion, as well as additional substances, could of course make the signaling more selective.

Compared to the results of analyses of volatile cephalic secretions of *Andrena* bees (Tengö and Bergström 1976, 1977), we can say that the latter have been found to contain more substances than those found in *Colletes*. On the other hand, no single compound was so dominant and present in such amounts as the linalool of *Colletes*. Perhaps the need for species-characteristic secretions is higher in *Andrena* than in *Colletes*.

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INTERSPECIFIC RESPONSE TO SEX PHEROMONES, AND CALLING BEHAVIOR OF SEVERAL *Attagenus* SPECIES (COLEOPTERA: DERMESTIDAE)[1]

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Abstract—Females of several *Attagenus* species demonstrated calling behaviors similar to those previously reported for *A. elongatulus*. *Attagenus rufipennis* females did not call until they were 12–14 days old. Other *Attagenus* species called at approx. 4 days of age. All species exhibited diurnal periods of calling activity. Interspecific pheromone responses were tested, with *A. megatoma megatoma*, *A. megatoma canadensis*, *A. megatoma japonicus*, and *A. schaefferi spurcus* showing equal cross-responses. Male response to females was demonstrated in *A. rufipennis* and *A. bicolor*. *Attagenus rufipennis*, *bicolor*, and *elongatulus* males responded only to female extracts of their own species.

Key Words—Coleoptera, Dermestidae, *Attagenus megatoma*, *canadensis*, *japonicus*, *schaefferi spurcus*, *bicolor*, *rufipennis*, *elongatulus*, behavior, sex pheromone, calling, bioassay, black carpet beetle, megatomoic acid.

INTRODUCTION

The first reported pheromone in the genus *Attagenus* was demonstrated by Burkholder and Dicke (1966). They described the response of male black

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carpet beetles [*A. megatoma megatoma* (F.)] to virgin female extracts. The identification of the *A. megatoma* sex pheromone, (*E,Z*)-3,5-tetradecadienoic acid, was reported by Silverstein et al. (1967). The compound was later synthesized (Rodin et al., 1970) and given the trivial name megatomoic acid. Barak and Burkholder (1977) reported the existence of a pheromone for *A. elongatulus* Casey, and the compound was identified as the (*Z,Z*) isomer of megatomoic acid by Fukui et al. (1977).

Interspecific responses for several species of dermestids of the genus *Trogoderma* were reported by Rodin et al. (1969) and Vick et al. (1970). A chemical basis for this interspecific excitation was presented by Cross et al. (1976). They reported that Porapak-Q collections of airborne volatiles from four species of *Trogoderma* yielded either (*Z*)- or (*E*)-14-methyl-8-hexadecenal.

The first reference to "calling" postures in the Coleoptera was by Woodroffe (1958) in *Ptinus clavipes* Panzer f. *mobilus* Moore. No inference was made relating this behavior to pheromone release. Similar calling was reported by Burkholder et al. (1974), Hammack et al. (1976), and Barak and Burkholder (1977) for the first times in the Dermestidae with several species of *Anthrenus*, *Trogoderma* and *Attagenus*. Hammack and Burkholder (1976) demonstrated photoperiodically entrained circadian rhythms of calling in *T. glabrum* and similar diurnal rhythms were reported for *Anthrenus flavipes* LeConte by Burkholder et al. (1974) and for *A. elongatulus* by Barak and Burkholder (1977).

We report here the interspecific pheromone responses, existence of calling behavior, and the diurnal incidence of calling behavior in five species of *Attagenus*. The species studied are the three Holarctic subspecies of *A. megatoma* (*A. megatoma megatoma*, *A. megatoma japonicus* Reitt. and *A. megatoma canadensis* Casey), and *A. schaefferi spurcus* LeConte, *A. bicolor* Harold, *A. rufipennis* LeConte, and *A. elongatulus* Casey (Beal, 1970). Woodroffe (1975) had proposed synonymy of *A. elongatulus* with the older, East European *A. brunneus* Fald. However, Beal (1970) was not willing to place the two species in synonymy without more rigorous cross-breeding and examinations of larval stages.

METHODS AND MATERIALS

Attagenus megatoma spp. were reared on the medium described by Burkholder and Dicke (1966), which was composed of ground, sifted Purina Laboratory Chow plus 5% by weight brewer's yeast. All other *Attagenus* species were reared on a similar diet fortified with dry milk, wheat germ, and mixed meat and bone meal (Hammack et al., 1973). Cultures were maintained in approx. 1-liter wide-mouth canning jars that were half full

of medium previously autoclaved at 121 °C and 15 psi for approx. 10 min. The lids were replaced with a round brass screen and were lined with filter paper. Cultures were initiated by adding 15–25 unmated pairs to the jars. Cultures were kept in a rearing room held at 27.5 ± 1 °C and 50 ± 10% relative humidity and under a 16:8 light/dark photocycle with lights on at 0700 hours, central standard time (CST). After appropriate developmental times, cultures were checked for pupae at intervals of 5–7 days by sifting through a U.S. standard 840 μm sieve. Pupae were segregated by sex, based on size. Female pupae were larger than male pupae. Female pupae were held in the rearing room and male pupae in a separate incubator, both in filter paper-lined petri dishes, under the same environmental conditions. The dishes were checked every two days for adult emergence. A conclusive sexing was done based on antennal sexual dimorphism (Beal, 1970). Male adults were transferred to another incubator in the bioassay room. Both the incubator and bioassay room were under environmental conditions similar to the rearing room and were kept free of females and female pheromone contamination.

Pheromone extracts of females for male bioassays were prepared by placing females of suitable age and number in 125 × 15-mm screw-top culture tubes that contained a known amount of hexane and approx. 4 ml of 4-mm glass beads. The tubes were vibrated on a vortex mixer until all insects were macerated. The extracts were diluted with hexane to provide 0.1 female equivalents (FE)/10-μl aliquot. The olfactometer used in the male bioassays was a small-vial type similar to that described by Vick et al. (1970). Methods of bioassay and response characteristics for all species were similar to those reported by Barak and Burkholder (1977).

To observe calling behavior, female insects were placed individually in 1.5-dr shell vials, the bottoms of which were lined with an antibacterial assay disk and fitted with a 5 × 20-mm piece of filter paper pleated at three points. The vials were placed on glass shelves in a white-painted room, lighted by cool fluorescent lights with an intensity of approx. 125 lx at the center of the room. The method of quantifying calling behavior was like that employed by Barak and Burkholder (1977).

To determine the effect of male age on responsiveness in *A. rufipennis*, two replicates of 10 males of each age group from 0–2 to 26–28 days old were bioassayed at 2-day age intervals.

Interspecific responses were observed by bioassaying 30–50 males of each species, in replicates of 5–10 males each, against 0.1 FE (female equivalent) of each species, plus a hexane control. Extracts were prepared from 6- to 10-day-old females, except for *A. rufipennis* females which were 22–26 days old. Six- to 14-day-old males were tested except for *A. rufipennis*, where males 20–28 days old were used.

The influence of age on calling in females of *A. rufipennis* and *A. bicolor* was determined by observing groups of aged females of 0–2 to 20–22 days old for *A. rufipennis* and 0–2 to 16–18 days old for *A. bicolor*. A second replicate was obtained by observing the same insects 2 days later. Observations were made between 0930 and 1500 hours.

To study the diurnal incidence of calling, 50 females of each species, except *A. schaefferi spurcus*, for which 30 females were used, were observed individually at 1-hr intervals beginning at 0700 hours, or lights-on. Females were 15–18 days old, except for *A. rufipennis*, which were 16–20 days old.

RESULTS

Influence of Age on Pheromone Response of A. rufipennis

Males less than 12 days old did not respond to female extracts. Response levels gradually increased and reached the highest levels after 20–22 days of age, as shown in Table 1. In further bioassays, males at least 22 days old were used.

Interspecific Response to Female Extracts

Males of each species responded to conspecific female extracts at similar levels (Table 2). The three subspecies of *A. megatoma*, and *A. schaefferi spurcus* showed similar cross-responses, but did not respond significantly to *A. elongatulus*, *A. rufipennis*, or *A. bicolor* female extracts. Likewise, *A. elongatulus*, *A. rufipennis*, or *A. bicolor* males did not respond significantly to the heterospecific female extracts.

TABLE 1. FEMALE CALLING, AND MALE RESPONSE TO PHEROMONE EXTRACT OF FEMALES IN *A. rufipennis* OF VARIOUS AGES

	Age (days)								
	10–12	12–14	14–16	16–18	18–20	20–22	22–24	24–26	26–28
Female calling (%)[a]	0	1	20	24	49	55			
Male response to female extract (%)[b]	0	15	20	45	60	75	85	85	85

[a] Count reflects percent of females calling at one time.
[b] Bioassay as two replicates of 10 males each on 2 different days.

TABLE 2. PERCENT RESPONSE* OF MALES OF SEVERAL SPECIES TO FEMALE EXTRACT OF SPECIES OF *Attagenus*

Males tested†	Source of female extract‡							
	A. megatoma megatoma	*A. megatoma canadensis*	*A. megatoma japonicus*	*A. schaefferi spurcus*	*A. elongatulus*	*A. rufipennis*	*A. bicolor*	Control
megatoma megatoma	86_1^{ab}	84_1^{a}	84_1^{a}	92_1^{a}	16_2^{ab}	4_2^{bc}	12_{23}^{a}	2_3^{ab}
megatoma canadensis	78_1^{a}	80_1^{a}	88_1^{a}	88_1^{a}	18_2^{ab}	6_{23}^{abc}	14_2^{a}	14_2^{b}
megatoma japonicus	78_1^{a}	82_1^{a}	80_1^{a}	90_1^{a}	6_2^{ac}	10_2^{abc}	12_2^{a}	2_2^{ab}
schaefferi spurcus	97_1^{b}	90_1^{a}	83_1^{a}	93_1^{a}	30_2^{b}	20_{23}^{a}	10_3^{a}	13_3^{b}
elongatulus	6_1^{c}	12_1^{b}	4_1^{b}	12_1^{b}	78	12_1^{ab}	6_1^{a}	8_1^{ab}
rufipennis	2_1^{c}	10_1^{a}	2_1^{b}	8_1^{b}	0_1^{c}	76	6_1^{a}	0_1^{a}
bicolor	6_1^{c}	8_1^{b}	4_1^{b}	6_1^{b}	0_1^{c}	0_1^{c}	76	0_1^{a}

* Percents in any vertical column followed by same letter, or any horizontal row followed by same number are not significantly different at the 0.05 level as determined by proportion test (Zar, 1974).

† Fifty males of each species were tested against each female extract and control, except for *A. schaefferi* for which 30 males for each extract were used.

‡ All female extracts at 0.1 FE per assay.

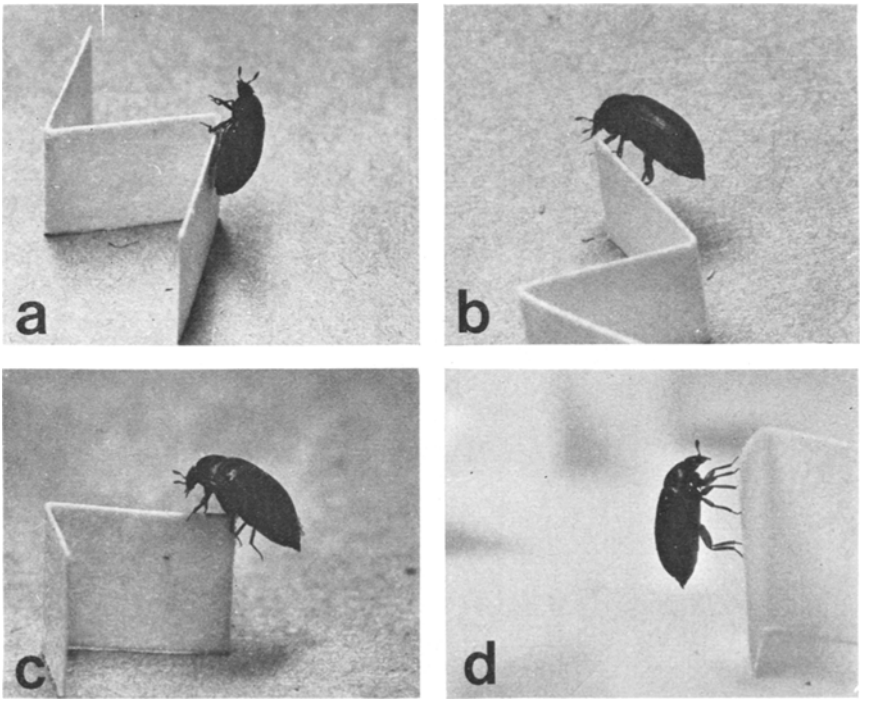

FIG. 1. Calling postures in four species of *Attagenus*; (a) *A. rufipennis*; (b) *A. megatoma megatoma;* (c) *A. schaefferi spurcus*; (d) *A. elongatulus*, from Barak and Burkholder (1977).

Evidence of Calling in Attagenus Species

Figure 1d depicts a female *A. elongatulus* in calling position. Similar behavior, during which a female would climb to a high place and extend its legs and antennae, was observed in the other *Attagenus* species. Although not identical, the postures are clearly similar. Figure 1a shows *A. rufipennis* in this posture. Females of this species climbed on pleated papers, but often only extended the anterior portion of the body over the top, leaving the prothoracic legs free. *Attagenus megatoma megatoma* had a higher incidence of calling and most commonly crawled to the top of the pleated papers (Figure 1b), as would *A. schaefferi spurcus* (Figure 1c), although *A. schaefferi spurcus* did not typically have such prominent leg extension. *Attagenus elongatulus* (Figure 1d) usually called on a vertical surface with prominent leg extension. *Attagenus bicolor* was similar to *A. elongatulus* in posture. *Attagenus megatoma japonicus* and *canadensis* did not demonstrate such

TABLE 3. FEMALE CALLING IN GROUPS OF *A. bicolor* OF VARIOUS AGES

	Ages of females (days)								
	0–2	2–4	4–6	6–8	8–10	10–12	12–14	14–16	16–18
Number of females observed	46	31	60	80	75	69	62	57	27
Percent calling		16	62	70	72	65	76	82	59

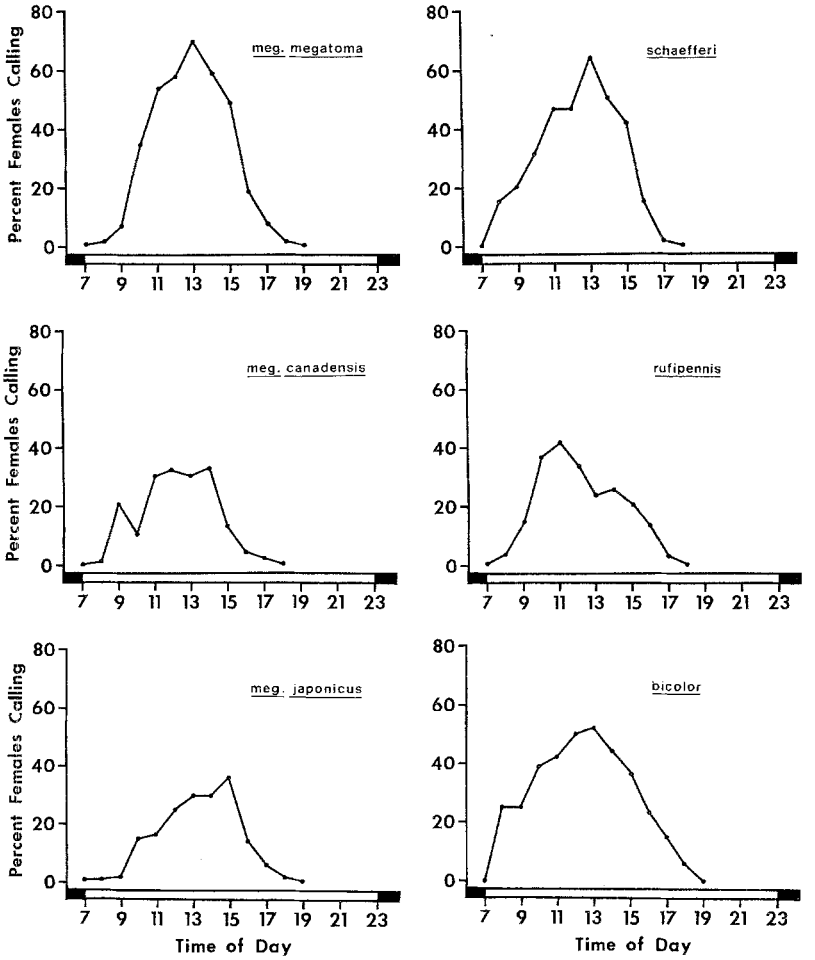

FIG. 2. Diurnal calling activity of several *Attagenus* species (dark bar indicates scotophase).

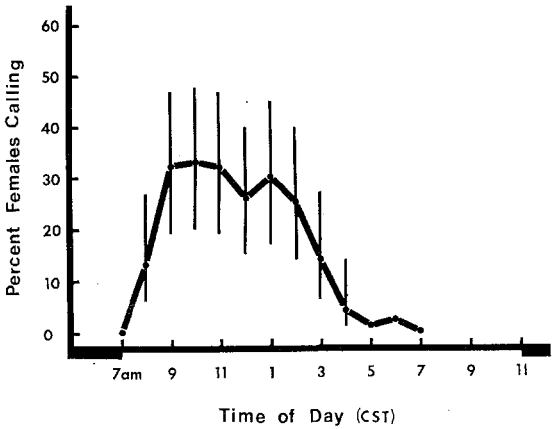

FIG. 3. Diurnal calling activity in 50 *A. elongatulus* virgin females 6–8 days old. Vertical lines represent the 0.95 binomial confidence interval about each point. (From Barak and Burkholder, 1977.)

obvious leg extension and usually stayed on the lower vertical surfaces. Rarely were the headstand or upside-down postures, seen in *Anthrenus* (Burkholder et al., 1974) and *Trogoderma* (Hammack et al., 1976), evident in *Attagenus*.

Calling in Aged A. rufipennis and A. bicolor Females

Attagenus rufipennis females younger than 12–14 days old did not call. Calling frequency gradually increased with age until about 18–20 days (Table 1), approximately the ages for maximum male response. *Attagenus bicolor* females began to call at 2–4 days of age, and the greatest frequencies occurred at 4–6 days of age (Table 3). Females at least 5 days old were used in later calling observations, and males at least 5 days old were used in bioassays.

Diurnal Incidence of Calling

All observed species showed a definite period of calling activity that occurred during the early to middle photocycle (Figure 2). *Attagenus megatoma megatoma*, *A. schaefferi spurcus*, and *A. bicolor* had the highest incidence of calling. *A. megatoma japonicus* had a calling curve which was skewed toward the later part of the activity period, while *A. rufipennis* called more during the earlier hours of the active period. Calling activity

was never observed immediately after lights-on, during the scotophase, or after 1900 hr of the photophase.

DISCUSSION

Attagenus rufipennis males were not responsive to female extracts until they were about two weeks old. This is quite different from *A. megatoma megatoma* males, where response first occurs at 4–5 days of age (Burkholder, 1970), and *A. elongatulus* males, where response first occurs at 3–4 days of age. As with *A. elongatulus*, the age at which male response was first observed in *A. rufipennis* coincided with the first exhibition of female calling behavior. *Attagenus bicolor* males were not bioassayed due to insufficient numbers. However, with *A. rufipennis* and *A. elongatulus* as precedents, *A. bicolor* males were used in bioassays if females of the same age called.

Attagenus megatoma megatoma, canadensis, and *japonicus* are considered subspecies (Beal, 1970), so it was not unexpected that they would respond equally to each others' female extracts (Table 2). *Attagenus schaefferi spurcus* also responded interspecifically with the *A. megatoma* complex. However, Beal (1970) suggested that *A. schaefferi* is most closely related to *A. elongatulus*, which utilizes the *Z,Z* isomer of megatomoic acid. *A. elongatulus* responds only to its own females, while *A. schaefferi* males did not respond strongly to *A. elongatulus*. If the other isomers of megatomoic acid function as pheromones in *Attagenus*, the possibility exists that *A. rufipennis* and *A. bicolor* may utilize them, since each responded to its own female's extract but not to female extracts of the other species.

Hammack et al. (1973) localized the probable pheromone-producing tissues in the 7th abdominal (last visible) sternites of six *Trogoderma* species. It seems likely that calling behavior is a means of facilitating the dispersal of volatile pheromones in the atmosphere by lifting the sternites away from the substrate and exposing pheromone-producing regions to air currents.

Calling behavior in *Trogoderma* has been shown by Hammack et al. (1976) to be circadian in nature and photoperiodically entrained. It seems probable that in the *Attagenus*, calling behavior is a circadian, photoperiodically entrained rhythm. Comparisons of diurnal calling in the *Attagenus* (Figures 2 and 3) show a basic 12-hr calling period with peak times varying by as much as 4 hr between species. *Attagenus elongatulus* and *A. rufipennis* called most during the earlier hours; *A. megatoma megatoma, A. canadensis, A. schaefferi spurcus,* and *A. bicolor* called most during the middle of the period; and *A. megatoma japonicus* calling reached a peak near the end of the period.

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ATTRACTANTS FOR SYNANTHROPIC FLIES
Ethanol as Attractant for *Fannia canicularis*[1] and Other Pest Flies in Poultry Ranches

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Abstract—Fermented molasses or sucrose solutions are known to attract several species of filth-breeding flies. To identify the volatile attractants produced in fermenting sucrose solutions with yeast, these solutions were fractionated, and the chemical constituents identified and bioassayed against filth-breeding flies including *Fannia canicularis* (L.), *Muscina stabulans* (Fallén), and *Musca domestica* (L.). Distillation of a fermented sucrose solution gave an active distillate and an inactive residue. Gas chromatographic analysis of the distillate showed the presence of acetaldehyde, ethyl acetate, ethanol, 1-propanol, 2-methyl-1-propanol, and 3-methyl-1-butanol. Ethanol constituted by far the greatest proportion of compounds present in the distillate. An aqueous solution of ethanol exhibited the same level of attractancy as the distillate, the fermented sucrose solution, and a reconstituted distillate containing all compounds identified. Ethanol was thus identified as the sole attractant emanated from fermented carbohydrate solutions that elicited positive responses in pest flies, especially in *F. canicularis*.

Key Words—Attractant, ethanol, fermented sucrose, little house fly, *Fannia canicularis*.

INTRODUCTION

The little house fly, *Fannia canicularis* (L.), is a common and cosmopolitan species which equals *Musca domestica* (L.) as a predominent pest in residential and rural areas of southern California and elsewhere. *F. canicularis*

[1] Diptera: Muscidae.

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was found to carry *Shigella flexneri* Castellani and Chalmers and *S. dysenteriae* (Shiga) in Bukhara (Sychevskaia et al., 1959). The larvae of the eyeworm *Thelazia californiensis* Price were reported to be capable of completing their development in *F. canicularis* (Burnett et al., 1957). Despite its implication as vector of pathogens, control of the little house fly, with a few exceptions (Williams, 1973a,b), has not been the subject of major studies in recent years.

Fermentation products of carbohydrates have long been known either as feeding or as ovipositional-type attractants for carpophagous and xylophagous insects. Peterson (1925) was the first to evaluate molasses–yeast bait against the oriental peach moth *Laspeyresia molesta* Busck. Molasses baits were also known to be attractive to *Carpocapsa pomonella* L. (Eyer, 1931). Recently, it was noted that molasses solutions fermented with yeast were highly attractive to *F. canicularis* and other filth-breeding flies (W.F. Rooney and W.D. McKeen, unpublished data). Subsequently, we found that fermented sucrose solutions were equally attractive as fermented molasses solutions to the little house fly (unpublished data of the authors).

As part of our ongoing research program to identify the chemical nature of attractants for synanthropic flies (flies associated with humans or fostered by accumulation of organic wastes), the current investigations were initiated on the chemical attractants for *F. canicularis* and other filth-breeding flies. Here we report the isolation and identification of the specific attractant produced by fermented sucrose solutions, showing a high level of attractancy against pestiferous insects produced in poultry establishments.

METHODS AND MATERIALS

In preliminary studies, we found that fermented sucrose solutions were as attractive as fermented molasses solutions to pest flies. Therefore the former solution was employed in the chemical isolation and identification studies. A fermented sucrose solution was prepared by dissolving sucrose (200 g) and Fleischman's® "fresh active" yeast (8 g) in water (final volume 2 liters) in a 3.7-liter wine bottle and incubating the solution at 26 ± 2°C in a greenhouse room for 14 days. During the fermentation, the cap was loosely placed onto the bottle for releasing carbon dioxide.

To separate volatile active compounds from nonvolatile inert materials, the fermented sucrose solution (1 liter) was distilled under atmospheric pressure until a distillate (approx. 800 ml) and a residue (approx. 200 ml) were obtained. The distilate and the residue were both diluted with water to 1 liter each to obtain the original concentration. Both solutions were bioassayed and compared against the fermented sucrose solutions.

Because the distillate was shown to be attractive in bioassay tests, it

was analyzed with a Hewlett Packard Model 5750B Research Gas Chromatograph equipped with a dual flame detector. A 1.8-m × 0.64-cm OD (wall thickness 0.89 mm), stainless-steel column packed with 15% Carbowax® 600 on 60–80 mesh Chromosorb G was used for the analysis. The chromatographic conditions were: injection port temperature, 210 °C; detector temperature, 210 °C; column temperature, 77 °C; carrier gas (N_2) flow rate, 60 ml/min at 60 psi; hydrogen flow rate, 62.5 ml/min; air flow rate, 400 ml/min; electrometer range, 10^2; recorder attenuation, × 1; sample size, 2–4 μl; and recorder speed, 1.27 or 0.635 cm/min. 1-Butanol was used as internal standard for qualitative analysis. For quantitative analysis, the peak areas of the sample were measured by triangulation and compared with those of authentic compounds with known concentrations.

To confirm the activity of the authentic compounds present in the distillate, they were reconstituted in water in the proper proportions. The resulting solution was designated as the reconstituted distillate and bioassayed. Because ethanol was the main product of fermentation, an aqueous solution of ethanol in the proper concentration was prepared and bioassayed.

To determine attractancy, the samples were bioassayed on poultry ranches in southern California where populations of *F. canicularis* prevailed from spring to midsummer. In addition to the little house fly, lower popula-

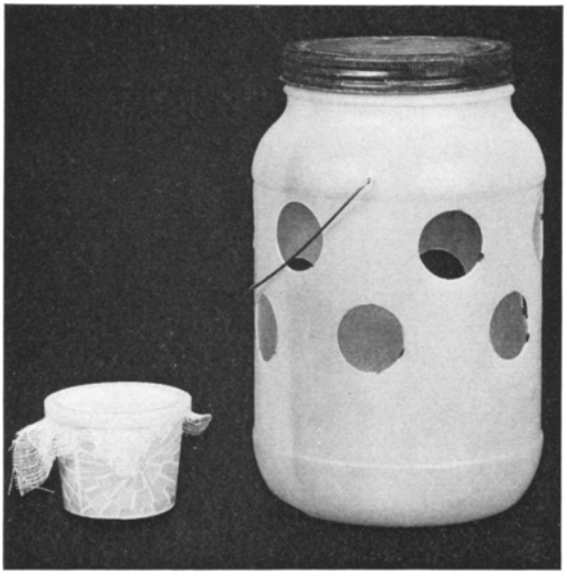

FIG. 1. A plastic jar trap and a Dixie cup. An attractant sample is placed in the cup which is covered by a layer of muslin. The cup is placed inside the jar trap.

tions of the false stable fly *Muscina stabulans* (Fallén) and the house fly *M. domestica* were also present at the testing sites. In bioassay tests, a 50-ml attractant sample was placed in a 120-ml Dixie® cup, which was in turn placed in a 3.8-liter plastic jar trap with two rows of holes (six 35-mm-diam. holes in each row) in its side for fly entry (Figure 1). To exclude the flies from the attractant samples, muslin covers were placed over the cups. Five-gram Golden Malrin® (sugar bait containing 0.09% dichlorvos and 0.25% ronnel) was sprinkled on the bottom of the jar traps for toxicant effect. The test materials were evaluated by the matched-pair test method, replicated five times. The traps in a pair were hung 5–8 cm above the ground 1.2–1.5 m apart from each other, and the pairs were separated at least 6 m apart from one another. The samples were exposed for 4–5 days. The flies caught were segregated to species and counted. Data were statistically analyzed by transforming the numbers to log $(n+1)$ and by using the matched-pair t test.

RESULTS AND DISCUSSION

On distillation of the fermented sucrose solution, the bioactive principles were carried over into the distillate (Table 1, test A). The distillate showed the

TABLE 1. ATTRACTANCY OF VARIOUS ATTRACTANT FRACTIONS AND SOLUTIONS AGAINST *F. canicularis*

Test pair	Material	Exposure period (days)	Average no./trap[a]		
			F.c.	M.s.	M.d.
A	Ferm. sucrose soln	4	237	46	14*
	Distillate	4	256	44	7
B	Ferm. sucrose soln	4	87*	30*	14*
	Residue	4	1	1	1
C	Distillate	5	283	195	44
	Reconstituted distillate	5	368	158	31
D	Distillate	5	815	100	38
	Aq. EtOH (5.25%, wt.)	5	899	160	29
E	Reconstituted distillate	5	590	121	5
	Aq. EtOH	5	570	117	14
F	Ferm. sucrose soln	3	1168	119	10
	Aq. EtOH	3	860	72	4

[a] Each test consists of five replicates. Average numbers with an asterisk are significantly different at 0.01 level in the matched-pair t test. F.c.: *F. canicularis*, M.s.: *M. stabulans*, M.d.: *M. domestica*.

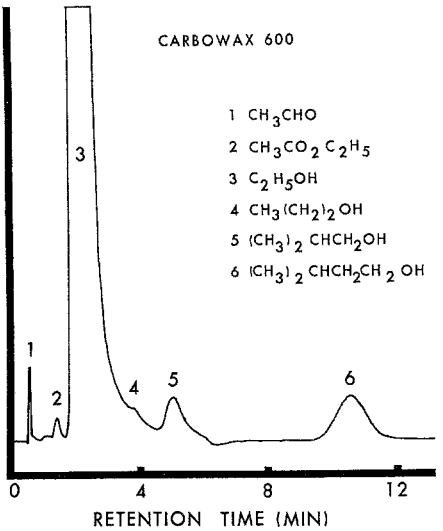

FIG. 2. The gas chromatogram of the distillate of the fermented sucrose solution on a Carbowax 600 column.

same level of attractancy as the original fermented sucrose solution against *F. canicularis* and *M. stabulans*. However, the number of *M. domestica* attracted by the fermented sucrose solution was significantly larger than that by the distillate. The residue of the fermented product exhibited no attractancy against the three species of flies as compared to the fermented sucrose solution (test B).

Gas chromatographic analysis of the distillate on the Carbowax 600 column showed the presence of acetaldehyde, ethyl acetate, ethanol, 1-propanol, 2-methyl-1-propanol (isobutyl alcohol), and 3-methyl-1-butanol (isopentyl alcohol) (Figure 2). The concentration of ethanol in the distillate was 5.25% (by weight), and those of the minor constituents ranged from 4 to 102 ppm (by weight) (Table 2). The results of this analysis were in agreement with those found for table wines (Shinohara and Watanabe, 1976). The gas chromatograms of the distillate and the reconstituted distillate were superimposable.

The reconstituted distillate showed the same attractancy as the distillate of the fermented sucrose solution against *F. canicularis* and other pest flies (Table 1, test C). Because the distillate mainly contained ethanol, an aqueous ethanol solution (5.25% by weight) was compared with other attractant solutions. The aqueous ethanol solution was as active as the distillate (test

TABLE 2. RELATIVE RETENTION AND CONCENTRATION OF COMPOUNDS IDENTIFIED FROM DISTILLATE OF FERMENTED SUCROSE SOLUTION

Compound	Relative retention[a]	Concentration (ppm by weight)
Acetaldehyde	0.076	30
Ethyl acetate	0.152	22
Ethanol	0.288	52,500
1-Propanol	0.515	4
2-Methyl-1-propanol	0.685	47
3-Methyl-1-butanol	1.424	102

[a] 1-Butanol is used as an internal standard.

D) and, at the same time, exhibited the same attractancy as the reconstituted distillate (test E). Also, the attractancies of the aqueous ethanol solution and the fermented sucrose solution were essentially the same (test F). The fermented sucrose solution, the distillate, the reconstituted distillate, and the 5.25% aqueous ethanol solution possessed the same degree of activity in luring the little house fly and other pest flies.

Originally, we suspected that the minor components might enhance the attractancy of ethanol produced on fermentation of carbohydrate–yeast solutions. The addition of the minor components (acetaldehyde, ethyl acetate, 1-propanol, 2-methyl-1-propanol, and 3-methyl-1-butanol) to the aqueous ethanol solution, however, did not enhance the attractancy of the latter. Both solutions showed the same degree of activity. These results implied that, in fermented carbohydrate solutions, it was ethanol that elicited a positive response in the little house fly and other pest flies.

During the period of field bioassays from spring to midsummer, insect populations in poultry ranches were predominantly *F. canicularis*. Although the male little house flies showed characteristic chasing behavior and swarmed in the air, flies attracted to the test samples were composed of about 67% females. The population of *M. domestica* was low during the early part of the year. In addition to the pest flies, the pestiferous moth *Niditinea fuscipunctella* (Haworth) was also attracted to the attractant samples.

F. canicularis was reported to be attracted to ammonium carbonate (Vanskaya, 1942). The *Hippelates* eye gnat attractants (Hwang and Mulla, 1971; Hwang et al., 1976), which contained an ammonium salt, attracted *M. domestica* (Mulla et al., 1977) but showed little attractancy against *F. canicularis* (unpublished data of the authors).

Ethanol, among other low-molecular-weight aliphatic alcohols, attracted the onion maggot *Hylemyia antiqua* Meigen and the seed corn maggot *H. cilicrura* Rondani (Peterson, 1924). It also attracted *Drosophila melanogaster* Meigen (Reed, 1938). Based on the findings of Richardson (1917) and Wieting and Hoskins (1939) that ethanol attracted *M. domestica*, Brown et al. (1961) developed an aqueous solution of malt extract, ethanol, skatole, and acetal which showed attractancy against the house fly. Ethanol was also reported to attract blow flies (McIndoo, 1933). Most of these parameters were studied in olfactometers against laboratory populations.

We have therefore documented for the first time that ethanol, emanated from fermented carbohydrate solutions, is the attractant for the little house fly *F. canicularis* and, to a lesser extent, for the false stable fly *M. stabulans* and the house fly *M. domestica*. The attractant thus identified can be used as a bait for monitoring fly populations as well as for the control of these pest flies in pest management programs.

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SEX PHEROMONE SPECIFICITY IN *Heliothis*[1,2]

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Abstract—Electric grid traps baited with *Heliothis subflexa* (Guenée), *H. virescens* (F.), or *H. zea* (Boddie) females captured conspecific males with few exceptions. *Heliothis subflexa* females reduced the attraction of *H. virescens* and *H. zea* males when used as bait simultaneously with females of either of these two species. Backcrosses were made with *H. virescens* males and female hybrids from a cross between *H. subflexa* females and *H. virescens* males. The backcross (BC) females and *H. virescens* females attracted approximately equal numbers of *H. virescens* males in field traps. BC males released in field cages were attracted to *H. virescens* females and to the synthetic pheromone of *H. virescens*. When laboratory-reared male *H. virescens*, BC males, *H. subflexa* males, and F_1 hybrid males were exposed to the synthetic pheromone of *H.virescens* in Plexiglas wind tunnels, *H. virescens* males and BC males responded to the pheromone, but *H. subflexa* and F_1 hybrid males did not. The peak activity of both *H. subflexa* and *H. zea* males occurred approx. 4 hr after sunset. Male *H. zea* were active throughout most of the night; male *H. virescens* were most active approx. 6 hr after sunset.

Key Words—Sex pheromone, *Heliothis subflexa*, *Heliothis virescens*, *Heliothis zea*, hybrid sterility.

INTRODUCTION

Recent studies suggested that it might be possible to utilize the sterile hybrid

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

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males (Laster 1972) resulting from matings between female *Heliothis subflexa* (Guenée) and male *H. virescens* (F.) in a sterile-release program to control *H. virescens*. Laster et al. (1976) therefore presented a population model illustrating the decline of *H. virescens* populations that could follow release of various ratios of hybrid moths capable of transmitting genetic sterility to the population in this way. However, populations of *H. subflexa*, *H. virescens*, and *H. zea* (Boddie) may or may not occur in a particular area at the same time. Although both *H. virescens* and *H. zea* have many of the same host plants, *H. subflexa* is found almost exclusively on ground cherry, *Physalis* spp., and is not known to be a pest of any economic importance (Laster 1972, Kimball 1965). Also, little is known about how these species and introduced hybrids might interact in the field.

We therefore made field, field cage, and laboratory studies to determine whether there was cross-attraction or inhibition between *H. zea*, *H. subflexa*, *H. virescens*, the F_1 hybrid, and selected backcross (BC) moths.

METHODS AND RESULTS

Insects used for bait in traps in the field or released in cages were reared in the laboratory on artificial diet. The *H. subflexa* were taken from a culture started from larvae collected from the wild host, ground cherry, at Gainesville, Florida, in September 1975. The *H. virescens* were obtained as pupae from Oxford, North Carolina, and the *H. zea* pupae were obtained from laboratory cultures at Oxford, North Carolina, and Gainesville, Florida. The hybrid females from *H. subflexa* females and *H. virescens* males were backcrossed to normal *H. virescens* males and subsequent backcross (BC) generations were produced at the Gainesville laboratory. Male progeny from these matings were sterile and female progeny were fertile.

Field Studies

For one field study, one or two electric grid traps (Mitchell et al., 1972) were baited with 3 females each of either *H. subflexa*, *H. virescens*, or *H. zea* to determine attraction of these species to the pheromone (female-baited) traps. These traps were located in farming areas where host plants were available at either Hastings or Gainesville, Florida, and were operated from June 28 through July 24, 1975 (total of 37 trap nights for each species). Also, females from BC_8, BC_9, or BC_{10} were used in one trap and *H. virescens* in another as bait (3 females/trap) for *H. virescens* males (total of 49 trap

nights) at Gainesville. In a similar study at Gainesville, either *H. subflexa* plus *H. virescens* females (17 trap nights) or *H. subflexa* plus *H. zea* females (10 trap nights) were used in combination as bait (3 females of each species/trap) in grid traps to determine whether any inhibition existed between these species. Captured insects were collected and counted every 1 or 2 days. The paired *t* test at the 5% level of probability was used for mean separation.

Traps baited with *H. subflexa*, *H. virescens*, or *H. zea* females almost always captured males of the respective species. The trap baited with *H. subflexa* females captured 689 *H. subflexa*, 9 *H. virescens*, and 6 *H. zea*; *H. virescens* females attracted no *H. subflexa*, 766 *H. virescens*, and 2 *H. zea*; and *H. zea* attracted only *H. zea* (1028). BC females (from BC_8, BC_9, and BC_{10}) and *H. virescens* females attracted statistically equal numbers of *H. virescens* males (392 and 402, respectively), and only a few *H. subflexa* were captured by these baits.

In the test in which combinations of species were used as bait, the trap baited with *H. subflexa* captured 61% and the trap baited with *H. subflexa* plus *H. virescens* captured 39% of the total (456) *H. subflexa* collected. However, the catches were not significantly different at the 5% level under the conditions of the test. The trap baited with *H. virescens* captured significantly more *H. virescens* (71% of the 303 total) than the trap baited with *H. subflexa* plus *H. virescens*. Thus, the *H. subflexa* females apparently reduced the attraction of *H. virescens* females for *H. virescens* males when both species of females were present in the same trap.

Likewise, traps baited with *H. subflexa* captured 46% of the total (250) *H. subflexa*, and the trap baited with *H. subflexa* plus *H. zea* captured 54%. However, the trap baited with *H. zea* captured 73% of the total (370) *H. zea*, and the trap baited with *H. subflexa* plus *H. zea* captured only 27%. Thus, the presence of *H. subflexa* females in the same trap with *H. zea* females significantly reduced the attraction of *H. zea* males, although the presence of *H. subflexa* females in the same trap with *H. zea* females had no apparent effect on the attraction of *H. subflexa* males. Haile et al. (1973) found that when *H. virescens* and *H. zea* females were used as bait in the same trap, the number of males of both species that were captured was reduced. (We have obtained similar results in unpublished field tests.) However, they reported that the reduction of *H. virescens*, unlike the reduction in *H. zea*, was apparent only at a high density.

The field data therefore gave no indication of the interspecific sex attraction among *H. subflexa*, *H. virescens*, and *H. zea*, and there was some apparent inhibition. Mitchell et al. (1976) reported results indicating that individual components of a pheromone can be highly effective as mating

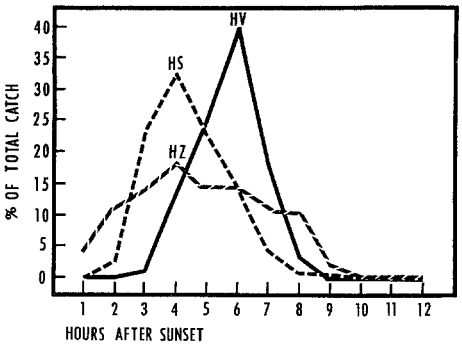

FIG. 1. Nocturnal activity of *Heliothis subflexa* (HS), *H. virescens* (HV) (Goodenough and Snow 1973), and *H. zea* (HZ) as determined with electric grid traps baited with virgin females.

inhibitors. For example, in small field tests, (*Z*)-11-hexadecenal, a component of the *H. virescens* pheromone, reduced the mating of *H. zea* females. Nevertheless, (*Z*)-9-tetradecenal, another component of the *H. virescens* pheromone, was ineffective against *H. zea* but this component reduced the mating of *H. virescens* females by 95%. (The pheromones of *H. subflexa* and *H. zea* have not yet been identified.)

The nocturnal activity of adult *H. subflexa* males was determined by operating a female-baited cylindrical electric grid equipped with an automatic sample changing device (Smith et al., 1973) from July 3 to 24, 1975, at Hastings in an area where host plants were available. Also, seasonal populations of *H. subflexa* and *H. virescens* were surveyed with female-baited grid traps at Gainesville from September 1975 to December 1976. In this case, traps (one for each species) were placed along the edges of fields in which host plants were present. (Trapping studies in process in this farming area guided us in location of these survey traps.)

The peak response of *H. subflexa* males to females occurred 3–5 hr after sunset (Figure 1); 78% were captured during this period, and an additional 14% were caught the following hour. The peak response of *H. zea* males to females occurred at the same time (approx. 4 hr after sunset), but activity remained relatively high throughout most of the night. Goodenough and Snow (1973) determined that *H. virescens* males were most active approx. 6 hr after sunset, and our data are in agreement.

When we subsequently surveyed populations of *H. subflexa* and *H.*

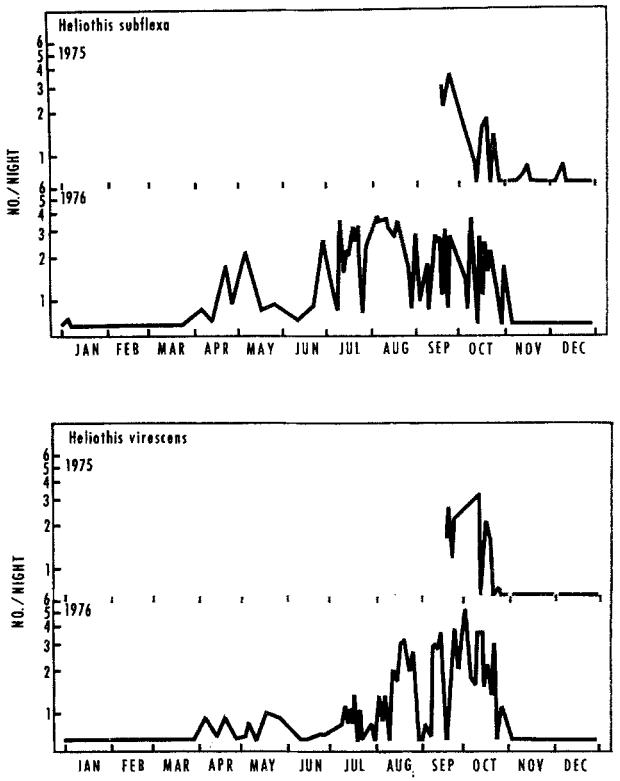

FIG. 2. *Heliothis subflexa* and *H. virescens* captured in female-baited electric grid traps, number [as $\log_e (n + 1)$]/night, Sept. 1975–Dec. 1976, Gainesville, Florida.

virescens at Gainesville, Florida, in an area where host plants were available to both species, we observed that population trends for these two species were similar (Figure 2). Diel and seasonal activities did not appear to be sufficient for reproductive isolation.

Cage Studies

Six cage studies were made (Table 1) in which *H. subflexa*, *H. virescens*, F_1 hybrids, or BC males were released in a 29 × 10-m arc-shaped cage with a maximum height of 3.5 m. Two electric grid traps were placed in the cage and baited either with females (3/trap) or with the synthetic pheromone of *H. virescens*, a 16:1 ratio of (*Z*)-11-hexadecenal and (*Z*)-9-tetradecenal (Tumlinson et al., 1975) dispensed in a Hercon® plastic strip (6.5 cm^2 of surface

TABLE 1. MEAN PERCENTAGE OF TOTAL MALES CAPTURED IN ELECTRIC GRID TRAPS (2/TEST) LOCATED IN A 29 × 10-m FIELD CAGE IN WHICH MALE MOTHS WERE RELEASED. TRAPS BAITED WITH *H. virescens* PHEROMONE OR *H. virescens*, F_1 HYBRID, OR BACKCROSS FEMALES, 3/TRAP, SIX TESTS)

Test	Released males	Bait females	$\bar{X}$ % of total males captured in indicated test
1A[a]	*H. virescens*	*H. virescens*	93[e]
		F_1 hybrid	7
B[b]	*H. virescens*	*H. virescens*	51
		BC_1	49
2[c]	F_1 hybrid	*H. virescens*	87[e]
		H. subflexa	13
3[d]	BC_1	*H. virescens*	72
		Pheromone	28
4A	BC_6	*H. virescens*	98[e]
		H. subflexa	2
B[b]	BC_6	*H. virescens*	64
		Pheromone	36
5	BC_7	*H. virescens*	67[e]
		BC_7	33
6A	BC_8	*H. virescens*	70
		BC_8	30
B	BC_8	*H. virescens*	59
		Pheromone	42

[a] Each treatment replicated three times unless otherwise noted.
[b] Four replicates per treatment.
[c] Six replicates per treatment.
[d] Two replicates per treatment.
[e] Means in the same test differ significantly at $P = 0.05$ level, Student's t test.

area on one side). Treatments were rotated daily between the two traps. Each treatment was replicated 2–6 times; a replication consisted of the catch for one night. Of the males released (200–400/test), approx. 40% were captured by the traps. The paired t test at the 5% level of probability was used for mean separation.

The results, although extremely variable, showed that the F_1 hybrid females attracted few *H. virescens* males, but the F_1 hybrid males and BC_6 males were more attracted to *H. virescens* females than to *H. subflexa* females. BC_1 and *H. virescens* females attracted approximately equal numbers of *H. virescens*; BC males were more attracted to *H. virescens* than to BC females.

Laboratory Studies

In the laboratory studies, the responses of *H. subflexa*, *H. virescens*, F_1 hybrid, BC_{1-5}, and BC_{10} males to the synthetic pheromone were compared in olfactometer tests. Laboratory-reared males that had been held in reverse photoperiod were released into three 30 × 30 × 350-cm Plexiglas wind tunnels (10–12/tunnel) used by Mayer (1973) and McLaughlin et al. (1974). The temperature and relative humidity in the wind tunnels were approx. 24–26 °C and 60%, respectively, and a light intensity of 0.5 lux was maintained. Moths were held in the downwind compartment at the beginning of each test. The pheromone (500 ng) was coated on the inside of glass tubes (Mayer 1973) and dispensed into the upwind compartment of each tunnel with filtered air at an airflow rate of 50 ml/min. Meanwhile, filtered air was passed through the tunnels at a rate of 0.25 m/sec. After the chemical had time to reach the holding compartment (calculated from air velocity and distance to holding compartment), the males were released and allowed 30 sec of free flight. Then dividers were inserted in the tunnels, and the

TABLE 2. MEAN CORRECTED PERCENTAGE RESPONSE (+SE) OF MALE *Heliothis virescens* (HV), *H. subflexa* (HS), AND CROSSES (F_1 AND BC) TO *H. virescens* PHEROMONE IN OLFACTOMETERS[a]

Insect species released in tunnel[b]	Mean % (±SE) in upwind compartment[c]
HV	32.8±5.0a
HS	1.1±1.1c
F_1	4.7±2.0c
BC_1	27.6±4.4ab
BC_2	36.7±5.9a
BC_3	30.8±8.0ab
BC_4	25.9±5.7b
BC_5	30.0±8.4ab
BC_{10}	38.2±2.6a

[a] Means followed by the same letter do not differ significantly at $P = 0.05$ level (Duncan's multiple range test).

[b] Pheromone = (*Z*)-11-hexadecenal + (*Z*)-9-tetradecenal (16:1), 500 ng of chemical dispensed into the tunnel with an airflow of 50 ml/min.

[c] Plexiglas tunnel 30 × 30 × 350 cm. Airflow 0.25 m/sec, 24–26°C, approx. 60% RH. Light intensity 0.5 lux.

number of moths in each compartment was recorded. Moths flying to the upwind compartment were considered to be responding to the pheromone. A control (no chemical released) was run each day that tests were conducted. All treatments were replicated 10 times.

Because the tests were conducted at three different times, the response of *H. virescens* to the synthetic pheromone (500 ng) was used as the standard in each test. Data are shown in Table 2 as corrected percentages (actual–control). The *H. virescens*, BC_{1-5}, and BC_{10} males responded to the *H. virescens* pheromone. However, the *H. subflexa* males did not respond, and only a few F_1 hybrid males (less than 5%) responded. This result was similar to that of the cage studies: more F_1 males were attracted to the trap baited with *H. virescens* than to the trap baited with *H. subflexa*, but few moths were caught in either trap.

The results of the olfactometer tests were therefore comparable to the results obtained in the cage and field studies, although all treatments were not made in each study. There was no evidence of cross-attraction among *H. subflexa*, *H. virescens*, or *H. zea*, and there was some evidence of inhibition. The F_1 hybrid males did not respond to the *H. virescens* pheromone, but BC males were attracted to both the pheromone and *H. virescens* females. Also, BC females and *H. virescens* females attracted equal numbers of *H. virescens* males. Thus, our results show that the sterile BC males will respond to *H. virescens* females and might be used, as Laster (1972) suggested, to suppress populations of *H. virescens*.

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OVIPOSITION-DETERRING PHEROMONE OF *Rhagoletis pomonella* A Kairomone for Its Parasitoid *Opius lectus*

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Abstract—We found that after the parasitoid *Opius lectus* has arrived on a fruit infested by eggs or early-instar larvae of its tephritid host *Rhagoletis pomonella*, the following stimuli act to retain it and elicit antennal tapping and oviposition probes: unidentified fruit chemical components; characteristic fruit shape, size and color; and *R. pomonella* oviposition-deterring pheromone. This is the first demonstration of an oviposition-deterring pheromone in a phytophagous insect serving as a kairomone to one of its parasitoids. *O. alloeus*, a parasitoid of late-instar larvae of *R. pomonella*, was not influenced by the pheromone. Possible use of the pheromone for management of *O. lectus* in a multi-faceted *R. pomonella* suppression program is discussed.

Key Words—Pheromone, kairomone, oviposition deterrent, parasitoid, host finding, *Opius lectus*, *Opius alloeus*, *Rhagoletis pomonella*.

INTRODUCTION

The Tephritidae includes a large number of species which oviposit into and develop as larvae in the flesh of growing fruits. Various studies have elucidated several host plant stimuli eliciting tephritid fly attraction and oviposition (Prokopy, 1977, for references), but less is known about the host-selection process of their egg or larval parasitoids.

The literature does indicate that the odor of fresh or decomposing uninfested tephritid host plant tissue is attractive to *Opius fletcheri* Silvestri, *O. oophilus* Fullaway, *O. incisi* Silvestri, and *Biosteres* (*Opius*) *longicaudatus* Ashmead, parasitoids of *Dacus*, *Ceratitis*, and/or *Anastrepha* spp. (Nishida,

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1956; Nishida and Napompeth, 1974; Greany et al., 1977). Also, oviposition by *O. fletcheri* and *O. melleus* Gahan (a parasitoid of *Rhagoletis mendax* Curran) is elicited by specific vibrations or movements of the host larvae (Lathrop and Newton, 1933; Nishida, 1956). Finally, Haramoto (1953) states that the egg parasitoid *O. oophilus* will not oviposit when the fruit surface is moist, the pulp exposed, or the host eggs exposed. He further reports that *O. oophilus* oviposition is not influenced by degree of fruit ripeness or host egg fertility or viability, but suggests (although presents no evidence) that a "liquid secreted by *D. dorsalis* Hendel females during oviposition is the primary stimulus that attracts *O. oophilus* to the ovipositional sites." He indicates that *O. oophilus* has a definite tendency to avoid superparasitism and is confirmed in this by Kaya and Nishida (1968).

Here, we deal principally with the chemical basis of host selection by *O. lectus* Gahan. As we determined through laboratory observation and host dissections, this parasitoid oviposits into eggs of the apple maggot, *R. pomonella* (Walsh). Garman and Townsend (1952) reported up to 15% parasitism of *R. pomonella* eggs by *O. melleus*, but we believe that the true identity of this parasite may have been *O. lectus*, as *O. melleus* parasitizes late-instar larvae. Laboratory and field observations (Smith and Prokopy, unpublished data) and circumstantial evidence (Rivard, 1967; Monteith, 1971; Dean and Chapman, 1973; Cameron and Morrison, 1977) suggest that *O. lectus* also oviposits in early-instar larvae of *R. pomonella*. It emerges as an adult (1/host) from overwintering *R. pomonella* puparia.

We were especially interested in whether *R. pomonella* fruit-marking pheromone might be a host-finding stimulus to *O. lectus*. This pheromone is deposited on the egg surface during egglaying (Prokopy, unpublished data), and on the fruit surface immediately afterward, and deters boring attempts by other *R. pomonella* females in that fruit (Prokopy, 1972; Prokopy et al., 1976). We also include information on host selection by *O. alloeus* Muesbeck, which oviposits principally into 3rd (= last) instar *R. pomonella* larvae in the fruit.

METHODS AND MATERIALS

R. pomonella is a North American species whose native host is hawthorne (*Crataegus*), but which has within the past 120 years shifted onto apple, sour cherry, and plum (Bush, 1969; Shervis et al., 1970). *Opius* and *R. pomonella* adults were obtained from puparia from infested haw and apple fruits collected in nature in Door County, Wisconsin, and Amherst, Massachusetts. They were maintained separately in cages at 26 ± 2°C, 50 ± 5% relative humidity, and a 16-hr photoperiod under cool-white fluorescent lamps.

The *Opius* were fed a 1:1 mixture of honey and bee-gathered pollen (honey alone may have been adequate) and the flies a 4:1 mixture of sucrose and enzymatic yeast hydrolysate.

Unless stated otherwise, all test fruits were standardized as either (1) real uninfested haws, 15 ± 2 mm diam., picked when ripe (dark red in color), stored at 3 °C, water-washed and air-dried before use, or (2) artificial haws, constructed of modeling clay formed into 15-mm spheres attached to a stiff wire for ease of handling, dipped in molten black-dyed ceresin wax, and then completely enveloped in a single layer of parafilm whose entire outer surface had been exposed to (i.e., stretched against) the surface of a real haw fruit at 25 °C for the preceding 6 days. Black rather than dark red-dyed wax was used because of the noticeable odor (to the human nose) of the red dyes available to us and because most, if not all, insects are apparently unable to visually distinguish between black and dark red.

In several tests, there were different treatments of the standard real or artificial haws, including: (1) one boring puncture by an *R. pomonella* female, without or with egg deposition, (2) 6 circles of the fruit surface by an *R. pomonella* female dragging her ovipositor after egg-laying (5–8 circles/female was the average number around a 14-mm fruit observed by Prokopy et al., 1976); (3) one pin prick with the point of a No. 0 insect pin to make a hole approx. 150 μm diam. (= diam. of fly boring puncture), (4) 6 circles of the fruit surface with a fine brush saturated with fresh haw juice. A boring puncture without egg was obtained by removing a female just as the egg entered the vagina. A boring puncture with egg but without subsequent ovipositor dragging was obtained by removing a female within 1 sec of the start of dragging. A fruit without boring puncture or egg but with marking pheromone was obtained by placing a small paper under the fore-tarsi of a female boring into another fruit and transferring her to the test fruit at the start of dragging. In addition, in some tests we used a fine brush to manually insert into a pin prick (1) one fresh *R. pomonella* egg taken from a ceresin wax artificial fruit (Prokopy and Boller, 1970), (2) one vigorous 1st instar *R. pomonella* larva newly hatched from an egg incubated in a petri dish, or (3) approx. 4 μl aqueous solution of *R. pomonella* marking pheromone. The pheromone was obtained by swishing 50 haws, exposed to approx. 100 mature females for 2 days, in 10 ml distilled water (a known solvent for the pheromone, Prokopy et al., 1976) for 1 min each. In some tests, fruits were kept dry at 26 °C, 100% relative humidity for 7 or 18 days from treatment until testing. After 18 days, infested fruits showed a characteristic amount of decay (extensive rotting of fruit interior, but fruit surface largely unaffected), while comparably held controls showed no decay.

For testing, mature (2- to 3-week-old) *Opius* females were gently trans-

ferred to empty 15 × 15 × 15-cm cages (1 female/cage) and allowed to rest approx. 1 hr. An untreated (i.e., control) haw was then offered by slowly bringing it to within approx. 5 mm of the front of the female and allowing her to walk on it. If she did so (acceptance), testing was begun by offering her, in random sequence, various treatments of fruit, with a 10-min interval between each. Data were recorded on duration of stay, duration of antennal tapping on the fruit surface, and number of ovipositor boring attempts. If she did not walk onto the initial control haw within 10 sec (rejection), she was offered a second one. If rejection again occurred, she was not assayed that day. In each experiment, a replicate consisted of acceptance of one fruit of each treatment by the same female. If at any time a real control haw was rejected, all data for that replicate were ignored. If an artificial haw was rejected, the data for that replicate were ignored only if the female failed to accept a standard untreated artificial haw offered at the end of the replicate. All of these procedures aided in standardizing assay females with respect to threshold level of host-seeking activity. Owing to the limited number of *Opius* available, we often assayed the same female on more than one replicate (but not more than four on the same day).

Van Lenteren and Bakker (1975) found that certain parasitoids could discriminate between parasitized and unparasitized hosts only after oviposition into an unparasitized one. By analogy, we considered that *O. lectus* and *O. alloeus* might be incapable of discriminating between control and host-infested haws unless they had prior ovipositional experience. Preliminary tests indicated this was not so. Hence, the assay females on any given day were not segregated according to previous experience.

Because the supply of real haws was limited, we were forced to use the same test fruit in more than one replicate (but not more than four). Price (1970) showed that some parasitoids deposit and recognize their own trail odors, enabling them to discriminate between presearched and unsearched areas and avoid the former. Preliminary tests using artificial haws suggested this was not the case for *O. lectus* (*O. alloeus* was not tested).

Deposition of oviposition-deterring marking pheromone following egg-laying is common among hymenopterous parasitoids (Vinson, 1976; Weseloh, 1976). Although direct evidence is lacking, this could be the mechanism mediating the tendency of *O. oophilus* to avoid superparasitism (Kaya and Nishida, 1968). It is unknown whether *O. lectus* deposits such a pheromone. But if so, its influence on *O. lectus* response through multiple presentation of the same fruit must have been minor owing to the infrequency of *O. lectus* oviposition (*O. lectus* often attempted boring into host-infested haws, but actual oviposition, as determined by dissection of host eggs and early larvae, was, for reasons unknown, less than 5% of boring attempts). Our experimental procedure did not take into account the possibility that *O. lectus*

might have deposited marking pheromone following boring attempts where no egg was laid.

All data were submitted to Student's *t* test or analysis of variance and Duncan's multiple range test at the 5% level.

RESULTS

The normal behavior of *O. lectus* after arrival on a haw infested by an egg or early larva of *R. pomonella* is very similar to that of *O. oophilus* (Haramoto, 1953). It consists initially of several seconds of walking, followed by tapping of the antennae on the fruit surface while continuing to advance, and finally, when the fly egg-laying puncture is found, extension of the ovipositor and boring into the fruit for approx. 3–130 sec. This is usually followed by additional bouts of antennal tapping and boring in or near the puncture, although some borings may occur in areas away from the puncture. The behavior of *O. alloeus* after arrival on a haw infested with late-instar *R. pomonella* larvae is similar, except that boring attempts are not centered around the fly egg-laying puncture.

Effect of Fruit Chemical and Physical Stimuli on O. lectus

Both chemical and physical characteristics of uninfested fruit were found to influence *O. lectus* behavior, as evidenced by the significantly greater duration of stay and/or antennal tapping on wax spheres enveloped by haw-exposed parafilm compared with clean parafilm, and on 15-mm black wax spheres compared with 15-mm white spheres (formed with undyed white ceresin wax), black rectangles, or 8-mm black spheres (Table 1, Exp. 1, 2). Very few *O. lectus* boring attempts occurred in any of the artificial fruits, possibly because certain stimulating fruit chemicals were absorbed in insufficient quantity by the haw-exposed parafilm. Interestingly, fruit chemical components, spherical shape, dark color, and appropriate fruit size also stimulate boring attempts by *R. pomonella* (Prokopy 1966, 1967; Prokopy and Bush, 1973).

Effect of Host Insect Stimuli on O. lectus

Stimuli associated with the presence of a newly laid host egg also influenced *O. lectus* behavior, as shown by the significantly greater duration of stay, antennal tapping, and boring attempts on a haw oviposited in and dragged on by *R. pomonella* compared with a control haw (Table 1, Exp. 3). A pin prick (presumably simulating a fly boring puncture) was somewhat

TABLE 1. BEHAVIOR OF *O. lectus* FEMALES ON FRUITS TESTED WITHIN 6 hr OF TREATMENT[a]

Exp.	Fruit	Treatment	No. repl.	Acceptance (%)	Mean duration (sec)[b]		Mean no. boring attempts[b]
					Stay	Ant. tapping	
1	R	Control	34	100	147.2a	60.2a	1.0a
	A	HEP	34	85	95.0b	15.2b	0.1b
	A	HEP+haw juice	34	68	62.7bc	7.8bc	0b
	A	CP	34	62	30.1c	1.0c	0b
2	A	Black sphere, 15 mm, HEP	33	85	94.3a	14.9a	0.1a
	A	White sphere, 15 mm, HEP	33	82	66.5b	9.5ab	0.1a
	A	Black rectangle, 16 × 20 mm, HEP	33	82	61.7b	2.7bc	0a
	A	Black sphere, 8 mm, HEP	33	68	33.0c	6.5bc	0a
	A	Black sphere, 15 mm, CP	33	42	19.4c	0.5c	0a
3	R	+bore, +egg, +drag	28	100	260.6a	69.3a	2.8a
	R	Control	28	100	88.9b	25.2b	0.3b
4	R	+bore, +egg, no drag	39	100	156.6a	49.1a	1.3a
	R	No bore, no egg, +drag	39	100	118.6b	38.8ab	0.6b
	R	Pin prick	39	100	79.2c	26.9bc	0.5b
	R	Control	39	100	67.1d	17.9c	0.1c

5	R	No bore, no egg, +drag by ♀ that oviposited in wax dome	29	100	160.0a	63.5a	1.4a
	R	Haw juice	29	100	101.9b	40.1b	0.6b
	R	Control	29	100	77.3b	27.5b	0.5b
6	A	HEP+drag	29	86	185.7a	26.2a	0.1a
	A	HEP	29	86	111.2b	15.7a	0.1a
	A	CP+drag	29	59	56.7c	1.6b	0a
	A	CP	29	62	35.3c	1.0b	0a
7	A	HEP+drag	12	100	94.6a	33.1a	0.1a
	A	HEP	12	100	49.3b	8.0b	0.1a
	A	CP	12	50	16.1c	0.3c	0a
8	R	Marking pheromone inserted manually in pin prick	34	100	272.0a	99.2a	1.2a
	R	+bore, +egg, no drag	34	100	253.1a	102.4a	1.2a
	R	+bore, no egg, no drag	34	100	251.4a	94.2ab	1.3a
	R	Egg inserted manually in pin prick	34	100	233.7ab	90.5ab	0.9ab
	R	1st-instar larva inserted manually in pin prick	34	100	181.2c	64.2cd	0.5bc
	R	Pin prick	34	100	202.9bc	80.3bc	0.6bc
	R	Control	34	100	117.1d	49.4d	0.2c

[a] Fruit: R = real; A = artificial. Treatment: Control = untreated haw; HEP = haw-exposed parafilm; CP = clean parafilm; bore, egg, and drag = boring puncture, egg deposition, and ovipositor dragging by *R. pomonella*.

[b] Any two means in the same column in the same experiment followed by the same letter are not significantly different at the 5% level.

TABLE 2. BEHAVIOR OF *O. lectus* FEMALES ON REAL FRUITS TESTED DIFFERENT TIMES AFTER TREATMENT[a]

Exp.	Treatment	Days, treatment to testing	No. repl.	Mean duration (sec)[b]		Mean no. boring attempts[b]
				Stay	Ant. tapping	
1	+bore, +egg, no drag (= 1st-instar larva)	7	25	162.0a	47.3a	1.7a
	+bore, +egg, no drag	0	25	140.0a	36.6ab	2.2a
	No bore, no egg, +drag	7	25	149.0a	28.5bc	0.5b
	Pin prick	7	25	98.9b	20.6cd	0.7b
	Haw juice	7	25	97.3b	15.2de	0.3b
	Control		25	68.5c	9.7e	0.4b
2	+bore, +egg, no drag	0	33	355.7a	134.2a	2.7a
	+bore, +egg, no drag (= 3rd-instar larva)	17	33	232.6b	57.9b	0.9b
	Control		33	197.3b	49.0b	0.6b

[a] See Table 1 for treatment code. 100% acceptance of all fruits.
[b] See footnote [b] of Table 1.

stimulating to *O. lectus*, but less so than a fly boring puncture containing an egg or depositions from fly ovipositor dragging (Table 1, Exp. 4; Table 2, Exp. 1).

Experiment 5 (Table 1) is conclusive proof that fly marking pheromone is the principal component of fly ovipositor dragging deposition which stimulates *O. lectus*, and that any haw juice deposited during such dragging would play a minor role. The stimulating effect of the pheromone lasts at least 7 days (termination of experiment) (Table 2, Exp. 1). Application of the pheromone to an artificial fruit enveloped by haw-exposed parafilm significantly enhanced *O. lectus* duration of stay and antennal tapping (Table 1, Exp. 6, 7). But when the pheromone was applied to artificial fruit enveloped by clean parafilm (Table 1, Exp. 6), or to clean 15-mm black wax spheres without parafilm (untabulated data), there was no significant stimulating effect on *O. lectus*.

Other evidence (Table 1, Exp. 8) suggests that fly marking pheromone (or some other water-soluble, fly-secreted substance) on the ovipositor and egg surface may be the principal insect-originating stimulus present in the boring puncture. Thus, a fly boring puncture without egg and a pin prick with marking pheromone solution inserted manually were both significantly stimulating to *O. lectus* compared with a pin prick alone. The water solubility of the pheromone or other fly substance would facilitate its diffusion from the egg surface to the tissue enveloping the boring puncture. A 1st-instar larva manually inserted in a pin prick proved no more stimulating than a pin prick alone.

Haws containing 1st-instar fly larvae originating from eggs laid therein elicited about the same level of *O. lectus* response as haws containing newly laid fly eggs (Table 2, Exp. 1). When haws containing 3rd-instar fly larvae originating from eggs laid therein were tested, they were no more stimulating than control haws and were significantly less stimulating than haws containing newly laid fly eggs (Table 2, Exp. 2). Less than 18 hr after testing, the larvae burrowed out of these haws to form puparia. In the approx. 1-hr interval between exit and puparial formation, several of these larvae were confined in a 5-cm-diam. petri dish with several *O. lectus* females, none of which tapped their antennae or attempted boring.

Effect of Host Insect Stimuli on O. alloeus

The types of host-insect-originating stimuli influencing *O. alloeus* (Table 3) were quite different from those influencing *O. lectus*. Thus, duration of stay, antennal tapping, and boring attempts of *O. alloeus* were all significantly greater on haws containing 3rd instar fly larvae than on control haws (Exp. 1). On the other hand, haws containing manually inserted fly

TABLE 3. BEHAVIOR OF *O. alloeus* FEMALES ON REAL FRUITS[a]

Exp.	Treatment	Days, treatment to testing	No. repl.	Mean duration (sec)[b]		Mean no. boring attempts[b]
				Stay	Ant. tapping	
1	+bore, +egg, no drag (= 3rd-instar larva)	17	12	490.8a	39.2a	15.8a
	Egg inserted manually in pin prick	0	12	269.9b	19.0b	1.8b
	1st-instar larva inserted manually in pin prick	0	12	275.1b	23.1b	2.3b
	Control		12	253.6b	14.5b	1.8b
2	+bore, +egg, no drag	0	41	260.1a	11.5ab	2.8ab
	No bore, no egg, +drag	0	41	269.5a	14.2a	3.4a
	Pin prick	0	41	254.7a	11.6ab	2.6ab
	Haw juice	0	41	267.1a	14.0a	2.7ab
	Control		41	265.2a	10.2b	2.2b
3	No bore, no egg, +drag	0	26	250.3a	22.2a	3.8a
	Control		26	270.4a	20.1a	4.0a

[a] See Table 1 for treatment code. 100% acceptance of all fruits.
[b] See footnote [b] of Table 1.

eggs or 1st-instar larvae, fly-inserted eggs, or pin pricks were no more stimulating than controls to *O. alloeus* (Exp. 1, 2). Haws surface-marked with fly ovipositor draggings stimulated slightly (although significantly) more *O. alloeus* antennal tapping and boring attempts than control haws in one test (Exp. 2) but not in another (Exp. 3). However, the ovipositor-marked haws were no more stimulating than haws circled by haw juice (Exp. 2). Hence, the true stimulus in this experiment may have been haw juice deposited during ovipositor dragging rather than fly marking pheromone, although we have no direct evidence that haw juice is in fact deposited during dragging.

DISCUSSION

Our findings show that after an *O. lectus* female has arrived on a fruit infested with a host egg or 1st-instar larva, a constellation of stimuli function to retain it and elicit antennal tapping and boring attempts. These include: unidentified fruit chemical components; characteristic fruit shape, size, and color; fly marking pheromone on the fruit surface; a real or simulated fly boring puncture; and fly marking pheromone or other fly-secreted substance in the boring puncture. The possible influence of fruit chemical and physical characteristics on the behavior of *O. alloeus* females was not tested, but such females were highly stimulated to tap their antennae and bore by the presence of a 3rd-instar larva.

This study on the influence of *R. pomonella* marking pheromone on *O. lectus* behavior is the first demonstration of an oviposition-deterring pheromone in a phytophagous insect serving as a kairomone (Nordlund and Lewis, 1976; Blum, 1977) to one of its parasitoids. The fly pheromone trail on the fruit surface is laid down in apparently random fashion, repeatedly crossing itself. This precludes the likelihood that an *O. lectus* female could readily follow the trail to its origin at the boring puncture. Indeed, we observed no such instance of apparent following of a pheromone-trail. Rather, the pheromone trail serves principally to retain *O. lectus* on the fruit and stimulate antennal tapping. The tapping and boring attempts are particularly intensive at or very near the fly boring puncture, which is the site of fly egg deposition and early larval instar feeding activity. The 7-day (and probably longer) duration of pheromone effectiveness is well beyond the approx. 3 days required for *R. pomonella* egg hatch and well into the approx. 5-day developmental period of 1st-instar larvae. While the pheromone is an important stimulus to *O. lectus*, it is not required for location of a fruit puncture or initiation of boring, as some boring was attempted in a pin prick in fruits devoid of insect-originating stimuli. Interestingly, the

pheromone proved stimulating to *O. lectus* only when on a surface emitting fruit chemical stimuli and not in the absence of such.

The pheromone had no detectable influence on the behavior of *O. alloeus*. This is not surprising, since the principal hosts (3rd-instar larvae) of *O. alloeus* are not present in the fruit until approx. 13 days after egg deposition, when the pheromone is likely to have somewhat dissipated.

The possible use of kairomones for management of entomophagous insects in pest suppression programs is currently being elegantly explored by Lewis, Jones, Nordlund, and Gross in their studies of *Trichogramma* responses to moth kairomones. They demonstrated that the amount of *Trichogramma* parasitization of moth eggs was markedly increased by spraying a test field with the kairomone tricosane (Lewis et al., 1975a). They further found that the kairomone increased parasitization by *Trichogramma* by retaining it in the field and releasing and continuously reinforcing an intensified searching behavior rather than by attracting it directly to the host (Lewis et al., 1975b). Our results suggest that the rather stable, weakly volatile oviposition-deterring pheromone of *R. pomonella* influences *O. lectus* behavior in a similar manner that tricosane influences *Trichogramma*. It is therefore conceivable that application of synthetic *R. pomonella* pheromone (as yet unidentified) to host plants would not only deter *R. pomonella* females from ovipositing (Prokopy, 1972; Prokopy et al., 1976; Katsoyannos and Boller, 1976) but might also increase *O. lectus* parasitization of the egg–early larval progeny of nondeterred females. In combination with traps to capture out deterred females (Prokopy, 1975a), this could constitute a multifaceted, noninsecticidal approach to *R. pomonella* management. This same approach might also be rewarding for management of other pestiferous tephritids of the genera *Rhagoletis*, *Anastrepha*, and *Ceratitis* wherein the occurrence of oviposition-deterring pheromone has been demonstrated (Cirio, 1972; Katsoyannos, 1975; Prokopy, 1975b; Prokopy et al., 1976, 1977 1978). *O. oophilus*, capable of parasitizing a high percentage of *C. capitata* eggs (Bess, 1953), would appear especially promising for evaluation in this regard.

While the above approach involving *O. lectus* in an *R. pomonella* suppression program is conceivable, it is not free of difficulty, and will require considerable further investigation.

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FACTORS INVOLVED IN THE RESPONSES OF MALE GERMAN COCKROACHES TO SYNTHETIC SEX PHEROMONE

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Abstract—The precopulatory response of male German cockroaches, *Blattella germanica*, to synthetic contact sex pheromone (compound A) and analoge I increases with concentration, is greatest during the dark phase of the photocycle, and is enhanced by isolation of males from females of this species.

Key Words—Sex pheromone, *Blattella germanica*, photocycle, habituation, behavior, bioassay.

INTRODUCTION

The two components (3,11-dimethyl-2-nonacosanone and 29-hydroxy-3,11-dimethyl-2-nonacosanone) of the sex pheromone of the German cockroach, *Blattella germanica*, have been characterized (Nishida et al., 1974, 1976a) and synthesized (Nishida et al., 1975, 1976b; Burgstahler et al., 1975, 1977; Schwarz et al., 1975; Rosenblum et al., 1976). All papers published to date have employed a behavioral bioassay to estimate the activity of both purified pheromones and synthetic products. In some cases analogs of the pheromone were tested as well as the pheromones in an attempt to determine which chemical groups are responsible for activity (Sato et al., 1976; Burgstahler et al., 1975). Bioassays of identical *B. germanica* synthetic sex pheromones by the above-mentioned research groups have yielded conflicting results which may be due to differences in bioassay methodology. We therefore

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report investigations on the effects of isolation (from normal colony conditions), photoperiod, and habituation as possible factors that influence the response of males and must be considered in designing bioassays for cockroach sex pheromones.

METHODS AND MATERIALS

Animal Maintenance

Adult male *B. germanica* were removed from laboratory colonies and maintained in isolation from females for 2, 9, or 24 days prior to testing. Experimental cockroaches were housed and tested in groups of 5 in $30 \times 19 \times 12.5$ cm clear-polypropylene-covered cages; the sides of the containers were coated with a 1:1 mixture of mineral oil and petroleum jelly to prevent escape. A constant supply of food (Purina Lab Chow Pellets) and water was available in the containers during the isolation and testing periods. Factors considered in determining the appropriate number of cockroaches per container were (1) minimizing group effects (Bell et al., 1974) and (2) facility of visual observation (Rust, 1976). All testing was performed in feces-conditioned containers to preclude stress of the type previously observed when male *Periplaneta japonica* were introduced into clean containers (Takahashi and Kitamura, 1972).

Experimental cockroaches were maintained and tested under a 12-hr light, 12-hr dark cycle at 22 ± 2°C and 60% relative humidity. Tests were performed in a room totally devoid of female cockroaches 2–4 hr after the onset of the dark period (unless otherwise stated); GE Ruby photographic lamps (595–680 nm) were used to illuminate the observation room (Barth, 1964).

Preparation and Application of Test Solutions

Synthetic sex pheromone (compound A = 3,11-dimethyl-2-nonacosanone) and an analog (3-methyl-2-heneicosanone) (Burgstahler et al., 1975, 1977) were prepared as stock solutions in CCl_4 at a concentration of 9.9 mg/ml; serial dilutions in CCl_4 of 1:10, 1:100, and 1:1000 were tested for each compound. Previous studies have shown that courting behavior in the German cockroach includes antennation of the female by the male, presumably allowing the latter to perceive sex pheromone on the cuticular surface of the female (Roth and Willis, 1952).

In bioassays used here freshly ablated *Periplaneta americana* adult male antennae, fixed to 20-cm glass rods with white (milk-base) glue, were dipped into the test solutions for 1–2 sec; these were allowed to dry for 30 sec prior

to use. A fresh antenna was prepared for each compound, dilution, cage, and 5 min test. Antennae ablated from *P. americana* males were used to eliminate chemical stimuli but to retain any nonchemical stimuli associated with antennae in general.

Antennation Bioassay

Cockroaches were observed for a 1-min basal period immediately preceding testing; no spontaneous precopulatory behavior (wing-raising, running, or turning) was observed during these periods. Antennae were dipped in CCl_4, allowed to dry, and touched to antennae of males; as above, no sexual behavior was elicited. After basal and control tests, fixed antennae dipped in pheromone solutions were touched for 1–2 sec to the antennae of each of the 5 males in a test cage; antennal contact was repeated at random once for each male during the first 20 sec of each min of testing. All assays were performed for a series of 5 sequential minutes. The number of individual males that exhibited wing-raising after contact with the manipulated antenna was recorded per minute of testing.

Techniques for Measuring Variation in Responses at Different Times during the Photocycle and After Repeated Exposure to Sex Pheromone

Experiments to determine the effects of repeated exposure to sex pheromone varied from the procedures described above as follows: (1) Male cockroaches used in these assays were isolated from female *B. germanica* for 7 days, (2) tests were performed between 2 and 4 hr after the onset of the dark or light phase of the photocycle. For tests of habituation, cockroaches were tested continuously during 5-min sequences; a 1-min interval occurred between testing periods, during which a new antenna for the next 5-min period was prepared. All tests for both photocycle and habituation experiments were performed with a stock solution of compound A at a concentration of 9.9 mg/ml.

RESULTS

Effects of Different Pheromone or Analog Concentrations and Duration of Isolation on Responses of Males

To analyze the data obtained (Table 1) a preliminary chi-square analysis was first performed to determine if the effects of concentration and isolation were independent. The null hypothesis that there was no interaction effects was retained, which showed that there was no multiplicative or interactive

TABLE 1. MEAN FREQUENCIES OF WING-RAISING PER CAGE OF FIVE MALE *B. germanica*[a]

	Days of Isolation			
Conc.	2	9	24	Total
Compound A				
1.00	5.2	11.6	16.7	33.5
0.1	5.6	7.8	10.2	23.6
0.01	3.2	4.3	8.0	15.5
0.001	0.0	.9	3.2	4.1
Total	14.0	24.6	38.1	76.7
Analog I				
1.00	1.4	6.3	17.1	24.8
0.1	.3	9.6	14.2	24.1
0.01	1.5	1.6	0.5	3.6
0.001	0.0	0.0	0.3	.3
Total	3.2	17.5	32.1	52.8

[a] 1.00 concentration equals 9.9 mg/ml. Mean frequency represents mean wing-raising for 5 males per minute of testing.

effects of these two parameters at the 0.05 level. Therefore, isolation time and concentration could be analyzed separately. If isolation time had no effect (null hypothesis), then responses should be equally distributed over the three test periods; similarly, if concentration had no effect, responses should be equally distributed over the four concentrations used. Chi-square analyses for each factor showed that each variable significantly enhanced responses of males ($P < 0.005$) for both the pheromone and the analog.

Responses during Photocycle and Effects of Habituation

To analyze the differences in responses during the light and dark time periods and to test for habituation, the following statistical measures were used. First, a Friedman two-way analysis of variance was performed to test the null hypothesis that over the 48-min period there was no change due to habituation to either dark or light groups. The hypothesis was rejected at the $P < 0.005$ level, showing that significant differences did exist over the 48-min testing period. Next, a Wilcoxson matched-pairs signed ranks test was used to determine significant differences between time periods for dark and light groups. The results (Table 2), show that there is a significant decline over the 48-min testing period even though there are not significant differences between all time periods.

TABLE 2. PERCENT RESPONSES[a] OF MALES TO SEX PHEROMONE A IN DARK AND LIGHT PHASES OF PHOTOCYCLE[b] AND UNDER CONDITIONS OF REPEATED STIMULATION[c]

	Periods of stimulation (min)					
Condition	0–5	6–11	12–17	27–32	33–38	43–48
Light	50.0^{a}*	60.4^{a}†	54.4^{a}‡	49.2^{a}*	47.6^{a}‡	41.2
Dark	65.6^{a}*	68.8^{a}*	70.0^{a}‡	56.8^{a}*	52.8^{a}‡	48.8

[a] Percent response equals the % of males wing-raising in a given 5-min period; for each period 50 males were tested, thus there was a possibility of 250 positive responses. Levels of significance between responses at each time interval; the following symbols represent significance values between the interval with the symbol and the following interval: *a = N.S., †b = $P<0.02$, †c = $P<0.05$.

[b] Tests were performed during hours 2–4 after the onset of either the light phase or dark phase of a 12:12-hr photocycle. Males were employed which had been isolated for 3–9 days. Synthetic compound A was used at 9.9 mg/ml.

[c] The same males were repeatedly tested over a 48-min period as described in the Methods and Materials, with 1-min interval between tests 1 and 2, 2 and 3, 4 and 5; 10-min intervals between tests 3 and 4; and 5-min intervals between tests 5 and 6.

A binomial test was used to determine differences in responses between the dark and light periods. Given the six time period groups, all responses were greater in the dark than in the light; therefore the test showed a significant difference ($P = 0.016$) between all responses in the dark vs. light.

DISCUSSION

In testing sex pheromone compounds in bioassays researchers often choose experimental conditions and test animals that tend to produce optimal results. In some insects the parameters important for responsiveness are well known and have been considered in designing such bioassays. This is true for the Lepidoptera, for example, where responsiveness by males of many species is known for such variables as age, time during the photocycle when tests are performed, and the possible effects of habituation (Shorey, 1976).

Some work has been completed along these lines in cockroaches. In *P. americana*, for example, responses of males to sex pheromone are significantly greater during the dark phase of the photocycle than in the light phase (Hawkins and Rust, 1977). It is also known that total habituation to sex pheromone (i.e., no response) occurs within 30 min of exposure in *P. americana* (Hawkins and Rust, 1977) and in *Byrsotria fumigata* (Bell et al., 1974). In *B. germanica*, habituation seems to have less of an effect than in the other two species studied, the responsiveness of males to sex pheromone never reaching zero during the 48-min test period.

An important biological question raised by this study concerns the duration of isolation of males from females. When making an assessment of

the efficacy of cockroach sex pheromones, should an effort be made to assay within normal thresholds of the cockroach? Assays that generate positive responses using males isolated for long periods of time are probably not valid in determining meaningful lower threshold responses (Silverman, 1977). Isolation itself, as shown by the data, is responsible for enhanced responses to pheromones. Perhaps more meaningful results could be obtained with males that are not isolated at all. For example, positive results would not have been obtained for undiluted analog I unless the test males were isolated for at least 9 days, and the lowest effective concentration for synthetic A was 0.099 mg/ml unless males were isolated for at least 24 days.

In conclusion, if results of bioassays are to be comparable, then there must be standardization with regard to duration of subject isolation, periods between testing, time of testing, and methods used in a bioassay. In cases where a pheromone is proposed for employment in biological control, the duration of subject isolation is especially important, since in natural habitats isolation from females probably does not occur, thereby leading to spurious predictions for trapping efficiency by pheromones and related compounds.

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HIGH-PRESSURE LIQUID CHROMATOGRAPHIC TECHNIQUES FOR THE SEPARATION AND QUANTIFICATION OF NORSESQUITERPENES FROM GYRINIDS

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Abstract—A rapid, sensitive, stable, and quantitative high-pressure liquid chromatographic technique was developed for the analysis of the norsesquiterpenes, gyrinidal, isogyrinidal, gyrinidone, and gyrinidione. Methods were developed for both normal-phase (μPorasil) and reverse-phase (μBondapak) columns. The applicability of this technique was demonstrated when the norsesquiterpenes of the defensive secretions of the water beetle *Gyrinus frosti* Fall were isolated and identified as gyrinidal and isogyrinidal. This method was then used for the quantification of the norsesquiterpene titer of *G. frosti* and *Dineutus assimilis* (Kirby).

Key Words—Gyrinidae, high-pressure liquid chromatography, norsesquiterpenes, *Gyrinus frosti, Dineutus assimilis,* gyrinidal, isogyrinidal, gyrinidione, gyrinidone.

INTRODUCTION

Four norsesquiterpenes have been identified from the pygidial defensive secretions of adult whirligig beetles (Coleoptera: Gyrinidae) as gyrinidal, isogyrinidal, gyrinidone, and gyrinidione (Schildknecht et al., 1972a,b; Wheeler et al., 1972; Meinwald et al., 1972; Miller et al., 1975). These norsesquiterpenes have been shown to act as feeding deterrents to several species of fish and to the common newt (Benfield, 1972; Meinwald et al., 1972) and have been demonstrated to have anesthetic and toxic activity to minnows (Miller et al., 1975; Miller and Mumma, 1976a,b). These norses-

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quiterpenes are unstable in aqueous solutions, when exposed to light, or at elevated temperatures in the presence of metal (Miller et al., 1975). Isolation techniques have usually involved thin-layer (TLC) and gas–liquid chromatography (GLC), but both techniques have disadvantages. With TLC gyrinidal is partially isomerized to isogyrinidal on exposure to light. All-glass GLC systems must be used to eliminate thermal degradation.

Quantitation of the levels of defensive compounds in gyrinids have been reported in a few species (Schildknecht et al., 1972a; Miller et al., 1975) and sexual differences in *Dineuteus assimilis* Kirby have been reported (Miller et al., 1975).

This paper reports the development of a high-pressure liquid chromatographic (HPLC) technique for the separation of the norsesquiterpenes. This technique was then used in the isolation and identification of the norsesquiterpenes of *Gyrinus frosti* Fall, a hitherto unstudied beetle. Application of this procedure was demonstrated by the determination of the defensive titer of the norsesquiterpenes from *G. frosti* and *D. assimilis*.

METHODS AND MATERIALS

Collection and Extraction

Two species of gyrinid beetle, *D. assimilis* and *G. frosti*, were collected by the hundreds from small ponds located around State College, Pennsylvania. The beetles were placed in a beaker of tap water and electrically shocked (milked) with 90 volts DC in order to obtain their defensive compounds (Miller et al., 1975). The defensive agents were then extracted from the water with chloroform and concentrated under nitrogen. *D. assimilis* were electrically shocked individually in separate containers, whereas *G. frosti* were shocked in groups of four or five beetles.

Chromatography

TLC employed a stationary phase of 0.25 mm of Supelcosil 12A plus a zinc silicate phosphor (Supelco, Inc., Bellefonte, Pennsylvania) applied to 20×20 cm glass plates. Two solvent systems were used: (1) benzene–ethyl acetate (70:30, v/v) and (2) cyclohexane–ethyl acetate (50:50, v/v). The compounds were detected with a H_2SO_4–methanol (50:50, v/v) spray and charred at 110°C or by spraying with a 0.4% solution of 2,4-dinitrophenylhydrazine in 2 N HCl. GLC was performed with a Microtek 220 instrument using flame ionization for detection. Separations were developed for both a 1% OV-210 phase on a 100/120 mesh Supelcoport support employing a

nitrogen flow of 60 ml/min and a temperature program of 170–180°C at 1°/min, and a 2% OV-1 phase on 100/120 mesh Supelcoport support employing the same nitrogen flow rate and a temperature of 160°C. All-glass columns (1.8 m × 4 mm ID) were used which contained 160 cm of packing.

HPLC was performed on a model ALC/GPC 244 high-pressure liquid chromatograph with model 6000A pumps, U6K injector and model 440 absorbance detector (Waters Associates, Milford, Massachusetts). The UV detector monitored 254 and 313 nm simultaneously. A 4-mm ID × 30-cm μPorasil column (Waters Associates), using 80% chloroform–20% hexane at a flow rate of 0.8 ml/min, was used for normal-phase separations. For reverse-phase separations, a 4-mm ID × 30-cm μBondapak C_{18} column (Waters Associates), using 35% acetonitrile–65% water at a flow rate of 1.5 ml/min, was employed.

All solvents were filtered (0.45 μ Millipore filter) and degased before use. The chloroform contained 1% ethanol as a preservative. Solvents were of highest purity (Burdick and Jackson Laboratories, Inc., Muskegan, Michigan).

Spectroscopy

A Gilford scanning spectrometer (model 250) was used to record the UV spectra of the compounds between 200 and 360 nm. Low-resolution mass spectra were taken on a LKB-9000 gas–liquid chromatograph interfaced mass spectrometer using a 9-ft × 3/16-in. OD glass column packed with 1% OV-210 on a 100/200 mesh Supelcoport support. A helium flow of 30 ml/min and a column temperature of 180°C were used. Mass spectra were taken at an ionizing potential of 70 eV.

Quantitation

The UV absorbance monitor of the HPLC was connected with a Spectra-Physics Autolab Minigrator used for determining peak areas. Known quantities of isolated purified norsesquiterpenes were prepared based on the reported UV molar absorbitivity (ε) values (Miller et al., 1975). These were used to construct standard curves used in translating the peak areas into microgram values.

RESULTS AND DISCUSSION

HPLC of Norsesquiterpenes

Various solvents, combinations of solvents, and types of columns were

evaluated for their affect on the HPLC of norsesquiterpenes from the gyrinid defensive secretions. These investigations have evolved two methods for the separation of four norsesquiterpenes, one employing a normal-phase column (μPorasil) and one employing a reverse-phase column (μBondapak C_{18}). Figure 1 shows the separation of gyrinidal, isogyrinidal, gyrinidone, and gyrinidione, which were isolated from the gyrinid *Dineutus assimilis*, by HPLC using an acetonitrile–water solvent and a μBondapak C_{18} column. These same norsesquiterpenes were also separated by HPLC employing a chloroform–hexane solvent and a μPorasil column (Figure 2). The latter method was the preferred method for the preparation of milligram quantities because of the ease of evaporation of the solvent. Both methods give good resolution, are rapid, do not result in any noticeable decomposition or isomerization, and can readily be used in quantification of norsesquiterpenes. The chloroform extract of the defensive secretion can be directly analyzed without any prior clean-up. These procedures should be applicable to the analysis of the defensive agents of hitherto unstudied gyrinids.

The HPLC method, employing a UV absorbance detector, is found to be much more sensitive to these norsesquiterpenes than are the flame ionization detectors of GLC. The UV detector, utilizing the high molar absorbitivity

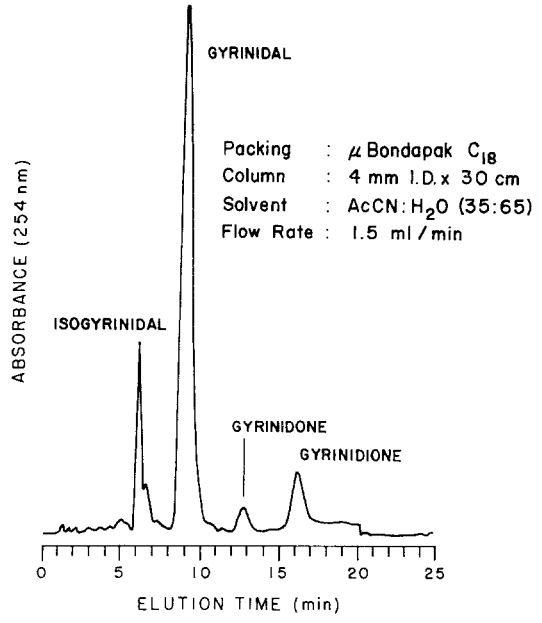

FIG. 1. HPLC separation of the norsesquiterpenes employing a μBondapak C_{18} column.

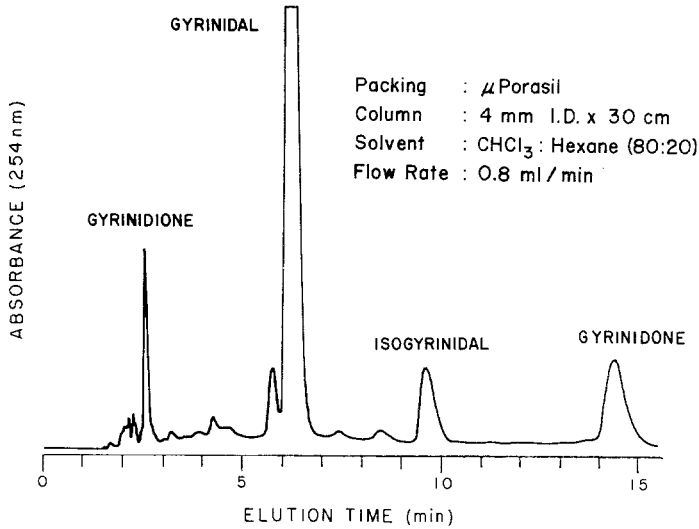

FIG. 2. HPLC separation of gyrinid norsesquiterpenes employing a μPorasil column.

(ε) values of these compounds, can detect norsesquiterpenes in the 5 to 10-ng range. The sensitivity of our flame ionization detector ranges from 50 ng for gyrinidone to 1000 ng for gyrinidal and isogyrinidal (Table 1).

Defensive Compounds of G. frosti

When electrically shocked, *G. frosti* secretes a light yellowish paste from its pygidial defensive gland, as is common of other gyrinids. Using the

TABLE 1. COMPARISON OF THE LOWER SENSITIVITY LIMITS FOR GYRINID NORSESQUITERPENES USING GLC AND HPLC

	Minimum detectable amount		
	GLC[a]	HPLC[b]	
Compound	(ng)	ng	nm
Isogyrinidal	1000	10	254
Gyrinidal	1000	5	254
Gyrinidione	100	10	254
Gyrinidone	50	10	313

[a] Flame ionization, 2% OV-1 column.
[b] μBondapak C_{18} column.

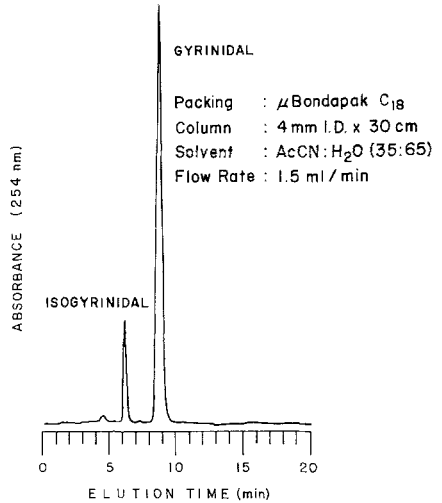

FIG. 3. Separation of the norsesquiterpenes isolated from *Gyrinus frosti* Fall.

HPLC methods described above, the chloroform extracts of these secretions were analyzed (Figure 3) and milligram quantities of the major components were collected for further analysis. Comparison of retention times with norsesquiterpene standards on both the μPorasil and μBondapak C_{18} columns suggests the presence of only two major components in *G. frosti*, gyrinidal and isogyrinidal. The two components from *G. frosti* also cochromatographed on GLC and TLC with authentic gyrinidal and isogyrinidal. The components from *G. frosti* gave ultraviolet absorptions of $\lambda_{max}^{EtOH} = 238.5$ and $\lambda_{max}^{EtOH} = 237.0$ nm which are consistent with gyrinidal and isogyrinidal (Miller et al., 1975), respectively. The mass spectra of these two components were also identical and congruent with the mass spectra of authentic gyrinidal and isogyrinidal. Collectively, these data indicate that the defensive secretions of *G. frosti* contain only two major norsesquiterpenes, gyrinidal and isogyrinidal, and no detectable amounts of gyrinidone and gyrinidione (<10 ng/beetle). These findings are consistent with results of other investigators (Miller et al., 1975; Meinwald et al., 1972; Schildknecht et al., 1972a) in that the genus *Gyrinis* has not been reported to possess gyrinidone and gyrinidione.

The quantitation of the norsesquiterpenes of *D. assimilis* and *G. frosti* by HPLC is presented in Table 2. Although *D. assimilis* were analyzed individually, no statistical differences between sexes were found as has been previously reported (Miller et al., 1975). Also, the relative amount of the four norsesquiterpenes in *D. assimilis* differ from previous studies (Miller et al.,

TABLE 2. QUANTITATION OF GYRINID NORSESQUITERPENES BY HPLC[a]

Species	Total per beetle (μg)	Norsesquiterpenes/beetle (μg)			
		Isogyrinidal	Gyrinidal	Gyrinidone	Gyrinidione
D. assimilis[b]	241.6 ± 32.5	5.1 ± 0.8	91.1 ± 8.8	9.1 ± 2.4	136.4 ± 24.5
G. frosti[c]	44.2 ± 8.6	1.7 ± 1.3	42.4 ± 7.7	ND[d]	ND[d]

[a] μBondapak C_{18} column, all compounds quantified at 254 nm except gyrinidone which was at 313 nm. Beetles collected May 3, 1977.
[b] Quantified individually, av. wt. = 78.7 mg.
[c] Quantified in groups of 5 beetles, av. wt. = 17.1 mg.
[d] Not detected (< 10 ng).

1975). Both isogyrinidal and gyrinidone are present in lower amounts and gyrinidione is present in somewhat higher amounts than previously reported. These differences may reflect seasonal variations in the defensive titer of this species as has been shown to occur in dytiscids (Miller and Mumma, 1974). It should be pointed out that both studies confirm the existence of a large variation in titer between individual *D. assimilis*. This large individual variation can also be seen in *G. frosti*.

Another finding of this quantitation study is the similarity of the amount of norsesquiterpene per weight of beetle for the two species. Although *G. frosti* is a much smaller beetle (average weight 17.1 mg) than *D. assimilis* (average weight 78.6 mg), it contains 2.6 μg norsesquiterpene per mg beetle weight compared to 3.1 μg per beetle weight for *D. assimilis*.

The development of this HPLC method has improved the analysis and purification of gyrinid norsesquiterpenes. It should now be applicable to the study of new gyrinid species and serve as a basis for further studies of the seasonal titer of these beetles.

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CEREAL LEAF BEETLE RESPONSE TO BIOCHEMICALS FROM BARLEY AND PEA SEEDLINGS. I. CRUDE EXTRACT, HYDROPHOBIC AND HYDROPHILIC FRACTIONS[1,2]

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Abstract—To identify contact feeding stimulants for adult cereal leaf beetles (CLB), *Oulema melanopus* (L.), compounds extracted from a host plant, *Hordeum vulgare* L. (cultivar 'Lakeland'), were bioassayed in 3% agar. Damage to agar gel with the hydrophilic fraction was dominated by narrow channels; the hydrophobic fraction gel was dominated by bites, rashes, and shorter channels. This difference probably reflected the functional nature of compounds in each fraction. Crude extract and the hydrophobic fraction of nonhost pea seedlings, *Pisum sativum* L., prevented CLB feeding behavior, but the hydrophilic fraction only reduced it. Combination of barley and pea crude extracts, by weight at 1:1 or greater, evoked a small feeding response which indicated a host-specific recognition factor in the barley.

Key Words—Cereal leaf beetle, barley, feeding stimulants, bioassay, *Oulema melanopus*, *Hordeum vulgare*.

INTRODUCTION

It is well recognized that phytophagous insects have a preferred range of host plants and that an understanding of this relationship ultimately would

[1] Michigan Agricultural Experiment Station Journal Article No. 8227.
[2] The use of a trade name in this paper does not constitute an official endorsement or approval by the USDA.

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be found at the molecular level (Verschaffelt, 1910; Dethier, 1947). Two views based on the chemistry of the plant have been proposed to explain this host specificity. Fraenkel (1959) suggested that the "secondary substances" variously associated with plant taxa were alone responsible for both susceptibility and resistance. This position became untenable as originally worded (Fraenkel, 1969) and was opposed by those who felt that the "sapid nutrients" (Thorsteinson, 1958) were heavily involved in the feeding response and host selection (Thorsteinson, 1960; Beck, 1965). The historical development of research and theory in this field is outlined in several reviews, i.e., Dethier (1947), Thorsteinson (1960), Kennedy (1965), Beck (1965), Hsiao (1972), Schoonhoven (1972a,b).

The cereal leaf beetle, *Oulema melanopus* (L.) (CLB), a chrysomelid pest of the plant family Gramineae, was introduced from Europe to Michigan in the late 1950s (Castro et al., 1956). The first published effort to clarify the chemical ties between the CLB and a host plant, barley, *Hordeum vulgare* L., was that of Panella et al. (1974). While their attempt to demonstrate an olfactory attractant for the CLB was unsuccessful, they did show that an agar-based bioassay could be used to investigate CLB contact feeding stimulants.

Under their conditions, the CLB distinguished among several sugars, showing a clear preference for sucrose, but their dichloromethane extract of barley seedlings was not fractionated to isolate feeding stimulants. The present study was therefore undertaken to clarify the phytochemical ecology of the CLB feeding response on seedling barley.

METHODS AND MATERIALS

The experimental procedure employed was to subdivide those fractions of barley seedling extract (*H. vulgare*, cultivar 'Lakeland') which evoked a feeding response in the CLB until individual stimulant compounds were isolated and identified. A nonhost, the garden pea, *Pisum sativum* L., was studied in parallel, partly as an experimental control. Five other nonhosts were each bioassayed once to confirm the specificity of positive responses to barley (see *Other Plants* below).

Beetles. The cereal leaf beetles used in this study were provided by the Entomology and Small Grains Laboratory of the U.S. Department of Agricultural, Agricultural Research Service, East Lansing, Michigan. Adults, newly emerged from the pupal cell (except as noted), were collected daily at about 5:00 PM. They were maintained overnight under 10-cm-square plastic refrigerator boxes (fewer than 200 per box) inverted over, but separated from, a clean glass plate by a piece of nylon screen. An 11-cm circle of filter

paper was placed between the inverted box and the screen, and a slight excess of distilled deionized water was applied to the paper at 5:30 PM and again at 9:45 AM the following day. A 75-watt incandescent bulb controlled by a 24-hr automatic timer was used to provide a 16-hr photophase.

Barley. Lakeland is a winter feed barley released by Michigan State University in 1967. Approximately 60 seeds of this cultivar were sown per plastic pot (8.9 cm diam.) containing a mixture of three parts soil, three parts peat, and one part sand, which had been sterilized for 2 hr by injected steam. The pots were placed in a greenhouse with a 16-hr daily photophase at 17°/21°C day/night temperature. The seedlings were taken to the laboratory when the second leaf was approx. 1.0–1.5 cm in length, usually 8–10 days after planting. All harvests were made at 11:00 AM $\pm$ 5 min, just prior to extraction.

Pea. Five seeds of the cultivar, 'Yellow Wonder,' were planted per pot (8.9 cm diam.) and grown in the same conditions as the barley. When 6–8 cm in length, all above-ground structures were harvested just prior to extraction.

Other Plants. Leaves from several nonhost plants were obtained from garden grown crops raised free of pesticides. These nonhosts were, pepper, *Capsicum annum* L.; broccoli, *Brassica oleraceae italica* Plenck; kohlrabi, *B. oleraceae* var. *gongylodes* L.; sunflower, *Helianthus annuus* L.; and tomato, *Lycopersicon esculentum* Mill. The leaves were frozen at —20°C until used.

Bioassay. Except where noted, experiments were conducted in a randomized, complete block design with blocks being days 1 through 4. There was one replication (test unit) per treatment per day.

Treatments consisted of a specific plant fraction alone or combined with some other chemical(s) at a known concentration in a 3% agar gel. Each day, fresh test units were prepared from these treatments for bioassay.

A test unit consisted of a plastic Petri dish (13 $\times$ 90 mm) inside of which two gel strips (approx. 6.5 $\times$ 0.15 $\times$ 0.6 cm) were positioned in the center to form a closed elliptical circle. Generally, one strip was a control and the other was a test strip, although two strips from the same treatment were sometimes used. Three "X" marks were applied to the lower exterior of the dish and the test strip was placed over these marks.

To prepare a treatment gel, 1.5 g of Bacto-Agar was added to 52 ml of hot distilled deionized water (less the volume added with an aqueous extract) at approx. 90°C. The test solution was added to the boiling agar and the mixture was stirred until solvent odor could not be detected. It was determined that approx. 2 ml of water vaporized in preparation of the gel to give a final weight, less the added extract, of 51.5 g.

The hot agar was poured into a glass Petri dish (15 $\times$ 100 mm) and immediately covered with the top in reversed position to prevent excess

condensation from collecting on the inside of the cover. After the gel had set, the cover was positioned normally and the dish refrigerated at 4–6°C. Treatments generally were made up 2–3 hr before use, occasionally on the evening before. Control gel was prepared by adding pure solvent equal to the greatest volume of test solvent used in that particular experiment.

At 12:50 PM ± 5 min daily, 25 newly emerged and unfed (except as noted) adult CLBs of undetermined sex were placed in the center of the agar-gel ellipse. The cover was positioned and the test units were then completely enclosed in a shallow cardboard box (capacity of 6 test units), the lights of the windowless room turned off, and the doors closed. Room temperature was approximately 23°C. At the end of each hour for three consecutive hours, a tally was made of beetles with at least the head in contact with each agar strip. Counts were recorded separately for the test and control strip.

The first count was made in the darkened room by light from a 25-watt bulb in a darkroom lamp equipped with a Kodak Safelight No. 2 filter which passed only light above 640 nm. The remaining two counts were quickly made in normal room light and the room was darkened again after the second of the three counts.

The mean of the hourly counts per treatment during the test period was determined and this average provided one index of response. Except as noted, another index was obtained by examining the gel strips under a dissecting microscope after each day's test and rating the damage in 0.1 units on the following scale:

0 = not damaged
1 = less than 1/4 of one side showed feeding damage
2 = approx. 1/4 of one side showed feeding damage
3 = approx. 1/2 of one side showed feeding damage
4 = most of one side well damaged
5 = most of one side plus 1/2 second side showed damage
6 = 80–100% of entire strip damaged

That average value for the experiment provided the activity index per treatment.

Curvilinear curve fitting was required for CLB count response to concentration for the crude extract and also the hydrophobic and hydrophilic fractions of barley. A program (No. A309362 46A) from the Hewlett-Packard BASIC Program Library Handbook was modified for a Digital PDP-11/40-RSTS computer by one of the authors (RAL) for this purpose.

Extraction of Plants. All organic solvents used were analytical grade which had been glass-distilled. Extracts in organic solvents were stored under nitrogen below 0° until analyzed. Aqueous samples were refrigerated at 4–6°C.

Crude extract. Seedling barley (10–12 g) was homogenized for 2-min in a blender with isopropanol (120 ml) to deactivate lipolytic enzymes (Kates, 1957). The homogenate was filtered through Whatman No. 1 paper and 250 μg of butylated hydroxytoluene added as an antioxidant (Johnson, 1971). The residue and filter paper were reextracted with 120 ml of chloroform–methanol, 1:1 (v/v), for 2 min and filtered (Kates and Eberhardt, 1957), then with chloroform–methanol, 2:1 (v/v), for 2 min (Christie, 1973). The three combined extracts were filtered through a "c" sintered glass funnel and taken to near dryness in vacuo at 30–35°C. Compounds taken up in 25 ml of chloroform–methanol, 2:1 (v/v), were termed the crude extract.

This method did not provide for separation of the hydrophobic and the hydrophilic compounds. A variation of the procedure was therefore developed. At the third homogenization, chloroform–methanol–water, 4:2:1 (v/v/v), was used (120 ml), and a fourth extraction with methanol–water, 1:1 (v/v), followed.

To these combined extractions in a 1-liter separatory funnel, approx. 150 ml of chloroform were added to develop two phases. The lower organic layer was removed to another separatory funnel where it was washed three times with 40 ml of water, and these washes were added to the aqueous layer. Repeated chloroform washes of the aqueous phase were made and combined in a third separatory funnel and backwashed with 2×20 ml water washes which were discarded. Both chloroform washes were then combined. The organic and aqueous phases were concentrated in vacuo at 30–35°C and 40–56°C, respectively.

RESULTS

Crude Extracts. Field-collected CLBs did not respond to crude extracts of nonhost plants when offered a choice of barley extract; nor were these nonhost extracts effective stimulants in the presence of a control gel (Table 1).

In Figure 1, the summarized response to concentration of barley crude extract is shown along with control data from several experiments. As recommended (Sokal and Rohlf, 1969) for count data including the value zero, the counts were transformed by $(Y + \frac{1}{2})^{\frac{1}{2}}$ for analysis of variance and sorted by Duncan's multiple range test. Plotted data are untransformed.

The response to the extract at 100 ppm or less was significantly lower than that from 300–1000 ppm. The response decreased above 1000 ppm (confirmed in several experiments not reported herein) so that a fifth-degree polynomial equation gave the best fit. No visual analyses were made for gel damage in these experiments.

Figure 2 shows the results when pea seedling extract was bioassayed

TABLE 1. RESPONSE OF FIELD-COLLECTED, ADULT CEREAL LEAF BEETLES TO CRUDE EXTRACT OF BARLEY AND NONHOST PLANTS INCORPORATED INTO 3% AGAR[a]

Test unit combination	Extract conc.	Response[b] (beetles/strip)
Barley (*H. vulgare*)	7669	6.3 ± 2.0
vs.		
Pepper (*Capsicum annum* L.)	9211	0.5 ± 0.7
Blank	0	1.8 ± 1.8
vs.		
C. annum	9211	1.5 ± 1.4
H. vulgare	7969	5.9 ± 2.1
vs.		
Broccoli (*B. o. italica* Plenck)	5903	1.0 ± 0.8
Blank	0	0.8 ± 1.0
vs.		
B. o. italica	5903	1.3 ± 1.2
H. vulgare	6289	3.3 ± 1.8
vs.		
P. sativum	7082	1.0 ± 0.9
H. vulgare	6289	3.0 ± 1.6
vs.		
Kohlrabi (*B. o.* var. *gongylodes* L.)	5777	1.3 ± 1.2
H. vulgare	6289	3.8 ± 1.3
vs.		
Sunflower (*Helianthus annuus* L.)	6233	0.3 ± 0.7
H. vulgare	4469	3.1 ± 1.4
vs.		
Tomato (*Lycopersicon esculentum* Mill.)	5648	0.1 ± 0.3

[a] 25 beetles (fasted 24 hr) per test unit.
[b] Average number of beetles responding to each strip after 1 hr. Ten replicates.

alone and then combined with barley crude extract which varied in concentration while the pea extract remained constant. There was a deterrent quality about the pea extract which prevented feeding on the pea extract–gel mixture (curve a). This effect was overcome, in part, by the presence of barley extract (curves a-1, b-1). Activity scores for curve a-1 indicated renewed feeding at a barley–pea ratio of approx. 1:1, and a ratio of 2.3:1 for curve b-1. In both cases, the attractive power of the combined extracts was enhanced at a 1:1 ratio over that of pea extract alone, based on the higher CLB counts on the mixtures.

Hydrophobic Compounds vs. Hydrophilic Compounds. Greater sensitivity was shown by the beetles to the barley hydrophobic compounds at the lower concentrations (up to 300 ppm), but the average count and activity for the hydrophilic compounds increased rapidly beyond this point to approximate

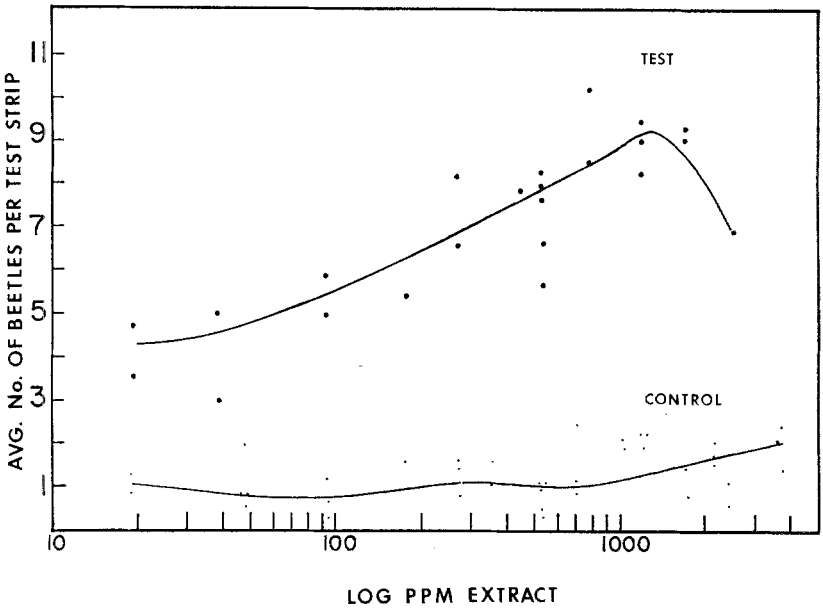

FIG. 1. Summarized response of laboratory-reared, adult cereal leaf beetles (sex undetermined) to barley seedling crude extract in agar-gel. Data plotted as mean of two hourly observations per day over four days.

equality with the hydrophobic fraction (Figure 3). Selected activity scores for control and test strips, left and right, respectively, are given in parentheses.

It was determined that the hydrophobic and not the hydrophilic materials were the major source of CLB deterrents in pea seedling extract (Table 2). There was some indication that the hydrophilic compounds did possess a small degree of deterrence although this was not investigated further. It was also confirmed that barley crude extract partially overcame the effect of pea crude extract when both were combined at approx. an 1:1 ratio barley–pea (Table 2).

Contrary to being strongly deterrent, the hydrophilic compounds of pea seedlings stimulated a low level of CLB feeding behavior (Table 3).

All gel damage took the form of superficial scarification classified as bites, rashes, or channels. Bites were individual pairs of holes produced by the insect's mandibles. Rashes were an amorphous series of bites too closely spaced to be individually discerned. Channels were narrow, linear zones of gel removal generally in a horizontal direction, but also in a vertical direction.

A difference existed in the type of damage evoked by the barley hydrophilic and hydrophobic fractions regardless of extract concentration. Damage to the gel mixed with hydrophilic compounds was dominated by channels;

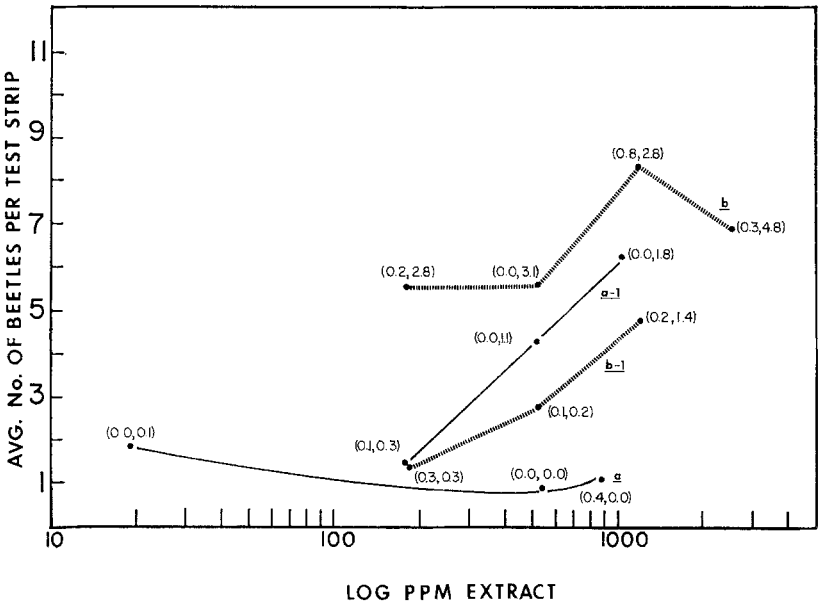

FIG. 2. Response of laboratory-reared, adult cereal leaf beetles (sex undetermined) which showed the deterrent effect of pea seedling crude extract. Tested compositions: (a) pea seedling crude extract; (b) barley seedling crude extract; (a-1 and b-1) 30 mg pea seedling crude extract in three concentrations of barley crude extract with log PPM based on barley extract concentration. Data points are means of three consecutive hourly observations per day replicated on four days. Activity (gel damage) values in parentheses (blank, left; test, right) are means from two replicate scores for lines a and a-1, and from four replicate scores for lines b and b-1 using the activity index scale 0 (none) through 6 (excellent).

that found in tests of hydrophobic compounds contained a larger proportion of bites and rashes in addition to shorter channels.

DISCUSSION

The bioassay was presumed to operate only through contact chemoreception for two reasons. First, the easily vaporized compounds were lost in preparation of the agar gel. Second, beetle counts on the test strip infrequently exceeded 50% of those in the Petri dish, which indicated a random dispersion from the test unit center and a chance encounter with either the test or control strip. However, a response-limiting crowding effect has not been ruled out.

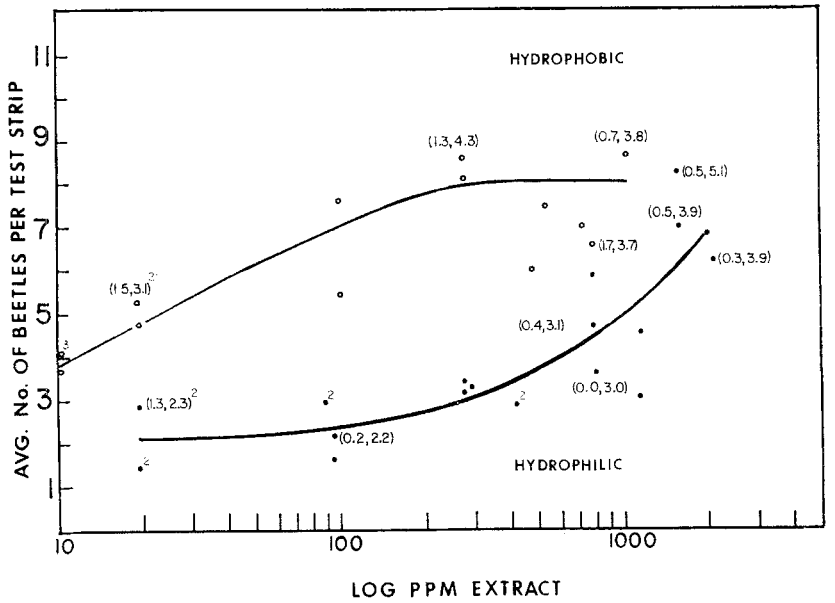

FIG. 3. Summarized response of laboratory-reared, adult cereal leaf beetles (sex undetermined) to barley seedling total hydrophobic and hydrophilic fractions in agar gel. Points are means of three hourly observations per day replicated on four days. Activity (gel damage) values in parentheses (control, left; test, right) were averaged from four daily scores: 0 (none) through 6 (excellent). Numerical superscript indicates replications if different from four.

TABLE 2. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO BARLEY CRUDE EXTRACT ADDED WITH EXTRACTS OF PEA SEEDLINGS IN 3% AGAR GEL[a]

Barley extract (ppm)	Pea extract (ppm)	Count response[b]		Activity index[c]	
		Control	Test[d]	Control	Test
0	0	1.3	1.1 (a)	0.4	0.7
485	0	1.2	8.0 (b)	0.6	4.6
485	R[e], 161	1.1	7.0 (c)	0.1	3.5
485	S, 388	1.1	3.3 (d)	0.1	1.9
485	T, 227	0.8	3.8 (d)	0.3	2.8
485	R+T = 388	1.5	2.6 (e)	0.2	1.7

[a] 25 laboratory-reared beetles, one control, one test agar strip per test unit; one test unit per concentration per day.
[b] Average of three hourly counts of beetles per day over four days.
[c] Average of four daily activity scores: 0 (none) through 6 (excellent).
[d] Means sorted by Duncan's MRT. Values opposite the same letter were not significantly different at the 5% level. Transformation: $(Y)^{\frac{1}{2}}$.
[e] R, pea hydrophilic compounds; S, pea crude extract; T, pea hydrophobic compounds.

TABLE 3. RESPONSE OF NEWLY EMERGED, UNFED, ADULT CEREAL LEAF BEETLES TO PEA SEEDLING HYDROPHILIC COMPOUNDS IN 3% AGAR GEL[a]

Concentration of extract (ppm)	Count response[b]		Activity index[c]	
	Control	Test	Control	Test
0	0.7	1.3	0.7	0.9
0	1.8	0.8	1.5	0.6
291	1.2	1.7	0.4	1.8
1164	1.0	2.5	0.2	1.3
1164	1.0	3.4	0.5	3.6
1746	0.7	2.2	0.2	2.0
1746	0.6	4.4	0.6	2.6

[a] 25 laboratory-reared beetles per test unit; one control and one test agar strip per test unit; one test unit per concentration per day.
[b] Average of three hourly counts of beetles per day over four days.
[c] Average of four daily activity scores: 0 (none) through 6 (excellent).

To assess response, the two indices, count and activity score, were not considered equally. Greater weight was given to the activity index as it seemed more closely related to feeding behavior than mere presence of the CLB. A difference of 0.7 in activity score was judged significant by the observer.

Using crude extracts, a CLB feeding response was stimulated only when the extract from a host plant was incorporated with it (Table 1). This result suggests that conclusions drawn from these studies actually reflect the molecular ecology of the CLB/host relationship in the field and, therefore, the chemical basis of CLB host selection.

The fact that the hydrophilic pea compounds stimulated a low CLB feeding response (Table 3) may indicate that substances widely distributed among plant forms will elicit feeding from the CLB in the absence of deterrents. Further examination of the hydrophilic compounds from other nonhosts are required to clarify this possibility.

The deterrent effect of pea crude extract and the fact that it was partly overcome by barley crude extract (Table 3, Figure 2) prompts two conclusions: First, the garden pea is not a host of the CLB, in part because it contains deterrent chemicals. This conclusion lends support to the contention of Jermy (1966) that the host range of a phytophagous insect is strongly limited by the distribution of deterrent chemicals. Second, there must be a host-specific, recognizable quality about the barley extract which caused the CLB to feed, at reduced activity, in the presence of the deterrent(s). This conclusion supports those who believe that host selection depends on both

the presence of stimulants and the absence of strong deterrents (Thorsteinson, 1958; Bate-Smith, 1972; Ishikawa et al., 1969).

We believe that the difference in the type of agar-gel damage found between the hydrophobic and hydrophilic barley fractions correlates with the respective functions of these compounds in the CLB feeding response. Our hypothesis is that the biting and rashing (hydrophobic compounds) indicated an excited biting response, but not a highly directed effort to feed. Channels (the major response to hydrophilic compounds) seem to represent the characteristic feeding pattern of the CLB as it chews through barley leaves in a direction parallel to the leaf veins. Therefore, the barley hydrophilic compounds may be the ultimate cause of continued CLB feeding. Based on a similar response to pea seedling hydrophilic compounds, this class of substances might generally induce continued CLB feeding if adequate deterrent compounds were not present.

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EVIDENCE FOR THE PRESENCE OF ACCEPTOR SITES FOR DIFFERENT TERPENES ON ONE RECEPTOR CELL IN MALE *Monochamus notatus* (DRURY) (COLEOPTERA: CERAMBYCIDAE)

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Abstract—Electrophysiological recordings confirmed the ability of *Monochamus notatus* to detect at least six terpenes, and showed that the sensilla basiconica are olfactory receptors for these compounds. There was no correlation between the degree of behavioral activity and the response of the sensory cells to terpenes. No odor specialists were found. A wide range of response spectra of the sensilla basiconica were recorded. One sense cell has acceptors for both limonene and pinene.

Key Words—Electrophysiology, acceptor, terpene, sensilla basiconica, *Monochamus notatus*.

INTRODUCTION

It has been known for some time that a single insect sensillum may have several receptor cells reacting to different modalities. In odor generalists, each of the cells has a unique, stable spectrum. Spectra of individual cells are different but may overlap (Schneider and Steinbrecht, 1968). Kaissling (1969) postulates that the acceptor sites for olfactory molecules are located on the dendritic membrane of the sensory neurone. He attributes the specificity of chemosensory neurons to these sites.

The question of whether different peripheral dendrites of a sense cell sever different olfactory qualities or whether the same acceptor site may be stimulated by more than one chemical is now being explored.

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Dickens and Payne (1977) have shown that the receptor neurons under the long sensilla basiconica of *Dendroctonus frontalis* have acceptor sites for different chemicals, including host tree terpenes, on the same cell. Seabrook (1977) has demonstrated that *trans*-11-tetradecenal and *trans*-11-tetradecenyl acetate bind to a common acceptor site in the eastern spruce budworm.

The antennae of *Monochamus notatus* Drury are well supplied with sensilla basiconica (Dyer and Seabrook, 1975). A study of the olfactory response of some of the cells of these sensilla to the terpenes found in host trees was undertaken in an effort to provide some insight into the function of these receptors.

METHODS AND MATERIALS

Specimens of known age were immobilized by exposure to cold (0°C) for a few minutes. They were then secured near the edge of a wax disk with a strip of plasticene around the neck, and another over the scape of the antenna. The reference electrode was passed through the scape or first flagellar segment into the wax below to immobilize the antenna. The treatment did not seriously injure the specimen. On release, the beetles resumed normal activity and could be reused for electrophysiological recording at a subsequent date. The preparation was placed under a dissecting microscope and illuminated with a quartz iodine lamp. The recording electrode, made of electrolytically sharpened tungsten wire, was inserted near the base of a sensillum with the aid of a Leitz micromanipulator.

Stimulating chemicals were presented in an airflow which passed through a flowmeter, a charcoal filter, an 18-in. column of water, and a three-way solenoid valve. From the valve the purified airflow was normally directed onto a sensillum. When the switch on the valve was closed, the airflow was passed over the sensillum through a second tube terminating in a replaceable cartridge containing a piece of filter paper with 1.0 μl of the stimulant. The entire preparation was evacuated.

From the electrodes, the signals were passed through a Tektronic 122 differential preamplifier and a 60-Hz filter to a Tektronic R5030 dual-beam oscilloscope. The signals were permanently stored on a stereo tape recorder. All recordings were carried out in a double-shielded room. A diagram of the apparatus is shown in Figure 1.

RESULTS

When terpenes were tested individually, no cells were found which

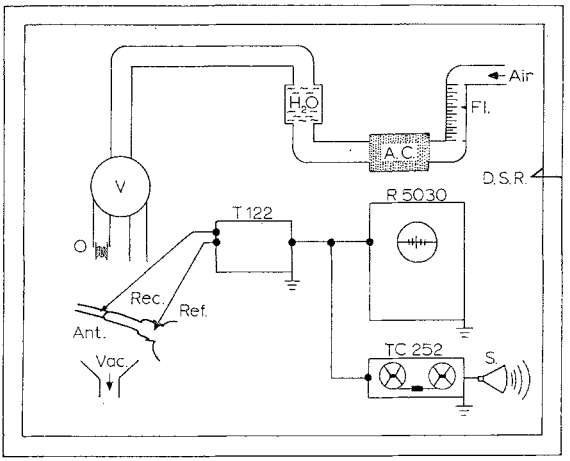

FIG. 1. Diagram of air delivery and recording systems. All electrophysiological tests were carried out in a double-shielded room (DSR). Air from compressors was filtered through charcoal (A.C.), bubbled through water, and channeled through a three-way electromagnetic valve (V) either into an open channel or over an odor source (0) adsorbed on filter paper in a parallel channel. The choice of channel was controlled by a switch. The air then passed over the antenna (Ant.) into a funnel connected to a vacuum line (Vac.). The reference electrode (Ref.) was in the hemocoel of the antenna and the recording electrode (Rec.) penetrated the base of a sensillum. The signal passed via a preamplifier (T122) to an oscilloscope and a tape recorder (TC 252) connected to a speaker (S). (R5030)

responded to only one terpene, and the responses of all cells tested to any one terpene were not identical. Some cells were inhibited by a particular terpene such as geraniol (Figure 2), while other cells associated with different sensilla basiconica of the same individual were stimulated by it (Figure 3).

A wide range of response patterns to terpenes was recorded, including complete inhibition of spontaneous background activity (Figure 2), discharge at the termination of the stimulus (Figure 3) and stimulation of one (Figures 2 and 3), two (Figure 2), or several neurons of a single sensillum. For any one cell, the responses to a repeated stimulation by a terpene usually were consistent (Figure 3). Occasionally a reduced response to a second stimulus

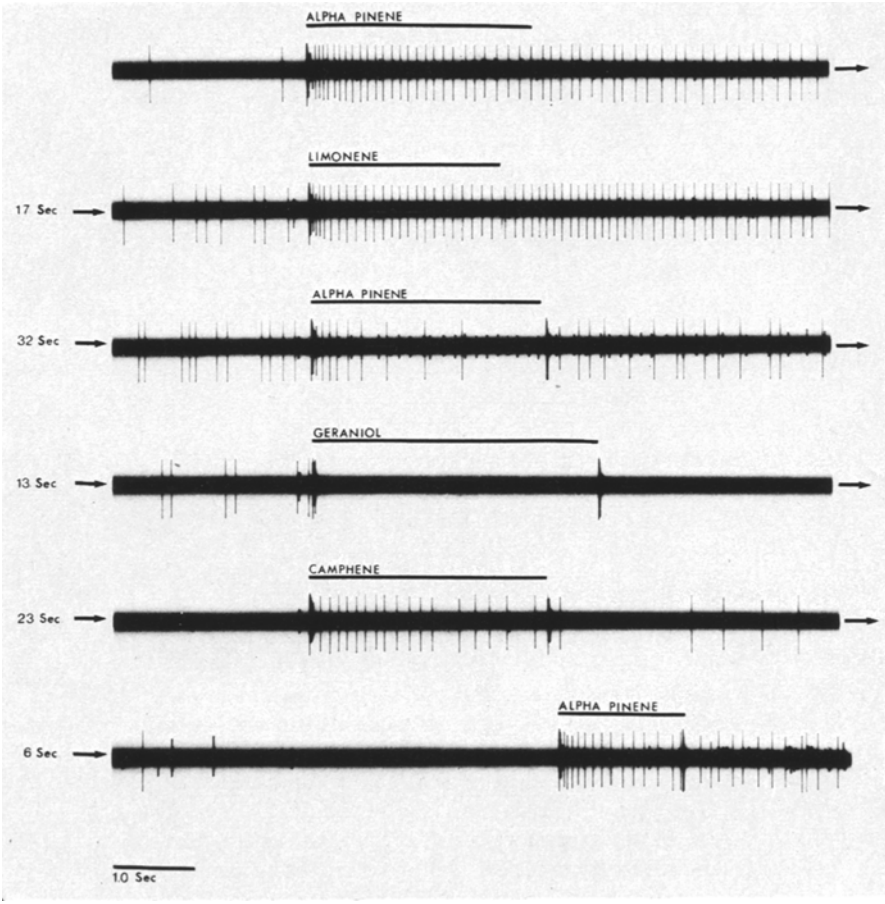

FIG. 2. Responses of cells of a single sensillum basiconicum of a 44-day-old male *Monochamus notatus* to successive stimulation with terpenes. The durations of this continuous record which were omitted are noted at the beginning of each trace. The response of a second cell is nearly obscured in these traces.

with the same terpene provided evidence of adaptation of the receptor (Figure 3).

The response to a terpene often continued for relatively long periods of time after the termination of the stimulus. In Figure 2, it can be seen that the response of one of the two neurons stimulated by alpha-pinene remained above background level for more than 20 sec after termination of the stimulus. The neurons of a single sensillum differed in their response patterns.

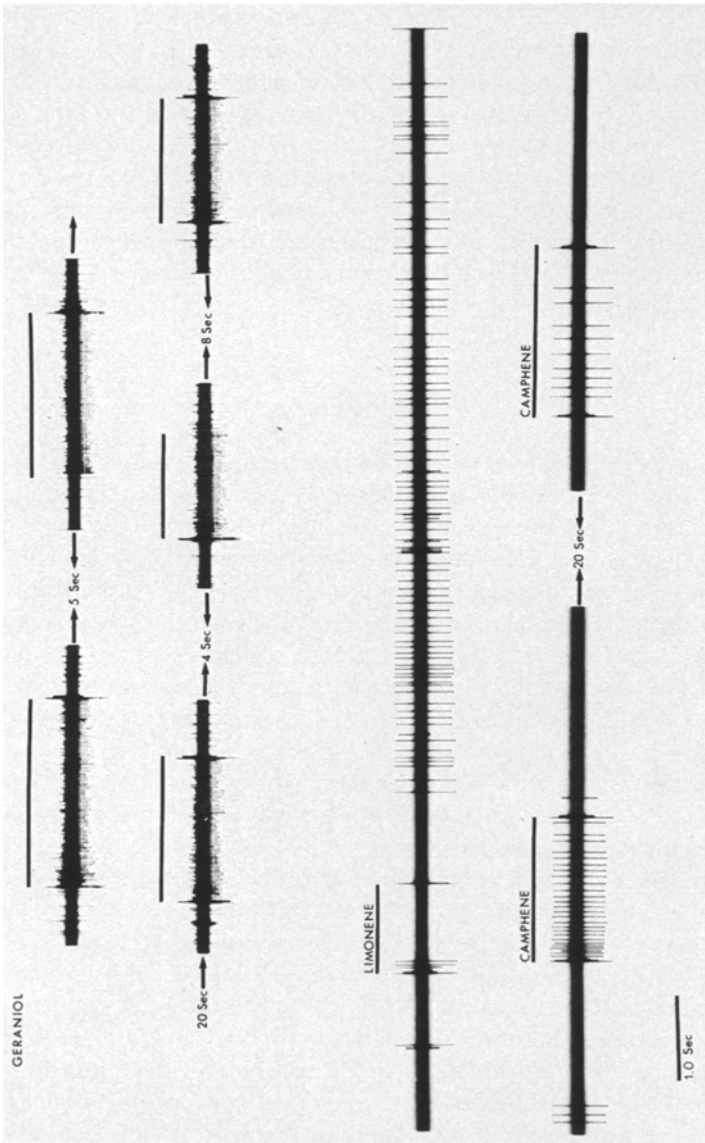

FIG. 3. Responses of cells of two different sensilla basiconica of the same individual used for Figure 2 to terpenes. Upper traces: response to five successive stimulations with geraniol. Lower traces: responses of another receptor to limonene and camphene.

The response of a neuron to a terpene may be altered by previous stimulation with another terpene. In one sensillum a 2-sec stimulation with limonene altered the response of one of the two neurons to alpha-pinene (Figure 2). The firing rate in response to alpha-pinene in this neuron dropped from thirteen spikes in the first second before application of limonene to seven spikes in the first second when the response followed a response to limonene. The firing rate of the second neuron, which did not respond to limonene, was unaltered at eight spikes per second in both situations.

Recordings from individual beetles at successive intervals over their life spans have been achieved in a few cases. A recording from one male made shortly after its emergence was very similar to the recordings made after a similar stimulus six weeks later.

DISCUSSION

From the data presented it can be concluded that not all receptor neurons have acceptor sites for all terpenes. Figure 2 shows that although two receptor cells under one sensillum responded to alpha-pinene, only one responded to limonene or camphene. Thus one of the two receptor cells did not have an acceptor for these terpenes and was neither stimulated nor inhibited by them. The background firing rate of the neuron was unaltered.

In the above case, one of the two neurons which responded to alpha-pinene may have an acceptor which responds both to limonene and alpha-pinene. This can be deduced from the fact that the response to alpha-pinene immediately following stimulation by limonene was altered for only one of the two neurons. The small spikes from the neuron which did not respond to limonene fired at the same rate of about eight per second in the first second in response to alpha-pinene whether the stimulus is after or before application of limonene. The firing rate of the other neuron in response to alpha-pinene is greatly reduced from thirteen spikes per second when there is no preexposure to limonene to seven per second in the first second after stimulation following the application of limonene. This could be explained either by assuming that the acceptor sites for alpha-pinene on the dendrite are already occupied by limonene in one neuron and not in the other or that the acceptor sites for both limonene and alpha-pinene are found on the same dendrite in one class of sensilla basiconica found in *Monochamus*. It may be that these two terpenes share a common acceptor; however, the above data does not conclusively prove this point.

It appears that there is no direct relationship between the behavioral response to terpenes in *Monochamus* (Dyer and Seabrook, unpublished) and the electrophysiological responses of receptor neurons. In view of the variety

of responses found in different receptor neurons, it is likely that behavioral activity requires central integration of sensory information from several sources.

The conclusion that the sensilla basiconica of *Monochamus* are odor generalists is in accord with the results obtained for many other insects (Schneider and Steinbrecht, 1968). Most odor specialists described to date are sensilla trichodea, sensitive to pheromones and not to food or host plant odors. Some sensilla which had been classified as odor specialists are now known to respond to a spectrum of compounds in the same way as odor generalists (O'Connell, 1975).

On the basis of the evidence available to date (Figures 2 and 3), there do not appear to be any sensilla basiconica in *Monochamus* comparable to the B_1 basiconica described by Mustaparta (1974) in the pine weevil, which were specific for only three terpenes. It should be noted, however, that the thick, heavily sclerotized cuticle of *Monochamus* presents serious obstacles to single-receptor cell recordings. Only a proportion of the sensilla was successfully penetrated, and it is possible that other receptor cell types were present but undetected.

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SYNTHESIS OF THE TWO COMPONENTS OF THE SEX PHEROMONE SYSTEM OF THE POTATO TUBERWORM MOTH, *Phthorimaea operculella* (ZELLER) (LEPIDOPTERA: GELECHIIDAE) AND FIELD EXPERIENCE WITH THEM

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Abstract—Male potato tuberworm moths are attracted by a mixture of *trans*-4,*cis*-7-tridecadien-1-ol acetate and *trans*-4,*cis*-7,*cis*-10-tridecatrien-1-ol acetate. The synthesis of both compounds is described. Overall yields were 14.4 and 9.5% after distillation. The products were purified by liquid chromatography. Mixtures of these compounds in several ratios and quantities were tested in potato fields in Australia, Peru, and Cyprus. The largest catches were obtained from water pan traps baited with rubber sleeve stoppers containing both components in ratios varying between 1:9 and 9:1. The stoppers were attractive over a period of several months even under hot weather conditions.

Key Words—*Phthorimaea operculella* (Zeller), potato tuberworm moth, sex pheromone, attractant, *trans*-4,*cis*-7-tridecadien-1-ol acetate, *trans*-4,*cis*-7, *cis*-10-tridecatrien-1-ol acetate.

INTRODUCTION

The potato tuberworm moth, *Phthorimaea operculella* (Zeller), is a serious pest in several areas of the world. Adeesan et al. (1969) showed that the female moths release volatile substances to lure the males. Knowledge about the chemistry of these substances could provide another weapon for controlling this insect. Recently two attractive compounds were isolated from female

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abdominal tip extracts. Roelofs et al. (1975) isolated, identified, and synthesized *trans*-4,*cis*-7-tridecadien-1-ol acetate (PTM 1). They also isolated another active component from the extracts but this was not identified. Independently Persoons et al. (1976a,b) found PTM 1 and also demonstrated that the second component of the pheromone system is *trans*-4,*cis*-7,*cis*-10-tridecatrien-1-ol acetate (PTM 2). They discovered that a mixture of PTM 1 and PTM 2 in the ratio 1:4 was much more attractive than each single compound. Other investigators have also tried to elucidate the pheromone system of the potato tuberworm moth (Voerman et al., 1977). Strangely enough Yamaoka et al. (1976) did not find PTM 1 in their extract from adult females and concluded that the pheromone should be one of the geometric isomers of the 4,7,10-tridecatrienyl acetates. A description of the synthesis of PTM 1 was given before (Roelofs et al., 1975; Henrick, 1977). This paper describes the synthesis of both PTM 1 and PTM 2, and the results from more extensive field experiments in Australia, Peru, and Cyprus.

METHODS AND MATERIALS

Synthesis of PTM 1 and PTM 2

The progress of all reactions was followed and all products were checked by gas–liquid chromatography using a column packed with 1.5% SP-2250/1.95% SP 2401 on Supelcon AW-DMCS 100/120 (glass, 2.1 m × 2.4 mm ID) and a column packed with 15.6% OV-275 on Chromosorb W AW-DMCS 100/120 (glass, 5.4 m × 2.4 mm ID). The end-products, PTM 1 and PTM 2, were ultimately purified by liquid chromatography on a silver-loaded resin (glass column, 200 cm × 0.8 cm, packed with Lewatit SP 1080, 170–200 mesh, Ag^+ form, eluent methanol, temperature 20–40°C) (Houx et al., 1974). Purity was checked with HPLC at 21°C (PTM 1) and 37°C (PTM 2) (Houx and Voerman, 1976). They were stored at —20°C under nitrogen after addition of 0.1% 2,6-di-*tert*-butyl-4-methylphenol as an antioxidant (Goto et al., 1974). The reaction sequences for the synthesis of PTM 1 and PTM 2 are shown in Figure 1.

2-(3-Bromopropyloxy)tetrahydropyran (I). To 83 g (0.59 mol) 3-bromopropanol-1 and 4 drops of concentrated HCl, 65 g (0.77 mol) of dihydropyran was slowly added. The mixture was stirred magnetically and cooled in ice water. After stirring for 2 hr at room temperature, 2 g of K_2CO_3 was added and stirring was continued for 1 hr. After filtration, the filtrate was distilled; main fraction 82.9 g (63%), b. 76°C/1.4 mm, n_D^{25} 1.4778.

2-(6-Hydroxy-4-hexynyloxy)tetrahydropyran (II). After stirring 0.2 g $Fe(NO_3)_3 . 9H_2O$ for $\frac{1}{4}$ hr in 600 ml NH_3, 5.6 g lithium (0.76 mol) was added in small pieces. When the blue color had vanished, 21.2 g (0.38 mol)

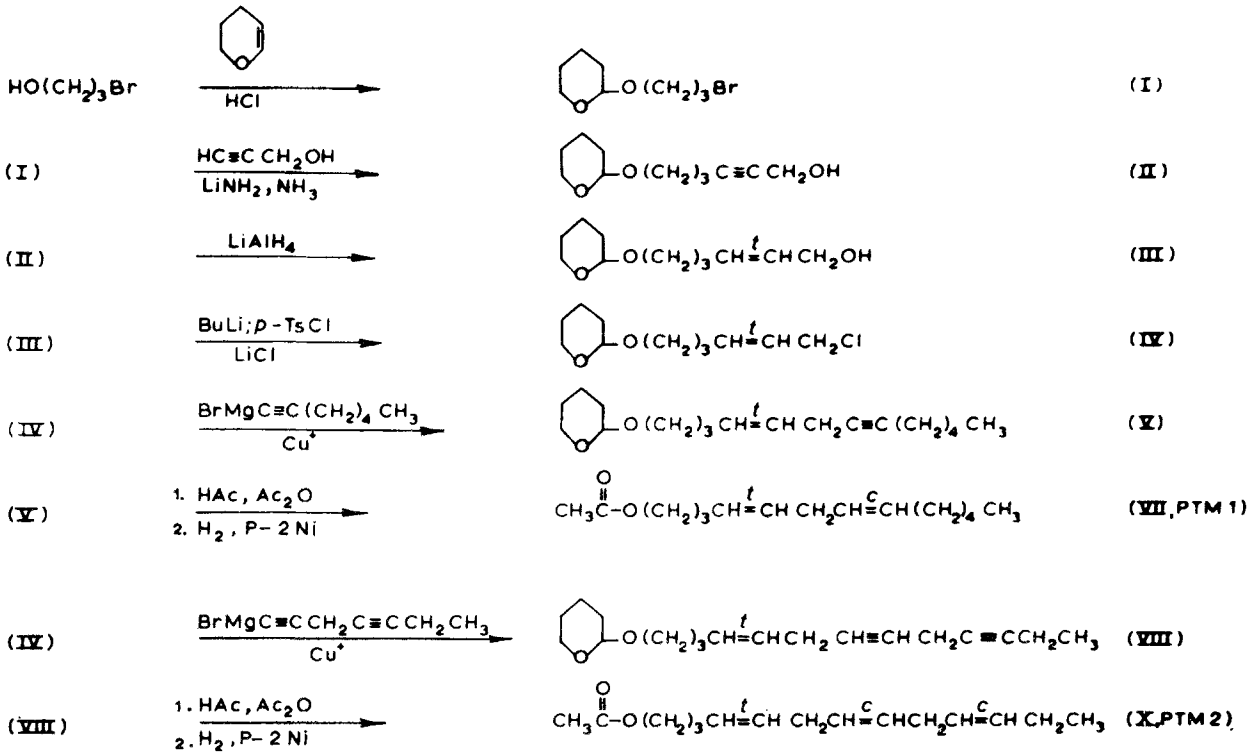

FIG. 1. Reaction scheme for synthesizing PTM 1 and PTM 2.

propargyl alcohol was added. After 2 hr of stirring, 53 g (0.24 mol) (I) diluted with 250 ml tetrahydrofuran was added. After stirring overnight, the mixture was worked up (Ames et al., 1963) and distilled. Main fraction 36.7 g (78.1%), b. 104°C/0.03 mm, n_D^{25} 1.4825.

2-(6-Hydroxy-trans-4-hexenyloxy)tetrahydropyran (III). To a cooled (—80°) mechanically stirred suspension of 4.3 g (113 mmol) $LiAlH_4$ in 90 ml dry ether, 25.0 g (126 mmol) of (II) was added fairly rapidly. After 1 hr of stirring, the cooling bath was removed and the mixture was allowed to warm up. It was gently refluxed for $3\frac{1}{2}$ hr, cooled again, and 12 ml ethyl acetate, 250 ml saturated NH_4Cl, and 200 ml 20% NaCl were added. Stirring was continued for several hours (Raphael, 1955). The organic layer was extracted with ether (some CH_3OH might be helpful to break the emulsion). The extract was washed with 20% NaCl, dried, and distilled; main fraction 18.7 g (74.5%), b. 99–101°C/0.05 mm, n_D^{25} 1.4720.

2-(6-Chloro-trans-4-hexenyloxy)tetrahydropyran (IV). The alcohol (III), 48 g (240 mmol) in 120 ml dry ether and 60 ml dry HMPT, cooled in an ice–salt bath, was provided with an equivalent of butyllithium in hexane as described by Stork et al. (1969). The mixture became brown-red at the equivalence point. After stirring some time at room temperature, the mixture was cooled again to 3°C and 48.6 g (255 mmol) *p*-TsCl dissolved in 120 ml ether and 60 ml HMPT was added followed by 30.9 g (720 mmol) LiCl. After stirring overnight, the mixture was worked up and the product distilled, main fraction 34.3 g (65.4%), b. 82°C/0.10 mm, n_D^{25} 1.4780.

2-(trans-4-en,7-Tridecynyloxy)tetrahydropyran (V). Ethylmagnesium bromide [from 4.9 g (0.20 mol) Mg and 16.3 g (0.15 mol) EtBr] in 70 ml THF was added to 15.4 g (0.16 mol) 1-heptyne in 15 ml THF. The reaction mixture was warmed on a waterbath at 60°C for $\frac{3}{4}$ hr. Afterwards it was decanted from the magnesium and 1 g dry CuCl was added. The suspension was stirred for $\frac{1}{4}$ hr before 21.8 g (0.10 mol) of (IV) and 5 ml THF were added. After stirring overnight at 35°C, the mixture was refuxed at 60°C for $\frac{1}{2}$ day. This resulted in a light green reaction mixture, which was poured out in 120 ml H_2O containing 20 g NH_4Cl and 4 g KCN. The product was extracted with ether, and the extract was washed with 20% NaCl until neutral. After drying on $MgSO_4/K_2CO_3$ and removing the solvent in a rotary evaporator, 26.1 g oil remained (see also Brandsma, 1971).

trans-4,7-Tridecyn-1-ol acetate (VI). 13.0 g of (V) in 50 ml HAc was stirred and heated at 80°C. Then 25 ml Ac_2O was added, and heating and stirring were continued overnight. The mixture was poured out in icewater and worked up in the usual way. Distillation gave 9.0 g (76.2% from IV) b. 82–83°C/0.05 mm, n_D^{25}1.4620.

trans-4,cis-7-Tridecadien-1-ol acetate (VII, PTM 1). To 1.25 g $Ni(OAC)_2 \cdot 4H_2O$ in 50 ml EtOH under H_2, was added 5.0 ml of a $NaBH_4$ solution

(prepared by filtering the solution resulting from 1 g $NaBH_4$, 24 ml EtOH, 1.25 ml 2 N NaOH) (Brown and Ahuja, 1973a,b). After the hydrogen evolution had ceased, 0.7 ml 1,2-diaminoethane and 9.0 g (38 mmol) of (VI) were added. After 959.4 ml H_2 had been taken up, under vigorously stirring, the reaction stopped. The reaction mixture was filtered, diluted with a 20% NaCl solution, and extracted with ether. The extract was washed with 20% NaCl, dried, and distilled giving 7.1 g (78.9%) PTM 1, b. 73°/0.02 mm, n_D^{25} 1.4528. This oil was purified further by liquid chromatography (Houx et al., 1974) giving pure PTM 1, n_D^{25} 1.4542, with satisfactory elemental, GC and LC analyses, and consistent MS, NMR, and IR spectra (Persoons et al., 1976b).

1,4-Heptadiyne. A Grignard reagent, made from 12 g (0.5 mol) magnesium and 39 g (0.36 mol) ethyl bromide in 175 ml THF, was decanted from the excess of magnesium and provided with 30 g (0.56 mol) 1-butyne in 100 ml dry THF (Brandsma, 1971, p. 52). After stirring the mixture $\frac{3}{4}$ hr at 60° and $\frac{1}{4}$ hr at room temperature, 2.0 g dry CuCl and 38 g (0.32 mol) 3-bromopropyne were added. The mixture was stirred overnight. Then a water solution of 52 g NH_4Cl and 6 g KCN was added to the green suspension. The ether extract of the latter was washed with 5% KCN and 20% NaCl until neutral and was then dried and distilled. Main fraction 15.3 g (55%), b. 34–41°/22 mm, n_D^{25} 1.4488 (Kraevskii et al., 1964, found b. 62–63°/80 mm, n_D^{20} 1.4440).

trans-4-en,7,10-Tridecadiyn-1-ol acetate (IX). A Grignard reagent, prepared from 5.0 g Mg (0.21 mol) and 18.3 g (0.17 mol) ethyl bromide in 100 ml THF, was decanted from the excess of magnesium and slowly added to 20.4 g (0.22 mol) 1,4-heptadiyne in 60 ml dry THF. The mixture was warmed at 65°C for $\frac{1}{2}$ hr. Then it was stirred for some time with 2 g dry CuCl after which 32 g (0.146 mol) (IV) was added. The reaction was slightly exothermic, and the mixture became light green. After 36 hr of stirring at room temperature and 1 hr at 55°C, the mixture was poured out into 250 ml water containing 50 g NH_4Cl and 6 g KCN. After extraction with ether and working up as described above, 39.0 g of a red–brown oil was obtained (VIII). From this oil, 16.5 g was converted into the acetate (IX) with 60 ml HAc and 32 ml Ac_2O. The product was distilled; main fraction 7.6 g (53%), b. 108–114°/0.05–0.07 mm, n_D^{25} 1.4830.

trans-,4,cis-7,cis-10-Tridecatrien-1-ol acetate (X, PTM 2). To 2.5 g $Ni(OAc)_2.4H_2O$ in 100 ml EtOH under H_2 was added 10 ml of a $NaBH_4$ solution (see preparation of VII), and after the H_2 evolution ceased, 1.4 ml 1,2-diaminoethane was added. In this mixture 7.6 g (IX) (32.8 mmol) took up 1680 ml H_2. The mixture was worked up as described above. Distillation gave 5.8 g (75.1%) colorless oil, b. 75°/0.02 mm, n_D^{25}1.4650. Part of this oil was purified by liquid chromatography giving pure PTM 2, n_D^{25}1.4660, with

satisfactory elemental, GC, and LC analyses and consistent MS, NMR, and IR spectra (Persoons et al., 1976b).

Field Experiments with PTM 1 and PTM 2

Field trials were undertaken in Australia to determine the influence on male trap captures of baits containing various mixtures of both female pheromone components. The effects of differing dosage levels in baits and the possible role of the components in bringing males to pheromone sources were also examined. Other field tests were carried out in Cyprus on component ratios and male catches. There was also an extended trial in Peru to test the longevity of a single blend.

In Australia, trials were located in and around an experimental potato field. The traps consisted of plastic boxes ($20 \times 10 \times 5$ cm) containing 200 ml of water and 2% wetting agent. The pheromone components, dissolved in methylene chloride, were applied to red rubber sleeve stoppers (Fisher Scientific Company, Pittsburgh, Pennsylvania, catalog No. 14-126A). These were suspended from the lids 2.5 cm above the water (Bacon et al., 1976). Traps were placed 9 m apart on the ground within the rows of potato plants. To obviate significant positional effects, traps were rotated regularly. Trapping methods in Cyprus and Peru were essentially the same as those in the Australian tests.

RESULTS

Ratio of Pheromone Components and Male Captures

PTM 1 and PTM 2 were applied singly or in combination to rubber sleeve stoppers in varying quantities as indicated in Table 1. In the first trial within the potato crop, traps were sampled regularly.

The totals for the entire period (Table 1) indicate that there was no significant difference in male captures at traps baited with blends of PTM 1 and PTM 2 ranging from 9:1 to 1:9, although all were significantly greater than either component alone. PTM 2 was generally significantly more attractive on its own than PTM 1. Statistical analysis of the results obtained during the first and last 15 days of exposure of the baits showed that this pattern of captures was consistent over the period of the tests, although the actual dosage level of each component would have decreased with time. Catches were greatest during the first few days of exposure of the pheromones in previously unsampled areas both in Australia and Cyprus. Adult numbers were high within the potato field and there were very significant differences between inner and outer trap locations within the potato field (mean catch

TABLE 1. MEAN NUMBER OF MALE POTATO TUBERWORM MOTHS CAPTURED WITH VARIOUS BLENDS OF PTM 1 AND PTM2[a]

Treatment		Mean catch per trap[b]	
PTM 1 (μg)	PTM 2 (μg)	Potato field	Pasture field
200	0	131 a	3 a
180	20	854 bc	13 b
160	40	1160 b	17 bc
140	60	782 bc	29 bc
120	80	983 bc	16 bc
100	100	932 bc	16 bc
80	120	1041 b	15 bc
60	140	591 cd	20 bc
40	160	658 bc	21 bc
20	180	748 bc	15 bc
0	200	252 d	5 a
0	0	20 e	0 d

[a] Australia, February 2–March 18, in potato field and March 18–April 5 1977, in pasture field, 5 traps per treatment.
[b] Means followed by the same letter are not significantly different at $P < 0.05$, Duncan's multiple range test $\sqrt{x}$ transformation.

per treatment: outer traps, 1590; inner traps, 674; significant difference at $P<0.05$). For this reason traps had to be redistributed randomly at regular intervals. Blank traps without rubber septa caught relatively few males (Table 1). Less than 3% of captures consisted of females; of these most were mated. Similar numbers of females were taken at all treatments.

It was thought that the similarity in numbers of moths captured at almost all combinations of both components may have been due to the large numbers of males available for capture in the relatively small potato field. However, a second series of tests with the same treatments undertaken in a pasture field several hundred meters away from the potato field produced similar results (Table 1), although overall captures were much lower.

Moth captures in Cyprus (Table 2) also followed this pattern and, as noted in Australia, there was no significant difference in captures at the beginning and end of the 64-day trapping period. The Cyprus tests also demonstrated that most treatments were as effective as virgin female traps (Table 2) each baited with two females.

Quantity of Pheromone and Male Captures

Varying quantities of both PTM 1 and PTM 2 were tested singly and in

TABLE 2. NUMBERS OF MALES CAPTURED WITH VARIOUS BLENDS OF PTM 1 AND PTM 2 (CYPRUS, APRIL 15–JUNE 18, 1977)

Treatment		
PTM 1 (μg)	PTM 2 (μg)	Mean total catch per field[a]
200	0	1290 a
180	20	3334 b
160	40	3895 bc
140	60	3602 bc
120	80	3852 bc
100	100	3694 bc
80	120	3747 bc
60	140	3845 bc
40	160	3856 bc
20	180	3844 bc
0	200	3487 b
0	0	151 d
100	300	6154 e
2 virgin females		4075 c

[a] Figures followed by same letter are not significantly different at $P < 0.05$. Only 1 replicate in each of 5 fields, therefore analysis done on percentage captures per treatment with arcsin transformation; Duncan's multiple range test.

two combinations (4:1, 1:4) in the field in Australia. The results (Table 3) indicate that over the 38-day sampling period maximum male captures were taken at sources containing a total of 1000 μg of both components in both combinations. At the highest dosages of 10,000 μg, catches decreased significantly and, although the pheromone quantities must have diminished with time, remained low throughout the trial. The reduction in captures with PTM 2 was particularly marked (Table 3). Captures at traps baited with only 10 μg of blends of both components were also lower than those containing 1000 μg (Table 3). The difference between captures at 10 and 100 μg was also consistent but not always statistically significant.

Both pheromone components remain active over a prolonged period in the field. Comparisons of relative capture rates at traps baited with 10 and 100 μg of both pheromone blends (4:1, 1:4 PTM 1 and PTM 2) in nine successive samplings over 38 days showed that even low dosages remained active throughout this period. Bait longevity was demonstrated most strikingly in the tests conducted in Peru. Two traps baited with a mixture of 100 μg of PTM 1 and 300 μg of PTM 2 caught nearly 87,000 moths in 4 months of high temperatures, and weekly captures exceeding 1000 males per trap

TABLE 3. QUANTITY OF PTM 1 AND PTM 2 PER TRAP AND MALE CAPTURES (AUSTRALIA, APRIL 13–MAY 20, 1977). MEAN OF 5 TRAPS

Treatment		
PTM 1 (μg)	PTM 2 (μg)	Mean catch per trap[a]
10	0	32 abc
8	2	63 cdef
4	6	125 efghi
0	10	118 efgh
100	0	32 abc
80	20	148 ghij
40	60	193 hijk
0	100	128 fghij
1000	0	42 bcd
800	200	219 jk
400	600	249 k
0	1000	81 defg
10000	0	11 ab
8000	2000	102 afgh
4000	6000	61 cdef
0	10000	7 a

[a] Mean figure represents total of 9 successive samplings per trap; figures followed by the same letter are not significantly different at $P < 0.05$, $\sqrt{x}$ transformation, Duncan's multiple range test.

were still being recorded at the end of this period. The baits were not refreshed in this period. A similar 1:3 bait used in the Cyprus trials also caught significantly more males than the other treatments, (Table 2) suggesting that this combination may be particularly effective; this blend was not tested in Australia.

Role of PTM 1 and PTM 2 in Trapping Males

A field test was undertaken to determine whether the individual components of the pheromone blend differed in their influence on male movements towards a source of the compounds. Concentric rings of cellulose acetate 15 cm apart were placed within a shallow metal tray containing water and 2% detergent; the overall diameter of the trap container was 150 cm.

TABLE 4. NUMBERS OF MALES TRAPPED AT VARYING DISTANCES FROM A PHEROMONE SOURCE (AUSTRALIA, MARCH 17–APRIL 15, 1977)[a]

Distance from pheromone source (cm)	Catches (%) with		
	PTM 1 + PTM 2 (100μg + 100μg)	PTM 1 (200 μg)	PTM 2 (200 μg)
0–15	395 (35)	13 (30)	31 (35)
15–30	264 (23)	12 (28)	14 (16)
30–45	180 (16)	8 (19)	13 (15)
45–60	165 (15)	5 (12)	17 (19)
60–75	133 (12)	5 (12)	13 (15)
Total	1137 (100)	43 (100)	88 (100)

[a] No significant difference between percentages across table; arcsin transformation; Duncan's multiple range test. $N = 3$, replication achieved by analyzing 3 successive samples from individual traps.

Rubber sleeve stoppers baited with PTM 1 and PTM 2 singly or in combination (1:4, 200 μg) were placed in the central area. Numbers of captured males in the various sectors were counted at regular intervals. It was considered that a compound acting solely as long-range attractant (Kennedy, 1977) would have produced a greater proportion of captures in the outer sectors; conversely a short-range substance would have resulted in a greater proportion of captures in the central sector—albeit with lower overall numbers than those taken by the long-range compound in the outer sectors.

The results (Table 4) show that although the total numbers captured differed as expected (Table 1) between the various pheromone treatments, there was no significant difference between the proportion of captures in the various sectors. In all three treatments, captures increased with proximity to the pheromone sources, and there was no evidence to suggest that the two components acted differently over short or long-range distances.

DISCUSSION

The Australian field results confirm and extend the earlier findings of Persoons et al. (1976a,b) and show that there is no obvious optimal ratio of PTM 1 to PTM 2 and that blends are more attractive than the single components. The Cyprus results are similar but, contrary to earlier field tests in that country (Persoons et al., 1976b), traps baited solely with PTM 2 caught as many males as those containing mixtures (Table 2). Both compounds were long-lived under field conditions, and the tests suggested that baits

with 400–1000 μg of both components produced larger catches than lower or higher quantities of the mixtures (Tables 2 and 3). Preliminary field tests failed to reveal any evidence that PTM 1 and PTM 2 had distinct individual roles. Males flew to sources containing either single compound, but when both compounds were present the numbers of moths attracted increased significantly.

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ATTRACTION OF VARIOUS TORTRICINE MOTHS TO BLENDS CONTAINING *cis*-11-TETRADECENAL

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Abstract—Blends containing *cis*-11-tetradecenal with the trans isomer or *cis*-11-tetradecen-1-ol were field tested against the tortricine species *Choristoneura conflictana* and *Croesia semipurpurana*. The results indicate that *cis*-11-tetradecenal alone is a very potent attractant for male *C. conflictana* whereas male *Croesia semipurpurana* were strongly attracted to blends of *trans*/*cis*-11-tetradecenal in the range 80:20–90:10. *Cenopis acerivorana* and *Choristoneura fumiferana* were also caught with mixtures of 11-tetradecenal isomers while *Choristoneura fractivittana* was very strongly attracted to the mixture of *cis*-11-tetradecen-1-ol/*cis*-11-tetradecenal in the ratio 95/5.

Key Words—Large aspen tortrix, oak leaf shredder, *Choristoneura conflictana*, *Choristoneura fumiferana*, *Choristoneura fractivittana*, *Croesia semipurpurana*, *Cenopis acerivorana*, *cis*-11-tetradecenal, attractant, field tests, *trans*-11-tetradecenal.

INTRODUCTION

Two lepidopteran pest species, the large aspen tortrix, *Choristoneura conflictana* Wlk., and the oak leaf shredder, *Croesia semipurpurana* Kft., are

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beginning to assume greater economic importance in the hardwood forests of Canada and the United States. The former pest occurs throughout the range of trembling aspen in North America with occasional outbreaks causing serious defoliation in Canada, the New England states, New York, and Michigan (Baker, 1972). The oak leaf shredder is indigenous to southeastern Canada and the northeastern and middle Atlantic states. During 1964 and 1965, this species severely defoliated approximately half a million acres of red oak in Pennsylvania (Baker, 1972). Heavy infestations causing serious defoliation were reported in 1977 in various locations in Ontario (Howse and McDowall, 1977). As a first step to controlling these pests, a sex pheromone or synthetic attractant was sought.

Results published in 1976 (Weatherston et al., 1976) indicated that *cis*-11-tetradecenal is attractive to male *C. conflictana*. The addition of the trans isomer or *cis*-11-tetradecen-1-ol did not increase the trap catches while the addition of *cis*-11-tetradecen-1-ol acetate drastically reduced the attractancy of the *cis*-11-tetradecenal.

This paper further documents the effect on trap catch by additions of these compounds to the attractant and reports the results of field testing various *trans*/*cis*-11-tetradecenal mixtures against *Croesia semipurpurana*.

METHODS AND MATERIALS

cis-11-Tetradecenal was either prepared on a polymer support by the method of Fyles et al. (1977) or obtained from Chemical Samples Corporation. *trans*-11-Tetradecenal and *cis*-11-tetradecen-1-ol were obtained from Chemical Samples Corporation. The compounds were purified by chromatography on Adsorbosil CABN (50/100 mesh) from Applied Science Laboratories Inc. Compound purity and isomer composition were determined by GLC on a 17-ft $\times \frac{1}{8}$-in. column of 10% Silar 10C on Gas Chrom Q (60/80 mesh) at 175° with a carrier gas flow of 15 ml/min N_2.

The 1977 field tests were conducted at four sites: (1) a natural stand of aspen (approx. 50%) mixed with maple, larch, oak regeneration, and some spruce near the limit of the trapping area; (2) an orchard of hybrid poplar with white spruce as windbreaks; (3) a stand of 70-year-old aspen; and (4 a natural stand predominantly maple with a small amount of balsam fir. Sites (1) and (2) were on Manitoulin Island, site (3) near Fairbanks, Alaska, and site (4) was in Sault Ste. Marie.

The test chemicals were dispensed in polyethylene vial caps (100 μg/cap unless stated otherwise) placed in the bottom center of Pherocon 1-C traps (Zoecon Corp.) except at site (3) where Pherocon 2 traps were used. Traps were randomly placed at a height of 1.5–2.0 m, each trap being 30 m from

its neighbors. At sites (1) and (2) the traps were set out June 10, inspected at weekly intervals, and collected June 30; at site (3) the traps were set out on July 15, inspected every 6 days, and collected on August 10; at site (4) the traps were set out on June 30, inspected twice weekly, and collected on August 8. Unless stated otherwise, 10 replicates were used in each test. Data were submitted to analysis of variance. The differences among the means were determined by Duncan's new multiple range test.

RESULTS

Choristoneura conflictana

The initial experiment carried out at site (1) was to test the effect of varying the amount of *cis*-11-tetradecenal on the trap catch. The results (Table 1) indicated that there was no statistical difference between the catches in traps containing 154, 770, and 1540 μg of the compound, whereas the catches in traps containing 1.54 and 15.4 μg were significantly lower.

In the 13 treatments carried out at sites (1) and (2) comparing the effectiveness of various *cis*-11-tetradecenal/*cis*-11-tetradecen-1-ol mixtures (Figure 2), the catch varied from three males with *cis*-11-tetradecen-1-ol to 754 with *cis*-11-tetradecenal. Analysis of variance of the trap catches indicated that there was no significant difference between the catch of 100% *cis*-11-tetradecenal [$\bar{X}$/trap 75.4 (1)] and that of a 95:5 mixture of *cis*-11-tetradecenal/*cis*-11-tetradecen-1-ol [$\bar{X}$/trap 56.7 (1,2)]. Catches with mixtures other than the 95:5 were significantly less than catches with pure *cis*-11-tetradecenal [90:10, $\bar{X}$/trap 50.4 (2); 80:20, $\bar{X}$/trap 50.2 (2)].

Comparing the catches of various isomer mixtures of 11-tetradecenals at

TABLE 1. CAPTURES OF MALE *Choristoneura conflictana* BY VARIOUS AMOUNTS OF *cis*-11-TETRADECENAL (10 REPLICATES)

Weight of *cis*-11-tetradecenal (μg[a])	$\bar{X}$/trap[b]
1.54	3.4 (a)
15.4	7.9 (a)
154.0	21.4 (b)
770.0	23.3 (b)
1540.0	39.4 (b)

[a] The *cis*-11-tetradecenal used in this experiment was 100% isomerically pure.

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

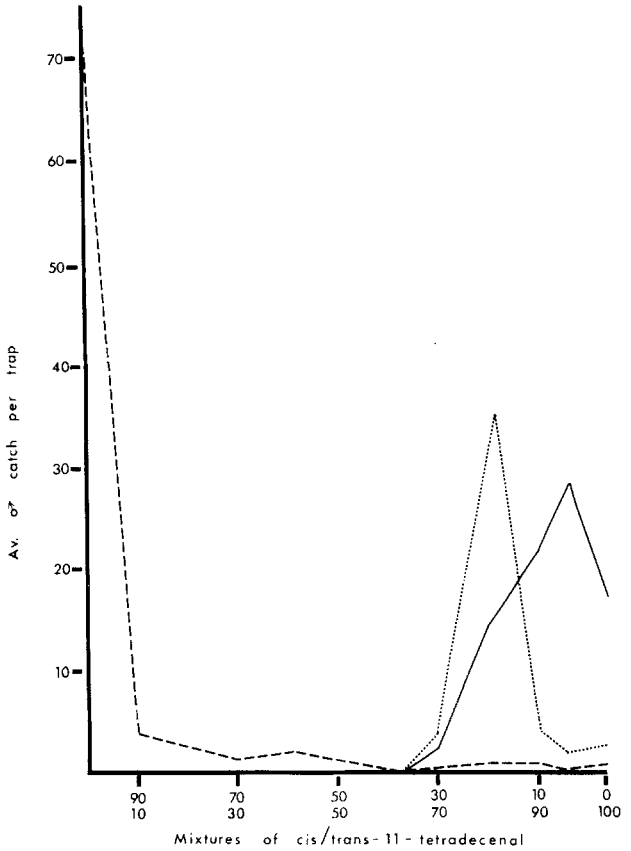

FIG. 1. The average number of male moths trapped with various blends of *cis*- and *trans*-11-tetradecenal. *Choristoneura conflictana* (– – –), *Croesia semipurpurana* (- - - -), *Choristoneura fumiferana* (———).

the Manitoulin sites (Figure 1) showed that catches with the 100% cis isomer [$\bar{X}$/trap 70.8 (1)] were significantly greater than all other baits.

The second largest catch, by the 95:5 cis–trans mixture [$\bar{X}$/trap 28.3 (2)], was also statistically greater than all of the other mixtures. There was no significant difference among the other treatments.

At the Alaskan site where the *C. conflictana* population was very low, the total male catch ranged from 0 to 16 for mixtures of *cis*-11-tetradecenal/*cis*-11-tetradecen-1-ol. Pure *cis*-11-tetradecenal caught 9 males whereas the 95:5 mixture caught 16. In the test with geometrical isomer mixtures the total male catch ranged from 0 to 28, pure *cis*-11-tetradecenal catching 28

males whereas the next best lure, the 95:5 cis–trans mixture, caught a total of 3 males.

Croesia semipurpurana

Trapping data from both Manitoulin Island and Sault Ste. Marie showed that blends of the isomers of 11-tetradecenal were very attractive to male *C. semipurpurana*. The Manitoulin Island data (Figure 1) obtained over a period of seven days show that an 80:20 trans–cis mixture of 11-tetradecenals was by far the best mixture tested. At the Sault Ste. Marie site data collected over 30 days (4 replicates) also indicated that the 80:20 mixture of isomers was highly attractive although the 90:10 trans–cis mixture was equally attractive.

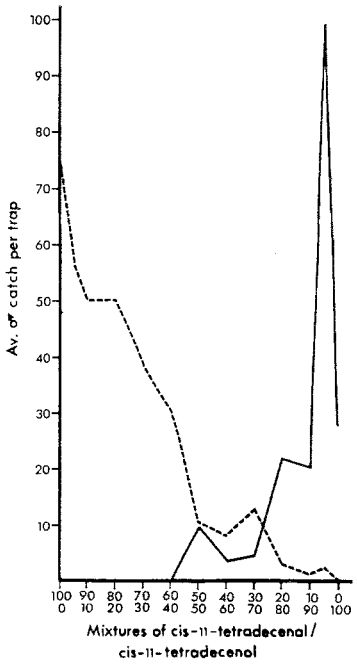

FIG. 2. The average number of male *Choristoneura conflictana* (– – –) and *Choristoneura fractivittana* (———) caught with blends of *cis*-11-tetradecenal and *cis*-11-tetradecen-1-ol.

Other Tortricine Species

Two other species were caught with mixtures of the tetradecenal isomers. The maple leaf roller, *Cenopis acerivorana* (MacK.) appeared to be attracted to a mid-range mixture of the isomers. Although the numbers were small, the attractiveness peaked in the 80:20–70:30 *cis*/*trans*-11-tetradecenal range [4 replicates, site (4)]. Eastern spruce budworm, *Choristoneura fumiferana* (Clem.), were caught at the Manitoulin Island site (1) with *trans*-11-tetradecenal synergized with a small amount of the cis isomer (Figure 1).

The polyphagous species *Choristoneura fractivittana* Clem. was caught at site (1) with various *cis*-11-tetradecen-1-ol/*cis*-11-tetradecenal mixtures (Figure 2). *cis*-11-Tetradecenal was unattractive to *C. fractivittana* males, but as can be seen in Figure 2, a small percentage (5%) greatly enhanced the attractiveness of *cis*-11-tetradecen-1-ol.

DISCUSSION

The *C. conflictana* trapping results, from both Ontario and Alaska, are in good agreement with the preliminary data published by Weatherston et al. (1976) and show that *cis*-11-tetradecenal by itself is a very potent attractant for males of this species and is a good candidate for monitoring populations of the pest.

No sex pheromone is known for any species of the genus *Croesia*, although Arn et al. (1974) have reported trapping male *Croesia holmiana* L. with codlemone (*trans*,*trans*-8,10-dodecadien-1-ol) plus *trans*-11-tetradecen-1-yl acetate. Since no *C. holmiana* were caught by codlemone alone, the authors (and subsequently Inscoe and Beroza, 1976) interpret this as indicating that *trans*-11-tetradecen-1-yl acetate is attractive to *C. holmiana* males. In the case of *C. semipurpurana*, data from both the Sault Ste. Marie and Manitoulin Island sites show that blends of the isomers of 11-tetradecenal are very attractive to males of this species. Blends in which the cis isomer predominates are unattractive, attractancy increasing with decreasing amounts of *cis*-11-tetradecenal until an optimum blend is reached with the 80:20–90:10 trans–cis mixtures. Verification of this mixture as the sex pheromone of *C. semipurpurana* must await the results of studies now in progress.

The catches of eastern spruce budworm *C. fumiferana* with isomer blends containing predominantly *trans*-11-tetradecenal concur with the results of Sanders and Weatherston (1976). In contrast to the oak leaf shredder and the spruce budworm, which are attracted to 11-tetradecenal blends rich in the trans isomer, males of the maple leaf roller *Cenopis aceri-*

vorana are best attracted to blends in which the cis isomer is present in the 70–80% range.

Roelofs and Comeau (1971) reported that *C. fractivittana* gave standard EAG responses to *cis*-11-tetradecen-1-ol and was attracted to this compound in the field. The field observations were substantiated by Weatherston and MacDonald (unpublished) in 1976, but no *cis*-11-tetradecen-1-ol/*cis*-11-tetradecenal mixtures were tested. Our results show that the aldehyde enhances the attractiveness of the alcohol.

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TRANSFER OF THE SEA ANEMONE PHEROMONE, ANTHOPLEURINE, BY THE NUDIBRANCH *Aeolidia papillosa*

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Abstract—After the nudibranch *Aeolidia papillosa* eats the sea anemone *Anthopleura elegantissima*, anthopleurine, an alarm pheromone from the sea anemone, persists in the tissues of the nudibranch. For at least five days following such a meal, nudibranchs are capable of evoking alarm responses in anemones without touching them, presumably by releasing anthopleurine into the water. The anemone's alarm response to anthopleurine is to withdraw the tentacles and oral disk, the preferred sites of attack for *Aeolidia*. This leaves exposed to attack the anemone body regions with the highest anthopleurine concentrations. Specimens of *Aeolidia* collected near sources of *Anthopleura* are more likely to contain detectable amounts of anthopleurine than those more distant; some nudibranchs collected 0.5 m from *Anthopleura* contained enough anthopleurine to evoke alarm responses in anemones they approached. These findings suggest that the predator helps in the transmission of anthopleurine, which may reduce the severity of predation on *Anthopleura*.

Key Words—*Anthopleura elegantissima*, *Aeolidia papillosa*, anthopleurine, alarm pheromone, anemone, nudibranch.

INTRODUCTION

Tissues of the sea anemone *Anthopleura elegantissima* contain large amounts of the betaine, anthopleurine, a compound that may function as an alarm

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pheromone (Howe and Sheikh, 1975; Howe, 1976a). Dilute solutions of anthopleurine activate specific receptors on the tentacles of *A. elegantissima* to evoke a stereotyped alarm response, consisting of rapid bending and shortening of the tentacles and depression of the oral disk, followed by constriction of the upper margin of the oral disk around the tentacles and oral disk (Howe, 1976a,b). The alarm response appears to have been integrated into the behavioral repertoire of *A. elegantissima* in an adaptive way (Howe, 1976c).

Any role for anthopleurine in intraspecific communication is likely to have two characteristics. First, because the anthopleurine in an anemone's tissues appears to be released only upon mechanical injury (Howe, 1976a), such a role probably requires tissue damage. Second, because the rates of diffusion of waterborne pheromones are low (Wilson, 1970) and the habitats of *A. elegantissima* are relatively turbulent (Ricketts and Calvin, 1962), anthopleurine cannot be expected to transmit useful messages more than a few centimeters from its source.

A potential function for anthopleurine in intraspecific communication that meets these two criteria is that anthopleurine released when a predator attacks *Anthopleura* evokes alarm responses in nearby anemones, thereby rendering them less vulnerable to predation. The present study is an experimental evaluation of that hypothesis.

The predator chosen for study was *Aeolidia papillosa*, an anemone-eating nudibranch gastropod that is regularly found in association with *A. elegantissima* and that appears to prefer *A. elegantissima* as prey to other western North American sea anemones (Harris, 1973; Waters, 1973). Two potential routes of anthopleurine release incident to predation by *Aeolidia* were considered: (1) that anthopleurine diffuses from the wounds on anemones that have been attacked, and (2) that anthopleurine diffuses from nudibranchs that have recently eaten *Anthopleura*. When some nudibranchs collected for the initial experiments evoked alarm responses in individual *Anthopleura*, even though all anemone–nudibranch contacts were prevented by manipulating the nudibranchs with a glass rod, the latter of these two alternative routes was chosen for detailed examination.

METHODS AND MATERIALS

Aeolidia between 2 and 3 cm in length were collected in 5–10 m of water from beneath Municipal Wharf #2 at Monterey, California, and were maintained in running sea water without feeding for no more than four days before experiments. The populations from which *Aeolidia* were collected suffered parasitism from a rhabdocoel flatworm; only animals without obvious

parasites were selected for experiments. *Aeolidia* did not survive long in captivity. By the tenth day after capture, about 50% had died. This short laboratory life span provided an upper limit to the duration of experiments. In no case, however, did the particular conditions of an experiment appear to further reduce that life span; experimental animals survived as long, on the average, as animals collected at the same time that were not selected for experiments. Weights of live *Aeolidia* were measured in sea water to the nearest 0.2 mg with a torsion balance. *A. elegantissima* used as nudibranch food were from clones collected at Pacific Grove, California; anemones from the same clone were used to feed *Aeolidia* in any given experiment. Detailed methods for each of the experiments performed are described with the results of those experiments.

RESULTS

Identification of Anthopleurine in Aeolidia

Our initial observation that individual *Aeolidia* could evoke alarm responses in *Anthopleura* at a distance suggested that a substance diffusing from *Aeolidia* was responsible for those alarm responses. That hypothesis was confirmed by the results of the following experiment. A nudibranch (collected, then starved for four days) that consistently evoked alarm responses was homogenized with a motor-driven, Teflon pestle homogenizer in 10 volumes of sea water. The filtered (Whatman GF/C) extract was capable of evoking alarm responses in *Anthopleura* even when diluted $1:5 \times 10^3$ (v/v) with sea water.

The high specificity of the alarm response for anthopleurine (Howe, 1976a) suggested that the alarm substance from *Aeolidia* was anthopleurine. That hypothesis was tested by comparing the alarm substances in *Anthopleura* and *Aeolidia* chromatographically. Whole *Anthopleura* and *Aeolidia* were dried overnight at 90°C and ground in a mortar. Weighed portions of the resulting powders were extracted for 1 hr in 4 parts (v/w) of methanol. The extracts were filtered and spotted (2 μl/spot) onto Whatman No. 1 paper, then developed (ascending) in 1-butanol, acetic acid, and water (4:1:5). Purified anthopleurine (Howe and Sheikh, 1975) was chromatographed separately, next to the tissue extracts. Developed chromatograms were sprayed with Dragendorff reagent or cut into 5-mm vertical sections and assayed for anthopleurine. The center of biological activity (alarm) for both extracts fell between R_f values of 0.14 and 0.17, compared with an R_f of 0.17 for purified anthopleurine on the same chromatogram. This result provides support for the hypothesis that the alarm substance from *Aeolidia* is chemically similar to anthopleurine. Previous structure–activity studies (Howe,

1976a) showed that the alarm response was specific for the anthopleurine structure. In tests with a series of ten commercially available quaternary ammonium anthopleurine analogs, the compound most similar to anthopleurine in structure and most active in the alarm response bioassay, DL-carnitine, was 3×10^3 times less active than anthopleurine. That result suggests that the alarm substance in *Aeolidia* is indeed anthopleurine.

Feeding Experiments

Although anthopleurine had not been reported from any organism except *Anthopleura*, it remained possible that the anthopleurine in *Aeolidia* was synthesized by *Aeolidia*, rather than by *Anthopleura*, especially because the *Aeolidia* used for the preliminary observations were known not to have eaten *Anthopleura* for at least four days. If, on the other hand, predation by *Aeolidia* upon *Anthopleura* was responsible for the presence of anthopleurine in *Aeolidia*, one would predict that the concentration of anthopleurine in the tissues of an *Aeolidia* would decrease with time after consuming *Anthopleura*. To test that prediction, a feeding experiment was performed.

For this experiment, the anthopleurine concentration of *Aeolidia* tissue was monitored by preparing extracts of cerata (finger-like projections of the dorsal surface that contain extensions of the hepatopancreas). Not only were cerata convenient to remove, but their repeated removal from the same animals did not appear to affect the behavior or survival of the nudibranchs. For each test, 1–3 anterior, mid-dorsal cerata were removed and blotted briefly, then placed on glass cover slips and dried to constant weight at 90°C. Cover slips were then weighed to the nearest 0.01 mg before and after scraping the dried cerata into test tubes; the dry tissue weight (mean for all samples = 0.9 mg) was computed by difference. Dried cerata were ground with a blunt glass rod and extracted with 10^4 parts (w/w) of distilled water for 2–4 hr at 2°C, then filtered (Whatman GF/C). Each extract was serially diluted 2-, 4-, and 8-fold, for a total of four concentrations for each extract. Using the same anemones and procedures as for previous bioassays (Howe and Sheikh, 1975), 0.5 ml of each concentration was mixed into a bowl of anemones, and the percentages of anemones giving alarm responses within 30 sec were recorded. Median effective concentrations, estimated graphically from probability plots of the resulting response percentages, were divided into the median effective concentration for pure anthopleurine (Howe and Sheikh, 1975) to yield estimates for the anthopleurine concentrations in the cerata. There was no significant correlation ($r = -0.19$, $P > 0.5$) between the amounts of *Anthopleura* tissue consumed by *Aeolidia*, expressed as a percentage of each nudibranch's weight, and the mean anthopleurine concentration in cerata during the experiment.

Six specimens of *Aeolidia* of approximately equal size (reduced weight range: 34.4–46.0 mg) were starved for four days, then placed in individual containers. Cerata were removed for anthopleurine assays; then these *Aeolidia* were allowed to feed upon pieces of *Anthopleura*. After 24 hr, during which time the *Aeolidia* had increased their weights by an average of 23% (SE = 5%), the pieces of *Anthopleura* were replaced with pieces of *Metridium senile*, an anemone that is a normal component of the diet of *Aeolidia* (Harris, 1973) but does not contain anthopleurine (Howe, unpublished). Cerata were removed for anthopleurine assays at the end of the *Anthopleura* meal and at two-day intervals thereafter. The experiment was terminated seven days after the beginning of the *Anthopleura* meal because of the deaths of three of the *Aeolidia*.

Anthopleurine concentrations in extracts of cerata are plotted (open circles) as a function of time since a meal of *Anthopleura* in Figure 1. Immediately after the *Aeolidia* ate *Anthopleura*, extracts of their cerata became markedly more effective in evoking alarm responses, and that increased effectiveness persisted for the duration of the experiment.

The retention of anthopleurine in the cerata following an *Anthopleura* meal might explain why an *Aeolidia* starved for several days could evoke the alarm response. Results from a bioassay experiment using live caged *Aeolidia* confirm that interpretation. A cage was constructed by replacing the lower wall and bottom of a 2.5 cm diameter plastic vial with plastic window screening. An *Aeolidia* was gently lifted into the cage. Slots cut through the upper wall of the vial accommodated a 2-cm-wide strip of plastic that both prevented the nudibranch's escape and suspended the cage in an anemone bowl. The caged nudibranch was then washed for 30 sec in slowly flowing sea water. The sea water supply to an anemone bowl was stopped; 3 min later the *Aeolidia* cage was introduced, without touching an anemone, and

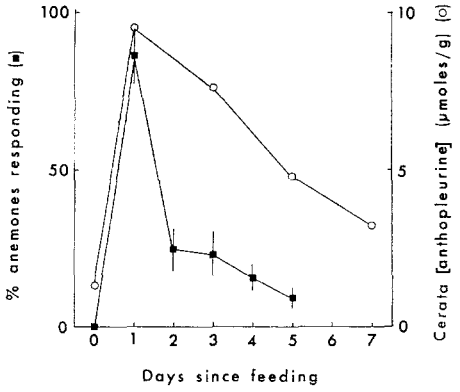

FIG. 1. Effects of an *Anthopleura* meal on anthopleurine concentrations in *Aeolidia* cerata (circles, right ordinate) and on the ability of live *Aeolidia* to evoke alarm responses (squares, left ordinate). Vertical lines through the squares indicate standard errors.

positioned near the center of the bowl, approximately 1 cm from the nearest anemone tentacles. At 30-sec intervals, the cage was gently rinsed with 20 ml of sea water from the bowl. The proportion of anemones giving an alarm response within 3 min was recorded.

Nine specimens of *Aeolidia* that had been starved for three days were tested with the cage bioassay. Of those nine, eight failed to evoke any alarm responses. These eight animals were placed in individual containers and offered pieces of *Anthopleura* for 31 hr (mean percent weight gain = 42, SE = 9.5), after which the food was removed, and the specimens of *Aeolidia* were tested for the ability to evoke alarm responses. The cage bioassay was repeated at 24-hr intervals for four more days. As is shown in Figure 1, the proportion of anemones giving an alarm response to live *Aeolidia* (squares) increased from 0 before the meal to 84% (SE = 10%) after the meal. This percentage dropped sharply by the second day of feeding, but still differed significantly from 0 ($t = 2.80$, $P < 0.05$) on the fifth day of testing. This experiment shows that for several days after a meal of *Anthopleura*, an intact *Aeolidia* releases enough anthopleurine to evoke alarm responses in nearby *Anthopleura*.

Site of Attack Preference by Aeolidia

Observations were made on feeding encounters between *Anthopleura* and *Aeolidia* to determine whether *Aeolidia* showed a preference for particular regions of the anemone. In the first experiment, 55 specimens of *Anthopleura* were placed in a shallow pan that had been coated with silicone grease to prevent their attachment and any behavioral responses that required attachment. Fifty nudibranchs 2–3 cm in length were introduced into the pan 24 hr after the anemones. After 5 hr, the nudibranchs were removed, and the anemones were relaxed with $MgCl_2$ and then surveyed for tissue damage. Thirty-three anemones showed tissue damage: 4 (12%) to the column, 11 (33%) to the pedal disk, and 18 (55%) to the oral disk and tentacles. Although the pedal disk would not normally be vulnerable to attack, these results suggest that *Aeolidia* that are allowed to select a site of attack prefer sites other than the surface of the column.

In a second experiment, nudibranchs were allowed to attack attached anemones. Fifty attacks were observed by specimens of *Aeolidia* longer than the column diameter of the attacked anemones. None of these nudibranchs evoked alarm responses at a distance. Thirty-six (72%) attacks were directed to the tentacles and oral disk, and 14 (28%) to the column. Several of the nudibranchs that did eat the column first attempted to reach the tentacles, but the anemones avoided contact between their tentacles and the nudi-

branchs by shortening the tentacles and then extending the column to take the tentacles beyond the reach of the predators. These results show that specimens of *Aeolidia* that do not evoke alarm responses as they approach *Anthopleura* are more likely to attack tentacles and oral disk than the column ($\chi^2 = 9.68$, $P < 0.005$).

Anthopleurine Bioassays on Field-Captured Aeolidia

Twenty specimens of *Aeolidia* from each of four different habitats were collected near Monterey Municipal Wharf #2. For each habitat, the mean distance to the nearest *A. elegantissima* was estimated to the nearest 0.5 m at the time of collection. Cerata were removed from the eight largest animals from each habitat (2–3 cm total length). These cerata were dried, weighed, and extracted in 10^4 parts distilled water as previously described. Each extract was then tested as before for the ability to evoke alarm responses in 14–18 anemones. In Figure 2, responses to cerata extracts for each habitat are expressed as a function of estimated mean distance from *Anthopleura*. For statistical comparisons, the positive responses elicited by the eight specimens of *Aeolidia* collected in each habitat were pooled. The percentage of anemones responding to extracts from *Aeolidia* 0.5 m away (47 of 130) is significantly greater than that from any other habitat ($\chi^2 > 30$, $P < 0.005$), and the percentage of responses to nudibranchs 1.5 m distant (6 of 129) is greater than that from the most distant habitat ($\chi^2 = 4.24$, $P < 0.05$). Although specimens of *Aeolidia* collected nearest those of *Anthopleura* showed considerable variability in their cerata anthopleurine concentrations, two of those nudibranchs had concentrations at least as high as the maximum recorded in laboratory feeding experiments.

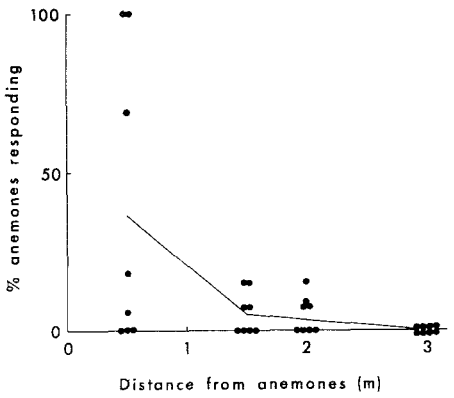

FIG. 2. Alarm responses to cerata extracts from four samples of eight specimens of *Aeolidia* collected at increasing mean distances from the nearest specimens of *Anthopleura*. Each point represents the percentage of 14–18 anemones that gave alarm responses to a cerata extract (0.2 mg/liter) from a single nudibranch. The line connects the pooled response percentages for each of the four collections.

TABLE 1. ANTHOPLEURINE CONCENTRATIONS IN DIFFERENT ANEMONE BODY REGIONS

Body region	Anthopleurine (μmol/g ± 95% CI)
Tentacles	23 ± 5
Oral disk and pharynx	58 ± 10
Upper column margin	63 ± 16
Upper column wall	66 ± 17
Lower column wall	106 ± 21
Pedal disk and lower column margin	121 ± 28
Mesenteries	29 ± 6

Location of Anthopleurine in Anemones

Identification of the anatomical sites where a pheromone is stored may provide useful information about the biological role of that pheromone (see Wilson and Pavan, 1959, for example). To locate anthopleurine in anemone bodies, a large, solitary specimen of *A. elegantissima* was dissected into the seven pieces described in Table 1. Pieces were dried to constant weight at 90°C, ground in a mortar, and extracted with 50 parts (w/w) distilled water at 20°C for 10 hr. Extracts were centrifuged, and the supernatant liquids were biologically assayed for anthopleurine. The results (Table 1) show that the apparent anthopleurine concentration is significantly higher in lower column margin/pedal disk and lower column wall than in other body regions and lower in the tentacles and mesenteries than in other body regions ($P < 0.05$). The results of two additional experiments suggest that these apparent differences in the anthopleurine activity of different tissues represent real differences in anthopleurine concentration.

To assess the possibility that enzymatic degradation of anthopleurine at different rates in different body regions contributed to the apparent differences in pheromone concentration, a second experiment was performed. Tentacles and lower column margin/pedal disk were removed from a single, live, clonal anemone, blotted briefly, then weighed. Each tissue type was homogenized in 50 parts (w/w) water and assayed for anthopleurine as before. In this experiment, the apparent anthopleurine concentration of lower column margin extract (0.24 μmol/ml) was about eight times that of the tentacle extract (0.03 μmol/ml). Three micro-moles of purified anthopleurine (Howe and Sheikh, 1975) in 0.3 ml water were added to 5 ml of each extract. Each extract was incubated 11 hr at 14°C, then assayed for anthopleurine. The anthopleurine concentration in each extract had increased by the expected 0.6 μmol/ml, suggesting that the tentacle extract could not degrade the added anthopleurine appreciably.

A final experiment was performed to examine the possibility that the low apparent anthopleurine concentration in tentacles could be due to binding of anthopleurine to specific anthopleurine receptors, previously shown to be located in tentacles (Howe, 1976b). Portions (10 ml) of the two extracts prepared for the preceding experiment were dialyzed (Spectrapore 3 membrane) against many changes of distilled water for 12 hr at 4°C. Volumes of the retentates were adjusted to 11 ml and each was assayed for anthopleurine. Neither had detectable levels (<2 nmol/ml). Purified anthopleurine (1 μmol) was added to each extract, and the extracts were incubated for 2 hr at 20°C, then assayed for anthopleurine. If anthopleurine receptors in the dialyzed tentacle extract bound some of the added anthopleurine, one might have expected it to have a lower apparent concentration than the lower column margin/pedal disk extract. Instead, the anthopleurine concentrations of the extracts did not differ significantly.

DISCUSSION

We have examined the hypothesis that an alarm pheromone transferred by a predator among members of a prey species may reduce the severity of damage by the predator. Our evidence supports that hypothesis in two major respects.

First, transfer of anthopleurine by *Aeolidia papillosa* can occur in the laboratory and probably does occur under natural conditions. Upon eating *Anthopleura*, *Aeolidia papillosa* accumulates anthopleurine in its cerata. The initial concentration (about 100 μmol/g) drops by about 10 μmol/g per day after feeding (Figure 1, circles), suggesting that *Aeolidia* can neither digest nor excrete anthopleurine rapidly. For at least five days after a meal of *Anthopleura*, *Aeolidia* is capable of evoking alarm responses in *Anthopleura* at a distance of at least 1 cm under experimental conditions (Figure 1, squares). Some nudibranchs collected near specimens of *Anthopleura* had concentrations of anthopleurine comparable to the levels observed in the early stages of the laboratory feeding experiments, a result that suggests that individual *Aeolidia* evoke alarm responses as they approach specimens of *Anthopleura* under natural conditions as well as in the laboratory.

Although the results from the two experiments summarized in Figure 1 are similar, a critical comparison reveals one difference: after the post-feeding peaks at day 1, the ability of *Aeolidia* to evoke alarm responses at a distance declines more rapidly than the anthopleurine concentration in the cerata. A possible explanation for this difference is that cerata may become saturated with anthopleurine after a meal. Any anthopleurine consumed in excess of the amount required to saturate the cerata might then be rapidly

lost to the medium, after which the anthopleurine concentration in the cerata might decline more slowly. That the concentration of anthopleurine in the cerata after a meal does not depend on the size of the meal (see results of first feeding experiment) supports this hypothesis.

Second, both the behavioral response to anthopleurine and the location of the pheromone in the body of an anemone suggest that *Anthopleura* benefits by responding to predator-borne anthopleurine. By enclosing the tentacles and oral disk, important but relatively delicate structures, within an envelope of column wall, the alarm response ensures that the preferred sites of attack for *Aeolidia* are less available for predation. Further, the regions of the anemone body that remain exposed to attack after an alarm response are those in which the anthopleurine concentration is highest (Table 1), ensuring that predation upon an alarmed anemone will result in maximal transfer of alarm pheromone to the predator. It seems especially advantageous that the lower column has the highest anthopleurine content, because this body region would be contacted first by predators, such as *Aeolidia,* that feed close to the substratum. Because *Aeolidia* apparently prefers to attack parts of the anemone body that are relatively deficient in anthopleurine, it is tempting to speculate that anthopleurine may be functioning both as an alarm pheromone and as a chemical feeding deterrent. No additional evidence supports that speculation at present.

In intertidal habitats, *A. elegantissima* undergoes longitudinal fission to form clones of genetically identical individuals (Francis, 1973), a reproductive pattern that should favor the evolution of an alarm pheromone communication system (Hamilton, 1963). We have considered only one set of circumstances under which such a system might operate. Other possible roles for anthopleurine in intraspecific communication remain to be investigated. For example, anthopleurine released when wave-borne logs (Dayton, 1971) or rocks damage anemones may cause alarm responses in nearby clonemates, making them smaller, less vulnerable targets. Anthopleurine released from a damaged, detached anemone could reduce the risk of being cannibalized by other anemones by evoking alarm responses instead. Apart from any other messages that may be conveyed by anthopleurine, it appears likely that anthopleurine transmission by eolid nudibranchs may help protect *Anthopleura.* Like the fabled mice who belled the cat to be warned of her approach, *Anthopleura* may have a means to ensure that it is forewarned of the presence of one of its major predators.

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BACTERIAL CONTENTS OF THE ANAL AND CASTOR GLANDS OF BEAVER (*Castor canadensis*)

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Abstract—Bacterial contents of both the anal gland and castor gland of the beaver (*Castor canadensis*) were determined. Using our culture methods, no bacteria were isolated from the castor glands, but the anal gland contained high numbers of the aerobe *Escherichia coli* and the anaerobe *Bacteroides fragilis*. The latter may be represented by several variants but facilities were not available for advanced anaerobic analysis. The relative numbers of each bacterial group and the group present were constant regardless of sex, age class, or colony of beaver. The bacterial fermentation hypothesis is rejected for castor gland section but remains possible for anal gland secretions based on variations seen in *B. fragilis*. The role of the products of both the castor gland and anal gland are discussed in relationship to scent communication in beaver.

Key Words—*Castor canadensis*, anal gland, castor gland, fermentation, scent communication.

INTRODUCTION

Canadian beaver (*Castor canadensis*) live in colonies composed of an extended family unit. Each colony occupies a discrete area not used by other colonies (Taylor, 1970; Svendsen, 1979). Scent mounds, a "mudpie" of mud and debris to which the beaver adds odoriferous body products, are conspicuous within the area used by a beaver colony. Beaver possess two pairs of large saclike glands which produce odoriferous products implicated in scent communication (Svendsen, 1978).

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A bacterial fermentation process producing volatile constituents used in chemical signaling has been proposed for several species of mammals (Albone and Perry, 1975; Michael et al., 1976; Gorman et al., 1974). The most detailed evidence to support the fermentation hypothesis and its role in conspecific recognition based on differences in fatty acid profiles are presented for *Vulpes vulpes* (Albone et al., 1974), *Macaca mulatta* (Michael et al., 1972), and *Herpestes auroputatus* (Gorman, 1976). In this paper we report the bacterial contents of the anal and castor glands of beaver and the relationship of these findings to chemical communication and the fermentation hypothesis.

METHOD AND MATERIALS

Samples of the contents of both the anal gland and castor gland were collected aseptically using a 1.0-ml sterile disposable syringe with a $1\frac{1}{2}$-in. 20-gauge needle. All samples were obtained from live beaver either anesthetized in the laboratory or restrained in a handling bag in the field. To obtain contents of the anal gland, the wall of the cloaca was swabbed with antiseptic, the anal gland pushed outward against the wall of the cloaca, and the needle inserted through the tissue into the lumen of the gland. A 1.0-ml sample was collected. Contents of the castor glands (=castoreum) were collected from anesthetized beaver. An incision was made through the skin to expose the gland, the gland surface swabbed with antiseptic, and castoreum drawn into a sterile syringe.

Viable counts of aerobic/facultative and anaerobic bacteria were determined by diluting the sample through 10^{-5} dilutions in prereduced thioglycolate broth, then 0.1-ml samples of the dilutions were plated on duplicate plates of sheep blood agar, chocolate agar, and eosine–methylene blue (EMB) medium. One set of plates was immediately placed in Gas Pac anaerobic jars and incubated for 48 hr at 37°C. The other set was incubated at the same temperature in air.

The isolation and enumeration of anaerobes from tissues and secretions presents special problems, foremost of which is the need to exclude oxygen from the collected material. In the case of the anal gland secretions, the problem is minimized during collection because the secretion is a highly viscous, fatty material which would retard 0_2 diffusion. The syringe used for collection was evacuated by forceful and full depression of the plunger and then immediately inserted into the lumen. A 0.5-ml sample of the secretion was emulsified in the thioglycolate diluent and placed on the solid media and placed under anaerobic conditions as rapidly as possible. Exposure to oxygen was kept to a minimum; none the less, since roller tube counts were

not feasible, we suspect that the viable counts of the anaerobes isolated by our procedure are underestimates of the numbers present in anal gland secretions.

After incubation, colony types were determined and counted. All colony types were Gram stained and aerobic/facultative Gram-negative rods were tested for oxidase production and characterized using the API 20E system for differentiating members Enterobacteriaceae or the API 20A system for anaerobic identification where appropriate.

RESULTS AND DISCUSSION

No bacteria were cultured from castoreum using the methods outlined. Types and number of bacteria cultured from anal gland secretion under the described conditions are shown in Table 1. In addition to the common isolates, *Escherichia coli* and *Bacteroides fragilis*, we occasionally isolated other types of bacteria in very low numbers. These random isolates were only found when the secretion was plated full-strength and were identified as *Staphylococcus*, *Bacillus*, *Proteus*, and in one case *Flavobacterium*. These random isolates most likely represent transient contaminants of the sample during collection.

The API 20A profiles of the anaerobic isolates are shown in Table 2. When different anaerobe colony types were detected in the same animal, each was tested individually by the API procedure. Different colony types from the same gland or animal are listed as "isolate number" in Table 2. The different colony types were found to give identical biochemical profiles in some cases, whereas in others the profiles were dissimilar in one or two reactions but still identifiable as *B. fragilis*.

Studies by Holdeman and Moore (1972) have shown that the anaerobe *B. fragilis* represents a "continuum of variants with many intermediate strains." Five subspecies of *B. fragilis* have been designated. The API procedure allows subspecies differentiation, but the variability seen among isolates in our samples makes subspeciation questionable without further confirmatory tests including gas–liquid chromatography. The isolates are readily identifiable as *B. fragilis* however, and we limited the interpretation to identification of the species, recognizing that the data indicate a number of variants were isolated.

In all cases, the bacteriological screening of the contents of the anal gland yielded only gram-negative facultative or anaerobic bacilli under our culture conditions. The absence of other species was striking, as was the relatively constant numbers of *E. coli* isolated from the different beaver. This suggests that the anal gland presents a unique niche with a well-defined normal bacterial flora. The possibility that other bacteria are present,

TABLE 1. BACTERIOLOGICAL SCREENING OF THE CONTENTS OF THE ANAL GLAND OF BEAVER IN SOUTHEAST OHIO

Beaver specimen			Bacterial flora			
			Facultative		Anaerobic	
Age class	Colony	Date collected	Organism	Numbers[a] (cfu/ml)	Organism	Numbers (cfu/ml)
Ad	A	3/17/77	*Escherichia coli*	1.13×10^8	*Bacteroides fragilis*	7.0×10^3
Ad	A	4/17/77	*Escherichia coli*	6.30×10^6	*Bacteroides fragilis*	7.0×10^5
Ylg	B	4/21/77	*Escherichia coli*	4.20×10^7	*Bacteroides fragilis*	9.8×10^4
Ylg	C	4/21/77	*Escherichia coli*	4.5×10^7	*Bacteroides fragilis*	8.0×10^5
Ylg	C	4/25/77	*Escherichia coli*	1.6×10^7	*Bacteroides fragilis*	1.2×10^4
Ylg	D	5/25/77	*Escherichia coli*	1.87×10^7	*Bacteroides fragilis*	6.0×10^5
Ad	D	5/26/77	*Escherichia coli*	2.2×10^6	*Bacteroides fragilis*	3.1×10^4
			Escherichia coli	6.7×10^6	*Bacteroides fragilis*	3.9×10^4
Kit	E	9/2/77	*Escherichia coli*	1.9×10^7	Anaerobe (unknown)	1.9×10^6

[a] Colony forming units (cfu).

TABLE 2. API 20A PROFILE OF REACTIONS OF *B. fragilis* ISOLATES FROM ANAL GLANDS

Animal	Isolate number	Biochemical tests																				
		GEL	ESC	GLY	CEL	MNE	MLZ	RAF	SOR	RHA	TRE	IND	URE	GLU	MAN	LAC	SAC	MAL	SAL	XYL	ARA
78/80	1	+	−	+	+	+	+	+	−	+	+	−	−	−	−	+	−	+	+	+	+
78/80	2	−	−	+	−	+	+	+	−	+	−	−	+	−	−	+	−	+	−	−	−
81/82	1	+	−	+	+	+	+	+	−	+	+	−	−	−	−	+	−	−	+	−	+
81/82	2	+	−	+	+	+	+	+	−	+	+	−	−	+	−	+	−	+	+	−	+
68/78	1	−	−	+	−	+	+	+	−	+	−	−	+	−	+	+	+	+	+	−	−
142/144	1	−	−	+	−	+	+	+	−	+	−	−	+	−	−	+	+	+	−	−	−
93/87	1	+	−	+	+	+	−	−	−	+	+	−	−	−	−	−	−	−	+	−	±
97/146	1	−	−	+	−	+	+	+	+	+	+	−	−	−	−	+	−	+	−	+	+
97/146	2	−	−	+	−	+	+	+	+	+	+	−	±	−	−	+	−	+	−	+	+
67/173R	1	−	−	+	−	+	+	+	+	+	−	−	+	−	+	+	+	+	−	−	−
67/173R	2	−	−	+	−	+	+	+	+	+	−	±	+	−	+	+	+	+	−	−	−
67/173L	1	−	−	+	−	+	+	+	+	+	−	−	+	−	+	+	+	+	−	−	−

especially anaerobes, cannot be completely excluded by our procedures, but our findings suggest that if other bacterial species are present they are likely to be in low numbers and a minor component of the normal flora.

These data provide further information in the examination of the fermentation hypothesis of chemical recognition based on different concentrations and combinations of chemicals derived from bacterial metabolism of sebum and other apocrine secretions. The assumption is that if the animals have different bacterial populations in the gland they will also have different chemical profiles and different odors (Gorman et al., 1974; Gorman, 1976). The data do not support a widely varied bacterial flora present in the anal gland regardless of sex, age class, colony, or geographic area (Table 1). Variation in biochemical reactions of *B. fragilis* does leave the possibility that subtle but distinct differences in secretion odor could result from this group, however (Table 2). *E. coli* is a ubiquitous microbe in mammalian digestive tracts and *B. fragilis* is a normal flora of rodent digestive tracts (Holdeman and Moore, 1972); thus both can invade the anal gland from the lower digestive tract.

Beaver construct scent mounds of mud to which castoreum is added. It is as yet undocumented if anal gland secretions are also added to the scent mound. Castoreum is a mixture of many compounds (Lederer, 1950), most of which are normal metabolic by-products in the urine. These high-molecular-weight compounds have low volatility and are widely found in mammalian species. Many of these compounds are by-products of digestion. Urine is drawn into the castor gland and carries the contents to the outside by way of the cloaca (Svendsen, 1978). Four compounds unique to castoreum and not found in beaver urine have been identified, these are castoramine (Valenta and Khaleque, 1959) *cis*-cylcohexane-1,2-diol (Valenta et al., 1961), and two yellow pigments (Lederer, 1949). Castoramine is a highly volatile, pungent chemical which characterizes the odor of beaver. Kacnelson and Orlov (1954) suggest that urine products interact with the lining of the castor gland and changes take place that give castoreum its characteristic composition. Both *cis*-cyclohexane-1,2-diol and the pigments are synthesizable from urine products, but castoramine is unique. In light of the apparent absence of a bacterial flora in the castor gland, one must assume that castoramine is a product of the cells lining the lumen of the gland.

The products of the anal gland are not known, and chemical analysis is underway. Whereas beaver do have a bacterial flora in the anal gland, two groups are present in high numbers and appear to be present in all sex, age, and colony classes. The only indication of variation which could be correlated to production of different chemical signals in individual beaver is among the variants of *B. fragilis*. Further analysis involving a complex anaerobic facility is needed to test this hypothesis however.

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VESTITOL: A PHYTOALEXIN WITH INSECT FEEDING-DETERRENT ACTIVITY

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Abstract—A major feeding deterrent for *Costelytra zealandica* larvae was isolated from the root of the resistant pasture legume *Lotus pedunculatus* and was identified as 3*R*-(−)-vestitol. This compound was also identified in feeding deterrent-active *L. pedunculatus* leaf extracts. (−)-Vestitol and a second *Lotus* isoflavan, sativan, have been reported to have phytoalexin activity, and the implications of this for the study and understanding of insect resistance are briefly discussed.

Key Words—*Costelytra zealandica*, *Lotus pedunculatus*, insect resistance, phytoalexin, feeding deterrents, isoflavan, sativan, vestitol.

INTRODUCTION

The subterranean larva of *Costelytra zealandica* (Coleoptera: Scarabaeidae) is the most serious agricultural pest in New Zealand. The economic impact of the insect is greatest when it attacks established ryegrass/white clover (*Lolium perenne*/*Trifolium repens*) pastures. Recent screening of numerous pasture species for resistance to *C. zealandica* larvae has led to the discovery of resistance in a few legumes and grasses (Farrell and Sweney 1972, 1974a,b). Particularly high resistance was found in the legume *Lotus pedunculatus*, a species already in use in some pasture situations, but other *Lotus* species, including *L. corniculatus* (birdsfoot trefoil), were not resistant. Interest in *L. pedunculatus* was heightened by the finding that pastures containing 1:1 mixtures of *L. pedunculatus* and perennial ryegrass were as resistant to *C.*

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zealandica as those containing only *L. pedunculatus* (Kain and Atkinson, 1977; East and King, 1977). Indeed, Kain and Atkinson (1977) found that pastures containing as little as 20% *L. pedunculatus* were also relatively resistant.

Our studies on the nature of the resistance of *L. pedunculatus* have revealed that the root of this plant is highly distasteful to third instar larvae (Sutherland, 1976). At least two feeding deterrents have been isolated from an active *L. pedunculatus* root extract and have been partially characterized (Sutherland et al., 1975). We report here the isolation and identification from *Lotus* root, of the isoflavan (−)-vestitol, which deters feeding of *C. zealandica* larvae.

METHODS AND RESULTS

Feeding Assay of L. pedunculatus Root

Preliminary assays of the effect of *L. pedunculatus* root on larval feeding were undertaken using $CHCl_3$/MeOH homogenates of fresh root from 4- to 6-month-old plants raised in a glasshouse (Sutherland et al., 1975). These extracts and fractions from the subsequent large-scale extraction were incorporated in a 4% agar–4% cellulose powder medium containing 0.1 M sucrose and 0.01 M ascorbic acid as standard feeding stimulants (Sutherland and Hillier, 1974). Test samples in solvents were evaporated onto cellulose powder, and this was mixed with hot agar containing the feeding stimulants to produce the feeding medium. Test disks (15 mm diam.) were cut from the cooled medium and offered to each of 20 third-instar *Costelytra zealandica* larvae, collected from the field and starved for 24 hr. Feeding-deterrent activity was assessed by comparing 24-hr fecal pellet counts of these larvae with similar counts of 20 larvae offered standard disks containing feeding stimulants only and of a third group offered blank disks prepared with distilled water. All root extracts and fractions were incorporated in the medium at a concentration equivalent to fresh root (1 g/ml) with the exception of the dose–response test.

Activity of Crude Extract of Fresh Root

The initial crude extract of *L. pedunculatus* root was highly active against larval feeding (Table 1, Figure 1). At fresh root concentration, feeding was reduced to less than the blank level. Whereas some chewing of blank disks was evident and ingestion from standard disks containing only stimulants was substantial, most larvae did not bite disks containing root extract plus stimulants. A dose–response curve was fitted by probit analyses

TABLE 1. EFFECT OF CRUDE $CHCl_3$–MeOH EXTRACTS OF *Lotus pedunculatus* ROOT AND TOPS ON RESPONSE OF THIRD INSTAR *Costelytra zealandica* LARVAE TO A STANDARD PHAGOSTIMULANT MIXTURE; TOTAL FECAL PELLETS PRODUCED BY 20 LARVAE IN 24 HR

		Standard and *Lotus*	
Blank	Standard	Root	Tops
101	324	85	
106	284		28

to the scaled bioassay data (Finney, 1952; Figure 1). The estimated amount of root required to reduce feeding by 50% (ED_{50}) was 0.05 g/g medium.

Isolation of Feeding Deterrents

Field-collected root material of 1-year-old *L. pedunculatus* (Grasslands 4702) was dried, milled, and extracted (2.6 kg) in a Soxhlet apparatus with methanol. The methanol extract was evaporated to dryness and partitioned

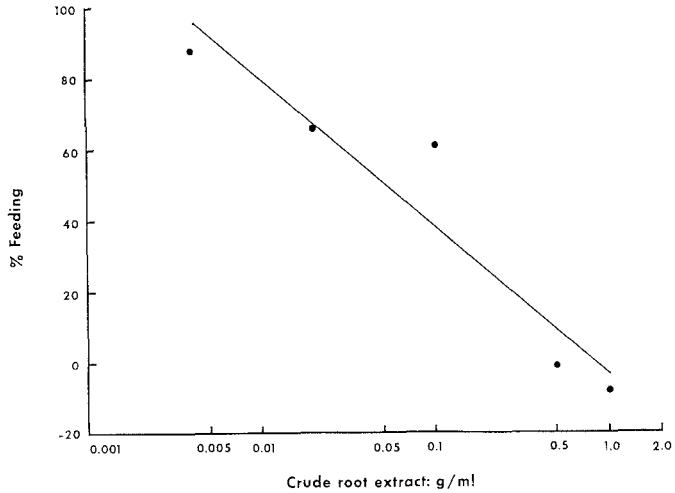
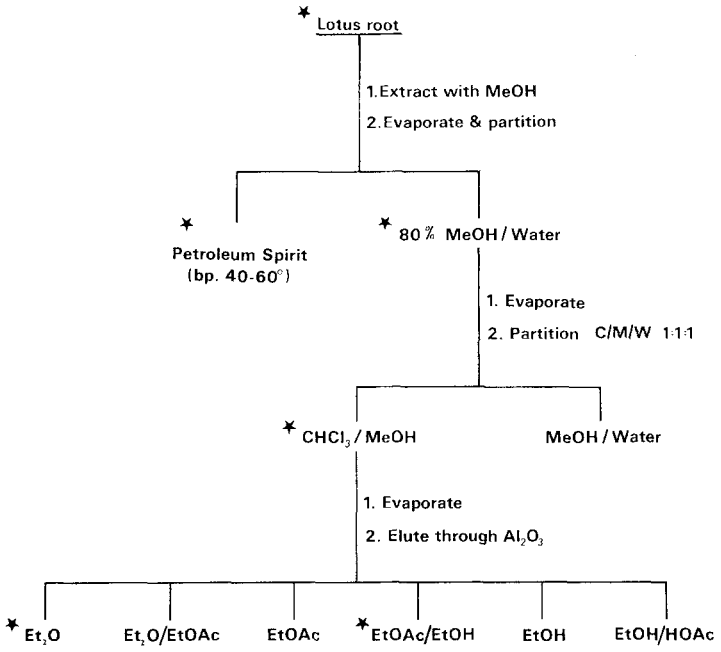


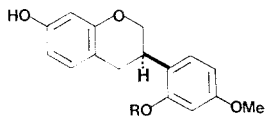
FIG. 1. Dose–response curve for the effect of a crude extract of fresh *L. pedunculatus* root on feeding by 3rd instar *Costelytra zealandica* larvae. Ingestion is expressed as a % of the standard response to the phagostimulant. Feeding on standard disks is taken as 100% and that on the corresponding blank disks as 0%.



SCHEME I

according to Scheme I. Each fraction was tested using the feeding assay and those which reduced feeding significantly are indicated by an asterisk. Although the initial partition between petroleum spirit (bp 40–60°) and aqueous methanol gave activity in both phases, the petroleum spirit layer has not been investigated further in the present work. The activity remained in the organic phase when the aqueous–methanol phase was further partitioned between $CHCl_3$–MeOH–H_2O (1:1:1). This phase was then evaporated to dryness and eluted through alumina (Woelm, 100 g, 10% water) with a sequence of solvents (250 ml). Deterrent activity was found in the diethyl ether and EtOAc–EtOH (1:1) eluates.

The EtOAc–EtOH eluate was evaporated to dryness (1.2 g) and chromatographed on silicic acid (Mallinkrodt, 100 g, 5% water) with $CHCl_3$, collecting 50-ml fractions. The active fraction was rechromatographed on LH20 Sephadex with $CHCl_3$–EtOH (9:1) to give two crystalline fractions. The major component (60 mg) had the same R_f (0.66) on thin-layer chromatography (Merck precoats, Silica gel HF_{254}, Et_2O–*n*-hexane, 5:1) as reported for vestitol (Bonde et al., 1973) while the minor component had R_f of 0.61 in the same solvent. Both gave a mauve color reaction with fast blue B salt



I R=H

II R=Me

solution. Feeding assays indicated that all the activity of the EtOAc–EtOH fraction could be attributed to vestitol (I).

The diethyl ether eluate was rechromatographed on silicic acid (50 g, 5% water) with *n*-hexane–Et_2O (4:1) to give a series of fractions. Feeding deterrent activity was located in the fraction which gave a spot on thin-layer chromatography (Et_2O–*n*-hexane, 5:1) with the same R_f (0.7) as that reported for sativan (II, Bonde et al., 1973). Rechromatography on silicic acid gave sativan as colorless crystals (5 mg), mp 120–122° (lit. 125–127°, Ingham and Millar, 1973). Sativan had $M^+ = 286$ and prominent fragments at *m/e* 164, 151, 149, 121 in the MS while the UV had λ_{max} (EtOH) 228, 280, 284 nm changing to λ_{max} (EtOH + NaOH) 248 *sh*, 280 *sh*, 285, 298 *sh* nm on addition of 0.1 N NaOH. Although sativan had some feeding activity, there was insufficient material isolated to confirm that it accounted for all the deterrent activity of the diethyl ether fraction.

Identification and Activity of Vestitol

Vestitol was recrystallized from MeOH, mp 144–145°, and it was identified spectroscopically. The mass spectral data gave $M^+ = 272$ and prominent fragments at *m/e* 150, 138, 137, 135 as reported by Bonde et al. (1973), while the UV absorption spectrum gave λ_{max} (EtOH) 228, 281, 285 nm changing to λ_{max} (EtOH + NaOH) 240 *sh*, 294 nm on the addition of 0.1 N NaOH.

The NMR spectrum of vestitol gave signals at δ 7.0 (d, J9, H-5 or 6′), 6.88 (d, J9, H-6′ or 5), 6.33–6.50 (m, H-6, 3, 8′, 5′), 4.28 (dd, J9, 4, H-2), 3.93 (d, J9, H-2), 3.70 (s, OMe), 3.46–3.60 (m, H-3), 2.90 (d, J8, H-4). The 3*R* configuration of vestitol was confirmed from the rotation, $[a]_D^{20} - 5°$ (C, 0.27, MeOH), which is consistent with that obtained by Bonde et al. (1973) for 3*R*-(−)-vestitol isolated from *L. corniculatus* leaf in response to infection.

A dose–response curve for 3*R*-(−)-vestitol is shown in Figure 2. This compound gives an ED_{50} with *C. zealandica* larvae of about 8.5 μg/g medium. Feeding was completely inhibited at about 100 μg/g.

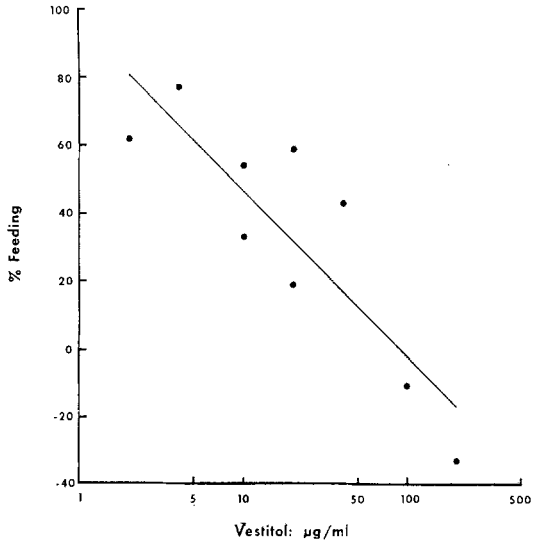


FIG. 2. Dose–response curve for the effect of 3*R*-(−)-vestitol on feeding by 3rd instar *Costelytra zealandica* larvae. Ingestion is expressed as a % of the standard response to the phagostimulant. Feeding on standard disks is taken as 100% and that on the corresponding blank disks as 0%.

Activity of L. Pedunculatus Leaf

Farrell et al. (1974) found that larvae of the grass caterpillar *Wiseana cervinata* (Lepidoptera: Hepialidae) fed very sparingly on *L. pedunculatus* leaves: a crude extract of these possessed strong feeding-deterrent activity (Farrell, personal communication). We tested the material against *C. zealandica* larvae although, being subterranean, they would not normally come into contact with foliage. A crude $CHCl_3$–MeOH extract showed strong feeding-deterrent activity (Table 1) as did each of the partition phases when tested separately. We have identified vestitol from the chloroform phase but other, more polar, active compounds are also present (Hutchings, unpublished results).

DISCUSSION

3*R*-(−)-vestitol (I) is an active feeding deterrent for *Costelytra zealandica* and may account for a significant part of the resistance of *Lotus pedunculatus*

to the insect. Certainly it was the major feeding deterrent in the crude extracts of the dried root. However, a comparison of the feeding deterrent activity of pure vestitol with the activity of crude extracts of fresh root indicates that the latter cannot be completely correlated with vestitol concentration. The active, unexamined, petroleum spirit fraction and the isoflavan sativan, which we have found to reduce larval feeding (Sutherland and Russell, unpublished results), must also account in part for the rejection of root by larvae. The existence of these feeding deterrents, together with the recently reported toxin from the same root (Sutherland and Greenfield, 1976), suggests a multifactorial basis for the resistance of *L. pedunculatus* to insect attack.

Both vestitol and sativan have been implicated in the disease resistance of *Lotus* species, and their production was induced in leaves of *L. corniculatus* by inoculation with a spore suspension of *Helminthosporium turcicum* (Bonde et al., 1973). The compounds are very inhibitory to mycelial growth of *H. turcicum*, and vestitol in particular, reduced growth by 50% at a concentration of 35 μg/ml. Its activity as an insect feeding deterrent with an ED_{50} of 8.5 μg/ml is of the same order of magnitude, and this dual function of vestitol as a phytoalexin and insect feeding deterrent is of ecological interest. It remains to be seen if other phytoalexins elicit a similar response from *C. zealandica*. Preliminary results with the pterocarpan, pisatin, indicate that this compound may deter larval feeding at similar concentrations (Sutherland et al., unpublished results).

Isoflavans have previously been isolated from leaf or hypocotyl tissues inoculated with fungus (Ingham and Millar, 1973; Ingham, 1977; VanEtten, 1973; Bonde et al., 1973), from *Derris* roots (Filho et al., 1975), and from *Dalbergia* and *Machaerium* species (Kurosawa et al., 1968). Whether they are always present in the tissue or are only produced in response to infection is unclear, but the issue is of considerable importance in regard to insect resistance. The concentration of vestitol in *Lotus* root was estimated at 3 μg/g fresh tissue, but the overall concentration may not be meaningful. If the compound is localized toward the root surface (Cruickshank et al., 1974) or is exuded from it, the effect on insect feeding would presumably be more pronounced. In any case, 3 μg/g may be a low estimate. Phytoalexin levels are dependant upon the health of the tissue examined and have been shown to increase 100-fold or more within a few days of fungal infection (Higgins and Smith, 1972; Deverall, 1972; Kuć, 1964). There are also studies which have demonstrated enhanced coumestan synthesis in response to attack by nematodes (Rich et al., 1977) and aphids (Loper, 1968), and it is not beyond possibility that enhanced synthesis of isoflavonoids occurs in response to insect feeding. Any such increase in the production of vestitol by *L. pedunculatus* root as a direct or indirect response to attack by *C. zealandica* would have a profound effect on the insect.

Since vestitol has been isolated from infected *L. corniculatus* leaves (Bonde et al., 1973), the roots of this plant may have a similar synthetic capability. The fact that it is not resistant to *C. zealandica* may reflect differing levels of vestitol concentration (Russell, unpublished results) and/or the importance of other deterrent factors.

In their discussion of allelochemics, Whittaker and Feeny (1971) emphasized the multiplicity of functions of some secondary plant substances and their role in the overall defense strategy of the plant, a theme that has been further developed by Feeny (1975) and Levin (1976). The dual role of the *Lotus* constituent, vestitol, as a weapon in the plant's defense against attack by disease microorganisms and at least one insect adds weight to their analysis which has major implications for the understanding of insect resistance in plants. Hitherto, disease resistance, insect resistance, and nematode resistance have for the most part been studied and considered separately; we believe that this division may be artificial and unhelpful.

Acknowledgments—We are most grateful to Mrs. Colleen Ford for collecting the many thousands of grass grubs in this study. We also thank W.J. Greenfield, C. Gnanasunderam and J.R. Hillier for technical help and Dr. D.R. Biggs for helpful advice.

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IDENTIFICATION OF BEHAVIORALLY SIGNIFICANT VOLATILE COMPOUNDS IN THE ANAL GLAND OF THE RABBIT, *Oryctolagus cuniculus*

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Abstract—An investigation of the anal gland volatiles of the male wild rabbit *Oryctolagus cuniculus* has been made in which the heart rate of adult male rabbits, positioned to sniff the effluent from a gas chromatographic capillary column, has been employed as an indicator for the presence of constituents of likely behavioral significance. The examination by this means of the headspace volatiles of homogenized anal glands using gas chromatography and gas chromatography—mass spectrometry has implicated a number of saturated and monounsaturated normal and methyl-branched aldehydes in the C_9–C_{12} range.

Key Words—rabbits, *Oryctolagus cuniculus*, cardiac responses, anal glands, aldehydes, headspace collection, gas chromatography, GC-MS.

INTRODUCTION

Earlier studies have established that the odor of anal gland secretions of the wild rabbit, *Oryctolagus cuniculus*, has a function in territorial marking (Mykytowycz, 1968). The general chemical composition of the secretions has been described (Goodrich and Mykytowycz, 1972), and more recently various fractions of secretions from this gland were tested for their behavioral significance using subadult and nestling rabbits (Hesterman et al., 1976). The acid and neutral fractions elicited the strongest behavioral response in the

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form of avoidance. Measurements of heart rate in nestlings showed a statistically significant correlation between deceleration of heart rate and strength of the behavioral response. These results suggested that changes in heart rate can be used to detect differential responses of rabbits to various chemical components of the same secretion.

In this study the change in heart rate in adult male rabbits located to sniff the outlet of a capillary column in a gas chromatograph and then in a gas chromatograph–mass spectrometer (GC-MS) unit, has been used to indicate the presence of volatile compounds in the anal glands, which may be behaviorally active.

METHODS AND MATERIALS

General Approach

In view of the low concentration of volatiles in the secretion from the anal glands of rabbits and the difficulty involved in collecting adequate amounts of the secretions, it was necessary to use pooled whole glands. These were homogenized in saline solution and headspace collections of the volatiles were made by techniques developed for the examination of food flavor volatiles.

Preliminary gas chromatographic examination of the headspace volatiles were made on a glass SCOT column coated with a polar phase (Carbowax 20M) with two human observers monitoring the effluent by nose. The presence of compounds with strong "rabbit-like" odors was thereby established. The next step was to use the heart-rate response of rabbits located to sniff the same column effluent to indicate the positions in the complex chromatogram (Figure 2) of compounds of likely behavioral activity. Guided by this information fractions were collected from the more significant areas in repeated runs of the chromatograph. These fractions were then rechromatographed on a glass SCOT column of lower polarity (Silicone SF96) which was coupled to a mass spectrometer and with the rabbit again positioned to monitor the effluent. The high resolution achieved by transfering narrow fractions from the polar to nonpolar columns ensured that the rabbit was exposed mostly to well-resolved constituents. Mass spectra recorded concurrently formed the basis of the identification of significant components.

Sample Preparation

Homogenates of pooled anal glands from adult male rabbits (20 g equivalent to approximately 20 glands) were prepared in saturated saline solution (40 ml) at 0°C using an Ultra-Turax high-speed homogenizer (Janke

and Kunkel Kg) for 5 min. These were stored at −20°C and used within one month.

Headspace Collection

The technique of collection of volatile compounds on Chromosorb 105 (Johns-Manville), has been described in detail (Murray, 1977). In the present work, a headspace collection involved the following procedure: the gland homogenate (25 ml) was diluted with an equal volume of distilled water and stirred magnetically in a 100-ml long-neck conical flask fitted with a 14/23 joint and a PTFE stopper with inlet and outlet tubes of 3.2 mm OD stainless-steel tubing, the whole being totally immersed in a water bath at 40°C. A flow of oxygen-free nitrogen (40 ml/min) passed downwards into the flask over the surface of the gently stirred homogenate and entered the first of two collection traps at a central point 2 cm above the sample surface. The traps were connected in line by PTFE tubing, the second being similarly connected to the outlet tube.

The traps of similar design to those previously described (Murray, 1977) consisted of a bed of Chromosorb 105 (50/60 mesh, 100 mg) packed into stainless-steel tubes (90 mm, 3.2 mm OD). They were preconditioned in a nitrogen stream initially at 225°C for 24 hr and thereafter, following each use, at 180°C for 30 min. After collection for 24 hr (60 liters) the traps were transferred to a dry flask and nitrogen flow continued for 2 min to purge the residual water from them. Both traps were then sealed with PTFE end caps and stored over solid carbon dioxide until required.

In the present work the headspace volatiles were introduced into a laboratory-constructed gas chromatograph (Figure 1) over a period of 10 min at an introducer temperature of 150°C. The column was a glass SCOT column 80 m, 0.6 mm ID coated with Carbowax 20M and was temperature programmed at 15 min at 70°C followed by 1.5°C/min rise to 180°C.

Monitoring the Column Effluent

Odors appearing in the effluent of the above GC column were monitored by nose as previously described (Murray et al., 1972).

The measurement of changes in heart rate of rabbits located at the column outlet were made using a miniature radio transmitter (Hesterman et al., 1976) to record heart beats (see Figure 1). Two small pin electrodes were inserted into the skin approximately 1 cm apart in the sternal region of the animal. The rabbits were restrained in a crouching position by means of a fiberglass cast and placed inside a soundproof timber box (75 × 30 × 30 cm) equipped with ventilation ports. An exhaust fan behind the rabbit drew

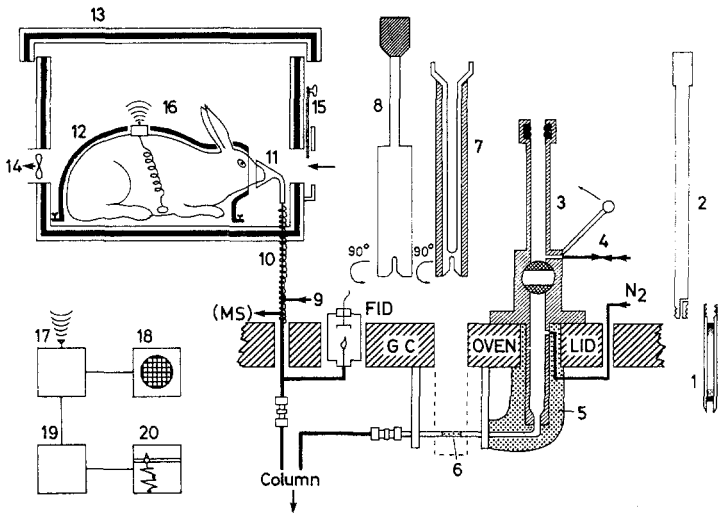

FIG. 1. Diagram of the radio transmitter and receiver equipment coupled to either the GC or GC-MS systems. 1 = Chromosorb 105 sample trap; 2 = probe for inserting (1) into introducer (3); 3 = introducer for sample traps (1); 4 = purge line toggle valve; 5 = asbestos tape lagging; 6 = section of line used for concentration of headspace volatiles from (1) prior to their introduction into capillary column. 7 = liquid N_2-cooled probe for cooling (6) and trapped material from (1); 8 = brass probe for rapid heating of section (6); 9 = make-up air for dilution of effluent; 10 = heated delivery tube for presentation of volatile components to rabbit; 11 = PTFE funnel; 12 = fiberglass cast for restraining rabbits; 13 = soundproofed box housing rabbit; 14 = exhaust fan used to remove odorous material from box (13); 15 = mechanical air-flow control valve; 16 = miniature radio transmitter. The signal is received and recorded on magnetic tape by a high-quality FM radio receiver–cassette recorder (17) connected to speaker (18) for simultaneous audio monitoring of the signal. The signals are amplified at (19) and transformed to a strip chart trace using a millivolt recorder (20).

laboratory air past the animal and prevented build-up of eluted material in the box. After a 15-min acclimatization period, the column effluent was introduced through PTFE tubing (2.5 mm ID), and a PTFE funnel was positioned close to the face of the animal. The tubing was heated and lagged to prevent condensation of eluted material.

Selection and Trapping of Active Fractions

Using a different rabbit for each run, five monitoring runs were made on identical headspace collections. Seven fractions of the volatiles (Figure 2) which showed the strongest and most consistent deceleration of heart rate

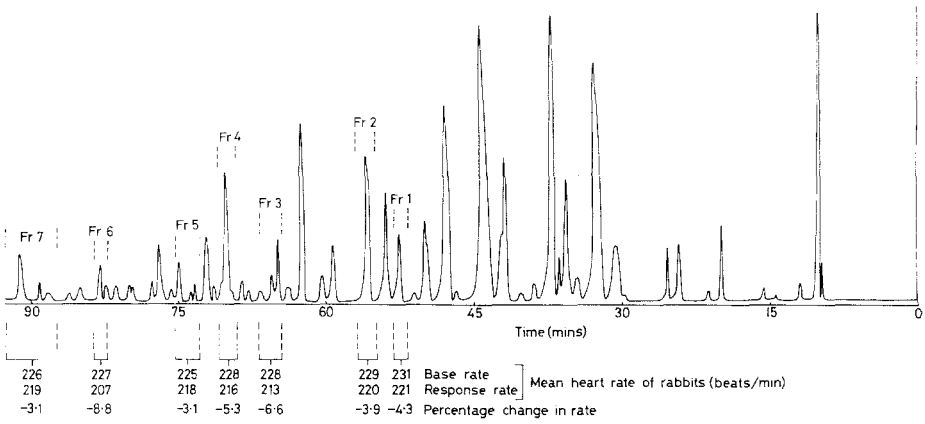

FIG. 2. Gas chromatogram on Carbowax 20M column of headspace volatiles above a saline homogenate of pooled male rabbit anal glands, with indication of the positions of fractions (Fr 1–7) selected for further studies together with their effect on heart rate in rabbits.

were selected. These were trapped in three additional runs by substituting a collection port for the effluent delivery line to the rabbit. Tubular traps, of the same design as the Chromosorb 105 trap, were used except that a bed of 10% Silicone SF96 on 30–40 mesh Chromosorb A replaced that of the porous polymer. The technique of trapping and unloading of these traps has been described previously (Murray et al., 1972; Murray, 1977).

Gas Chromatography–Mass Spectrometry

The three sets of seven fractions were examined by the GC-MS unit (Figure 1), using three different male rabbits to monitor each fraction. The GC unit was equipped with a glass SCOT column (80 m, 0.6 mm ID) coated with Silicone SF96 and isothermally heated at 80°C (fractions 1 and 2), 100°C (fractions 3 and 4), and 120°C (fractions 5, 6, and 7). Baseline resolution was obtained for most of the peaks in these fractions and high-quality mass spectra were concurrently recorded for resolved compounds to which the rabbit responded.

Spectra were also recorded for the more prominent "nonactive" components of the anal gland material.

Reaction Gas Chromatography

The mass spectra of compounds causing heart-rate deceleration in fractions 6 and 7 (Figure 2) suggested them to be unsaturated C_{11} and C_{12}

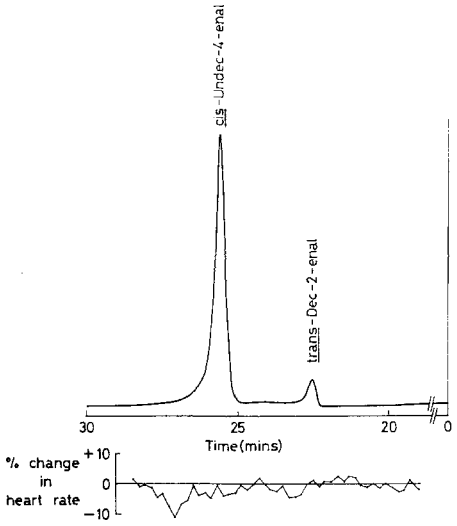

FIG. 3. The part of the gas chromatogram on Silicone SF 96 column of fraction 6 trapped from Carbowax 20M column (see Figure 2) indicating changes in heart rate in rabbits.

aldehydes of unknown structure. Samples of approximately 0.3 μg trapped in further runs under similar conditions as described above were submitted to hydrogenation and ozonolysis by the sealed-capillary technique of Stanley and Kennett (1973). The component from fraction 6 (Figure 3) on hydrogenation with Adam's catalyst at 160°C for 3 min yielded *n*-decane and *n*-undecane in the ratio of approximately 10:1, a result consistent with the hydrogenolysis of *n*-undecanal under the same conditions. Ozonolysis followed by pyrolytic cleavage of the ozonide yielded only *n*-heptanal: the dipolar fragment (a C_4 dialdehyde) did not elute from the capillary column. Identical treatment of two compounds from fraction 7 (with identical mass spectra) yielded on hydrogenation undecane and dodecane (10:1) and likewise *n*-heptanal on ozonolysis.

Synthesis of cis/trans-Undec-4-enals and Dodec-5-enals

The *cis*- and *trans*-undec-4-enals and -dodec-5-enals were synthesized by the unambiguous stereoselective route as outlined in Figure 4 for the C_{12} compounds. This involved the condensation of 1-bromohexane (commercially available) with respectively, the 4- or 5-carbon terminal acetylenic alcohols.

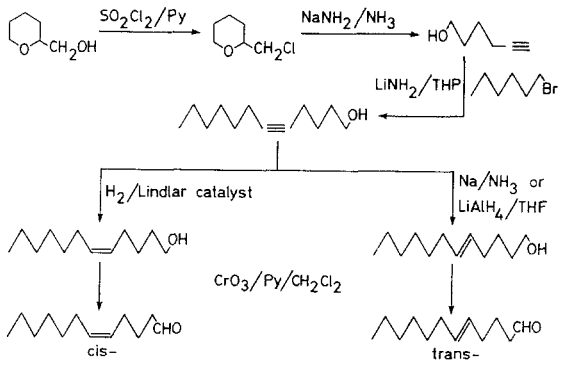

FIG. 4. Stereoselective route to the synthesis of cis and trans monounsaturated aldehydes isolated from the headspace above saline homogenates of pooled anal glands of male rabbits.

The latter were obtained by eliminative cleavage of the cyclic ethers having an *a*-chloromethyl group (Englinton et al., 1952), namely, 2-chloromethyltetrahydrofuran (Brooks and Snyder, 1955) and 2-chloromethyltetrahydropyran (Crombie et al., 1956). The C_{11} and C_{12} acetylenic alcohols (Paul and Norman, 1943; Wharthen and Jacobsen, 1968) were transformed by stereospecific reduction of the acetylenic bond to the corresponding cis (Lindlar, 1952) and trans (Birch, 1950; Magoon and Slough, 1967) olefinic compounds. Finally, these were oxidized (Ratcliffe and Rodehorst, 1970; Corey and Suggs, 1975) to the cis and trans aldehydes. They were purified by dry column chromatography on silica gel (Merck, Kieselgel 60) followed by small-scale preparative gas chromatography on a high-resolution open tubular stainless-steel column (150 m, 0.75 mm ID) coated with Carbowax 20M and operated under conditions capable of resolving isomeric material. Linear retention indices (LRI) were measured on this column and on the glass SCOT Silicone SF96 column. Infrared spectra were measured on a Perkin-Elmer 521 spectrophotometer. Low-resolution mass spectra were recorded by an Atlas CH4 instrument (source temperature 170°C).

cis-Undec-4-enal

IR spectrum (film) cm^{-1}: 3010, 2960, 2935, 2860, 2730 (CH ald.str.v.), 1720(s) (CO ald.), 1460, 1440, 725 (*cis*-CH=CH—).
LRI: 1638 (20 M), 1324 (SF96).
Mass spectrum: See Figure 5A.

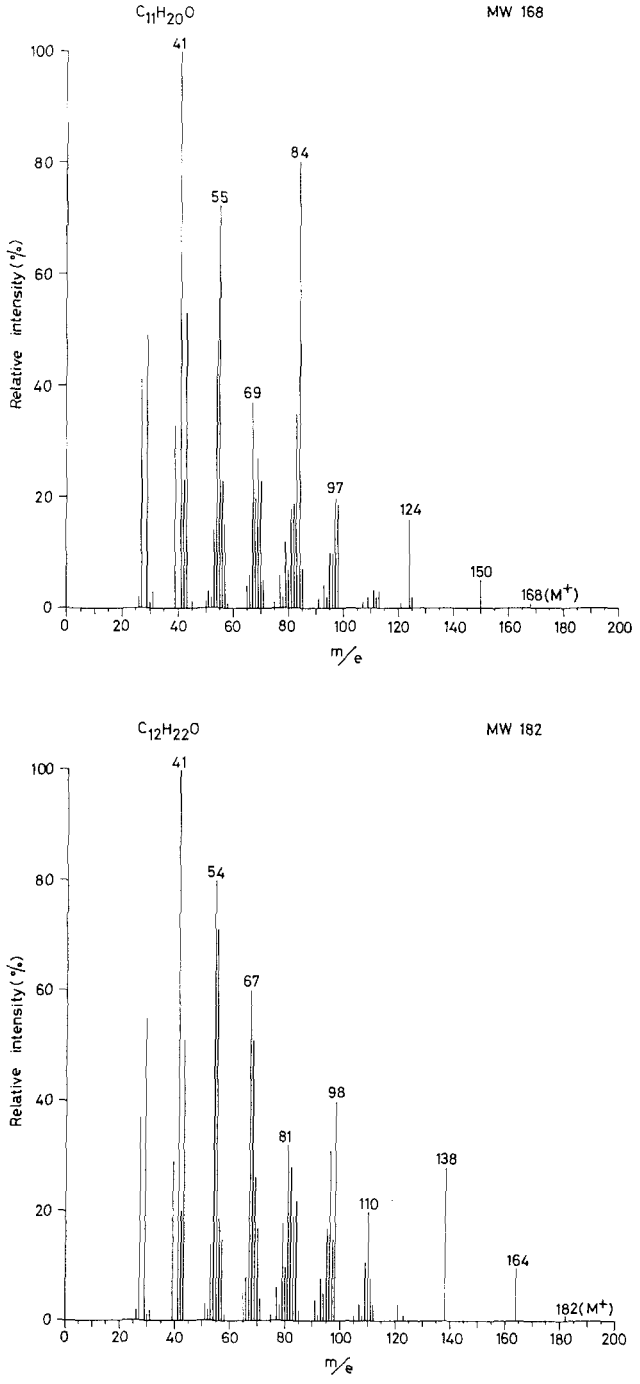

FIG. 5. Mass spectra of (A) *cis*-undec-4-enal, and (B) *cis*-dodec-5-enal.

trans-Undec-4-enal

IR spectrum (film) cm^{-1}: 3010, 2960, 2940, 2860, 2730, (CH ald.str.v.), 1720 (CO ald), 1460, 1440, 965 (s) (*trans*-CH=CH—).
LRI: 1636 (20 M), 1326 (SF96).
Mass spectrum: Indistinguishable from that of cis isomer. See Figure 5A.

cis-Dodec-5-enal

IR spectrum (film) cm^{-1}: 3010, 2960, 2930, 2680, 2720 (CH ald.str.v.), 1720(s) (CO ald.), 1460, 1440, 725 (*cis*-CH=CH—).
LRI: 1738 (20 M), 1423 (SF96).
Mass spectrum: See Figure 5B.

trans-Dodec-5-enal

IR spectrum (film) cm^{-1}: 3010, 2960, 2940, 2860, 2730 (CH ald.str.v.), 1725 (CO ald), 1455, 1440, 965 (*trans*-CH=CH—).
LRI: 1736 (20 M), 1425 (SF96).
Mass spectrum: Indistinguishable from that of cis isomer. See Figure 5B.

Preparation of Branched-chain Aldehydes

The C_{11} and C_{13} anteiso- and the C_{12} and C_{14} iso-methyl-branched aldehydes were prepared by a convenient one-step reaction from the corresponding acids. Reduction with lithium aluminium hydride in ether at 0°C for 15–20 min gave a mixture of aldehydes and alcohols in a ratio of approx. 1:1. Small-scale preparative gas chromatography (see above) afforded the pure aldehydes. The mass spectra are available on request.

RESULTS

The compounds which elicited a definite deceleration of heart rate in one or more of the rabbits in the GC-MS runs of the seven selected fractions are shown in Table 1 and the level of the rabbits' response to compounds emerging from the gas chromatographic column is illustrated in Figures 2 and 3. The C_9 and C_{11} *n*-aldehydes which are listed in Table 1 were not trapped in the selected fractions. However, they were identified in the anal gland headspace/volatiles in other runs and have released heart-rate deceleration in preliminary behavioral tests using pure materials on unrestrained

TABLE 1. ANAL GLAND CONSTITUENTS INDUCING HEART-RATE DECELERATION IN MALE RABBITS

Compound	Located in fraction no. (Figure 2)	Molecular weight	Evidence of identification[a]
6-Methylhept-5-enal	1	126	See text
6-Methyloctanal	2	142	ms gc+
7-Methylnonanal	3	156	ms gc+
8-Methylnonanal	3	156	ms gc+
n-Decanal	4	156	MS GC+
Non-2-enal[b]	5	140	MS GC+
Dec-2-enal[b]	6	154	MS GC+
cis-Undec-4-enal	6	168	MS GC++
cis-Dodec-5-enal[c]	7	182	MS GC++
trans-Dodec-5-enal[c]	7	182	MS GC++
n-Dodecanal	7	184	MS GC+
n-Nonanal	See text	142	MS GC+
n-Undecanal	See text	170	MS GC+

[a] MS = mass spectrum identical with that of the pure material; GC = LRI agrees with that of the pure material on one column (+) or two column (++) phases (20 M and SF96); and ms gc = mass spectra and retention indices agree with those characteristics of iso- or anteiso-methyl-branched aldehydes (see text).
[b] Probably trans.
[c] Ratio cis/trans approximately 5:1.

rabbits. Additional compounds identified in the anal gland headspace volatiles to which the rabbits did not respond are listed in Table 2.

The identification of the C_9 and C_{10} methyl-branched aldehydes in fractions 2 and 3 is not final as the pure compounds were not available for comparison. However, their mass spectra and LRIs indicate that they belong to the series of either iso- or anteiso-methyl-branched aldehydes. As shown by the spectra of the C_{11} and C_{13} anteisoaldehydes and the C_{12} and C_{14} isoaldehydes, these are characterized by the higher diagnostic ions of the normal saturated aldehydes (M-18, M-28, M-33, M-44) as well as M-15 and M-29 ions (strong in the anteiso series) derived from the terminal branched hydrocarbon chain.

The quantity of the heart-rate decelerating component of fraction 1 was insufficient to permit further study by reaction gas chromatography. Its provisional identification as 6-methylhept-5-enal is based on a mass spectral interpretation as an iso-methyl-branched C_8 monounsaturated aldehyde with the double bond not in the 2- or 3- positions. Further, it occurs (LRI, 973) in fraction 1 with 6-methylhept-5-en-2-one (LRI, 987) and the difference in

TABLE 2. ADDITIONAL COMPOUNDS IDENTIFIED IN ANAL GLAND HEADSPACE VOLATILES

Compound	Molecular weight	Evidence of identification[a]
(2+3)-Methylbut-2-en-1-ol	86	MS (mixed)
(2+3)-Methylbutan-1-ol	88	MS (mixed)
n-Hexanol	102	MS GC+
n-Heptanol	116	MS GC+
n-Octanol	130	MS GC+
Oct-1-en-3-ol	128	MS GC+
Linalool	154	MS GC+
3-Methylbut-2-enal	84	
6-Methylhept-5-en-2-one	126	MS GC+
n-Octanal	128	MS GC+
Citronellal	154	MS GC+
9-Methylundecanal	184	ms gc
10-Methylundecanal	184	MS gc
Tridec-5-enal	196	ms[b]
10-Methyldodecanal	198	MS gc

[a] See footnote *a* in Table 1.
[b] Spectrum indicates a C_{13} homolog of dodec-5-enal.

the LRIs is identical with the difference shown between *n*-octanal and its isomer octan-2-one on the same gas chromatographic phase.

DISCUSSION

The main aim of this study was to quickly locate the behaviorally active compounds in the anal gland volatiles by using the rabbit in the role of its own biological detector. Located in this manner and characterized by their mass spectra and in some cases by reaction GC-MS and synthesis, a number of "active" components were identified. It is noteworthy that all of these compounds are aliphatic aldehydes and belong to the normal and methyl-branched saturated and monounsaturated series in the C_9–C_{12} range. Saturated C_7–C_{10} *n*-aldehydes have been recently reported in the tarsal gland secretion of the reindeer *Rangifer tarandus* (Andersson et al., 1975), and C_4 and C_5 methyl-branched saturated aldehydes are among the volatile components of the anal sacs of the dog *Canis familiaris* and the coyote *Canis latrans* (Preti et al., 1976).

During the GC-MS examination of the fractions, it was observed that on several occasions the rabbit response was most evident at the front and on the tail of large gas chromatographic peaks. A clear example of this is

shown in Figure 3. For this and other reasons set out below, there has been no attempt to attribute quantitative values to the responses or to grade the behavioral significance of these aldehydes. First, the passage of a gas chromatographic peak, especially when eluting from a high-resolution column, is attended by rapid and wide changes in concentration. This can cause a pronounced change in the odor quality of the eluting compound, an effect often apparent to human observers who follow the practice of sniffing the column effluent when monitoring odorous constituents of volatile flavor materials. Thus it is probable that the rabbit responds to the odor character of the active aldehydes at low concentrations which approximate those in the anal gland secretion odor encountered under natural conditions. Second, the seven fractions chosen for GC-MS examination (Figure 2) were not the only zones in the chromatogram to which rabbits showed some reaction. One such zone, somewhat broad and diffuse, was beyond fraction 7. The response here could have been genuinely attributed to compounds in this region (C_{13} and C_{14} aldehydes) or to the build-up condensate of lower-molecular-weight aldehydes in the vapor line to the nose of the rabbit. Finally, as has been already noted, the C_9 and C_{11} *n*-aldehydes which were found to produce heart-rate deceleration activity in behavioral experiments were not trapped in any of the seven gas chromatographic fractions examined.

Changes in heart rate have been used by other investigators to reveal reactions of animals to various physical stimuli, including odors (Frisch, 1965; Lacey and Lacey, 1970; Wenzel and Sieck, 1972) and are accepted as demonstrations of orienting and fear responses. It has also been suggested that subsequent changes in heart rate during tests in which the subjects were exposed to light stimulation is related to the eliciting qualities or meaningfulness of the stimulus (Walter and Porges, 1976). Although the physiological mechanism and underlying changes in heart-rate response to external stimulation is not fully understood (Obrist, 1976), this technique in our experience appears to be a useful tool for the rapid screening of large numbers of volatile substances which may be of behavioral importance. The changes in heart rate of juvenile rabbits following exposure to the acidic, basic, and neutral fractions of rabbit anal gland secretions have been described previously (Hesterman et al., 1976). Also the ability of rabbits to differentiate between a number of synthetic volatile fatty acids has been observed with the aid of this technique (unpublished data) and indicated a difference in the response of male and female rabbits to the same compound. Considering the plasticity of the rabbits' territorial and sexual behavior, variations in the responses of different individuals to the same substances should be expected.

Heart-rate changes indicate not only that a substance has been perceived but also suggest the existence of an interpretation process which identifies and relates it to the behavioral situation. Thus it implies a state of

awareness in the animals. The question of the awareness of animals has recently attracted the attention of many biologists (Griffin, 1976).

The present study appears to be the first example of the use of a higher animal other than man for monitoring gas chromatographic effluents for the presence of compounds of possible biological significance. This technique, which is clearly capable of refinement, could provide a valuable tool for further olfactory–behavioral studies. The heart-rate technique is being currently used for the measurement of the response threshold of rabbits to the straight-chain saturated aldehydes. The results obtained will provide a basis for the extension of this work to studies on unrestrained rabbits. Observations based on human sniffing and rabbit response studies indicate that the odor of rabbit anal glands is very complex. A major volatile component whose odor is most characteristic of the anal gland was identified with *cis*-undec-4-enal, to which the rabbits also strongly responded. It is noteworthy that *trans*-undec-4-enal, which was not detected in the present studies but is most likely present in the anal gland secretion, and *trans*-dodec-5-enal have odors noticeably different from the cis isomers, having a slight additional "camphoraceous" note. The saturated normal and methyl-branched aldehydes in the C_{10}–C_{12} range were considered to have odors akin to *cis*-undec-4-enal.

This paper emphasizes once more the complexity of mammalian odors. Variation in the proportion of volatile constituents in the anal gland secretion may enable the rabbit to signal a wide range of messages. Considering a similar complexity of other odor-producing glands, for instance the inguinal and chin glands, one realizes that the species is well equipped for olfactory communication.

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NEMATODE SEX PHEROMONES

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Abstract—Current knowledge of the involvement of sex pheromones in the premating communication of free-living, plant-parasitic, and animal-parasitic nematodes is reviewed.

Key Words—Nematode sex pheromones, Nematoda, premating attraction, pheromone response, pheromone production.

INTRODUCTION

Pheromones are chemicals that are released to the external environment by an animal and that cause one or more specific behavioral or physiological responses among other individuals of the same species. With the advent of sophisticated microanalytical chemical techniques and with the appreciation that the need to communicate by chemicals through some distance in the environment may constitute a "weak link" in the life cycle of certain pest organisms, a great surge in research interest concerning pheromones has occurred during the past two decades.

By far, the largest amount of research has concerned those pheromones of insects that are used in communication between the sexes prior to mating (Jacobson, 1972; Birch, 1974; Shorey and McKelvey, 1977). The research results, accumulating through hundreds of new research publications each year, are revealing hitherto unexpectedly complex systems that have become adjusted through natural selection so as to maximize the likelihood that successful communication will occur under optimal environmental and physiological conditions.

Pheromone communication might be thought of as being especially

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appropriate for organisms that live in an aquatic or semiaquatic environment. Particularly at close range, the establishment of diffusion gradients that are relatively stable (compared to the poor stability of diffusion gradients in air) and the possibility of slow and uniform directional flow of the aquatic medium might allow fairly efficient transmission of the pheromone message from the releasing individual to potential responders of the same species. These factors might also allow the responders to sense the correct direction in which to move so as to reach the pheromone source.

Nematodes mainly live in an aquatic environment, although the details of this environment may differ greatly, considering that some nematodes are free-living, others are parasites of plants, and still others exist as parasites within animals. In 1964, Greet first demonstrated conclusively that pheromones released from either sex of the free-living nematode *Panagrolaimus rigidus* attracted members of the opposite sex. Since that time, nematode pheromones have attracted the interest of a number of research workers. We now know that pheromones are involved in the male–female interactions prior to mating for at least 23 species, including free-living, plant-parasitic, and animal-parasitic forms. In fact, with only one exception (De A.Santos, 1972), pheromones released from either (or both) males or females have been found for every nematode species for which such studies have been reported (Table 1).

As implied above, essentially all the literature dealing with nematode pheromones is restricted to the involvement of such chemicals in premating behavior, and almost all of these reports are addressed toward the role of the chemicals in stimulating one sex to move toward the other. In addition, some small amount of evidence indicates that in some nematode species pheromones may cause individuals to either approach or to leave the vicinity of other individuals of the same sex, a behavior whose significance can only be speculated upon. The possible role of pheromones in stimulating other "social" interactions among individuals of a nematode species is largely unexplored, and perhaps no other such interactions occur.

An aspect which has received little research attention, but which we predict will be found to be extremely important, is the role of primer pheromones in regulating nematode physiology and behavior. As defined by Wilson (1963), those pheromones which exhibit primer effects cause relatively enduring changes in the physiology of the receiving individuals. Such pheromones have been studied extensively for certain mammalian and insect species in which they have been found to have a number of biological roles. For example, primer pheromones have been found in some cases to regulate the onset of reproductive maturity or of sexual responsiveness of males or females (Birch, 1974). Some primer pheromones of insects or mammals may play an important role in regulating the population density by approp-

TABLE 1. SUMMARY OF NEMATODES EXHIBITING PHEROMONE-MEDIATED ATTRACTION

Species	Habitat[a]	Attracted sex(es)	Author(s)
Panagrolaimus rigidus	FL	M,F	Greet, 1964
Panagrellus silusiae	FL	M	Cheng and Samoiloff, 1971
Panagrellus redivivus	FL	M	Balakanich and Samoiloff, 1974
Pelodera teres	FL	M	Jones, 1966
Cylindrocorpus longistoma	FL	M	Chin and Taylor, 1969
Cylindrocorpus curzii	FL	M	Chin and Taylor, 1969
Cephalobus persignis	FL	M	Cheng and Samoiloff, 1971
Heterodera rostochiensis	PP	M	Green, 1966
Heterodera schachtii	PP	M	Green, 1966
Heterodera avenae	PP	M	Green and Plumb, 1970
Heterodera carotae	PP	M	Green and Plumb, 1970
Heterodera cruciferae	PP	M	Green and Plumb, 1970
Heterodera glycines	PP	M	Green and Plumb, 1970
Heterodera goettingiana	PP	M	Green and Plumb, 1970
Heterodera mexicana	PP	M	Green and Plumb, 1970
Heterodera tabacum	PP	M	Green and Plumb, 1970
Heterodera trifolii	PP	M	Green and Plumb, 1970
Pelodera strongyloides	FP	M,F	Stringfellow, 1974
Rhabditis pellio	FP	M,F	Somers et al., 1977b
Ancylostoma caninum	AP	M	Roche, 1966
Trichinella spiralis	AP	M,F	Bonner and Etges, 1967
Nippostrongylus brasiliensis	AP	M,F	Alphey, 1971 Roberts and Thorson, 1977a
Camallanus sp.	AP	M,F	Salm and Fried, 1973
Aspiculuris tetraptera	AP	M,F	Anya, 1976

[a] Habitat refers to that of the reproductive adults; FL = free-living; PP = plant parasite; FP = facultative parasite; AP = animal parasite.

riately modifying the survival, growth, or reproduction of some individuals under crowded conditions. Studies of this type with nematodes will possibly reveal similar important primer pheromone regulatory mechanisms. For example, the recent studies of Foor and his coworkers (1971, 1973, 1974) are suggestive of such primer mechanisms in some nematode species.

PRODUCTION AND RELEASE OF PHEROMONE

Site of Pheromone Production or Release

The anatomical location of pheromone production has received little attention. However, representative nematodes from all three ecological niches (free-living, plant-parasitic, animal-parasitic) have been at least

superficially studied. Green and Greet (1972) found that pheromone was released from the entire body surface of female *Heterodera schachtii* and *H. rostochiensis*, although the posterior vulvar region of *H. schachtii* exhibited the greatest activity. They suggested that the female hypodermis produced the pheromone or that the pheromone was distributed throughout the pseudocoelom after production at an unknown site.

Samoiloff (1970) implicated dissolved cuticular material, produced by females at ecdysis, as a sexual attractant for male *Panagrellus silusiae*. Later, following experiments with the developmental inhibitors hydroxyurea and actidione, Cheng and Samoiloff (1972) implicated the female gonads of *P. silusiae* in pheromone production. The gonads were suggested to either directly produce the pheromone or to regulate pheromone production at some other site via a hormonal mechanism, because inhibitor-treated animals exhibited a reduced level of pheromone production or release. Bone and Shorey (1978) obtained similar results with actidione-treated females of *Nippostrongylus brasiliensis*.

Marchant (1970) reported that smearing the vulvar area of female *Nematospiroides dubius* with silicone grease apparently prevented release of pheromone into saline solution. Males failed to exhibit a bursal flaring in response to these treated females but did respond to untreated females, which implicated the vulva as a release site for pheromone (Marchant, 1970). However, Croll and Wright (1976) failed to confirm the bursal flaring response, leaving some question as to the conclusiveness of Marchant's study.

Finally, the female pulvilar secretory cells were proposed as a site of pheromone production in *Aspiculuris tetraptera* (Anya, 1976).

It is obvious from the above reports that broad generalizations regarding the site of female nematode pheromone production or release would be premature. Only a single study has investigated pheromone production by those male nematodes that are attractive to their females, with the caudal glands of male *A. tetraptera* being suggested as the pheromone source (Anya, 1976).

Effect of Physiological Factors on Pheromone Production or Release

Pheromone production and pheromone release could be independent entities and production might precede release by some time if storage sites for the pheromone are present. On the other hand, pheromone release might occur simultaneously with production in the absence of pheromone storage. Several studies have been directed toward the influence of various physiological factors on the amount of pheromone released into the environment by the pheromone-producing sex. No studies have been conducted to deter-

mine whether the optimal conditions for pheromone release coincide with the optimal conditions for pheromone production.

Effect of Age. The onset of pheromone release by females is reported to coincide with about the time of that final larval molt in *Rhabditis pellio* (Somers et al., 1977b) and *N. brasiliensis* (Bone et al., 1977a). On the other hand, *P. silusiae* females were found to release pheromone while still in the fourth larval stage (Cheng and Samoiloff, 1971). The subsequent duration of pheromone release throughout life may depend on other factors that affect the reproduction and survival of the nematodes. Free-living females of *R. pellio* continued to release a fairly constant level of pheromone throughout life, with a tendency toward some reduction in release rate only being seen in the oldest tested females (Somers et al., 1977b). This is correlated with a high level of mating activity being seen in *R. pellio* females throughout most of their lives (Somers et al., 1977a). On the other hand, release of pheromone from females of *N. brasiliensis* decreased more rapidly with age, possibly representing a combination of female sexual senility plus immune reactions of the rodent host (Bone et al., 1977a; Roberts and Thorson, 1977a).

Effect of Time of Day. Pheromone released by females of *R. pellio* (Somers et al., 1977b) and both males and females of *N. brasiliensis* (Bone et al., 1977a) at various times of day and night was assayed to examine the possibility of a 24-hr periodicity. No such periodicity of release was found, although both groups of authors noted that subtle differences could have been missed due to the lengthy periods required prior to and during each test for pheromone collection and conduct of biossays. Bone et al. (1977a) suggested that a distinct, brief periodicity of female pheromone release (or male responsiveness) in *N. brasiliensis* might prove detrimental to mate location and successful copulation. This suggestion is supported by Phillipson's (1970) finding that *N. brasiliensis* males locate and copulate with previously established females within a short period after reinfection.

Effect of Mating. Most of the studies of nematode pheromone behavior have employed mated females as pheromone sources and have successfully demonstrated that such females are attractive to males. However, some investigators have employed virgin females and have shown that males were likewise attracted to them (Somers et al., 1977b). Cheng and Samoiloff (1971) demonstrated that males of *P. silusiae* were attracted to either virgin or mated females, although a larger degree of variability was associated with male attraction to the mated females. Based on this limited information, mating apparently may have little influence on the release of pheromone from female nematodes.

Effect of Crowding. Bone et al. (1978) held female *N. brasiliensis* in vitro at three densities ranging from 125 to 1250 per 0.5 ml of solution. Bioassays of the pheromone released into the solutions revealed no differences

in pheromone secretion per female attributable to the crowding, although one should realize that the in vitro holding situation was highly artificial and could have masked possible differences.

TRANSMISSION OF PHEROMONE IN THE ENVIRONMENT

Nematode sex pheromones must generally be transmitted from the emitting to the receiving individual through an aquatic medium. Thus, the resolution of questions relating to potential distances of communication or time required for the pheromone message to extend a certain distance from the emitter must be approached by analysis of the pheromone active space (Bossert and Wilson, 1963; Wilson, 1970). Such an analysis requires prior knowledge of three parameters: the emission rate of pheromone (Q), the response threshold for pheromone in the receiving individual (K), and the nature of movement of the pheromone through the environment.

Neither Q nor K has been determined (or even estimated) for any nematode species. However, some factors pertaining to movement of the pheromone in the environment have been explored or can be surmised. As is indicated later, our knowledge of the chemical nature of nematode sex pheromones is virtually nonexistent. Greet (1964), in the first report of nematode sex pheromones, showed that these substances were soluble in aqueous agar. This aqueous solubility has been confirmed for all subsequently investigated nematode species through direct or indirect experimental design. If the active space is totally dependent on diffusion of the pheromone through water, then relatively long time periods to achieve the maximum active space would be required, compared to the situation with airborne pheromones, because chemicals tend to exhibit a thousand-fold or more decrease in diffusivity in water compared to the same chemicals when evaporated in air (Wilson, 1970). Thus, during the minutes or hours following release of pheromone from a nematode, the active space might only extend for distances of a few centimeters. However, such a short distance, in comparison with the sometimes very long active spaces of sex pheromones of insects, might be completely appropriate to the way of life of many nematode species, in which males and females might be found in relatively high densities, being brought together by other environmental factors even before the beginning of a sex-pheromone communication sequence.

Several factors can operate to increase the pheromone active space. One of these is the release rate of pheromone (Q) from the emitter. If the pheromone is highly soluble in water, a relatively high Q is possible, in comparison with the situation in many terrestrial organisms where Q may be dependent on the volatility of the pheromone. If the aqueous medium in

which the pheromone emitter is located is flowing, then the pheromone message may take the form of an elongate trail, and the active space may thereby be considerably lengthened. Such an effect of medium flow may have been observed by Roche (1966) in his studies of the migration of surgically introduced hookworms, *Ancylostoma caninum*, in the dog. He found a greater migration of males to anteriad females than to posteriad females and suggested that the female pheromone travels in the direction of intestinal flow. Another factor, which might operate in the soil environment, is volatilization of the pheromone, from the aqueous film that surrounds soil particles and any nematodes present, into the air spaces that exist between the soil particles. A volatile component of the pheromone of the cyst nematodes *H. rostochiensis* and *H. schachtii* has been implicated in the premating attraction of males to females of these species (Greet et al., 1968). Such a volatile chemical message, which presumably would be reabsorbed in the aqueous film at some distance from the pheromone source, might enable the establishment of increased active spaces.

Some factors might also act to degrade the pheromone message and thus cause active spaces to reach smaller than anticipated distances from the pheromone sources. These factors, including possible chemical or microbial decomposition of the pheromone, might enhance orientation of responding individuals to particular pheromone sources by accentuating "fresh" pheromone concentration gradients (Green, 1966). Somers et al. (1977b) found no increase in the amount of pheromone in a medium containing females of *R. pellio* for 24 hr, as compared with 2 hr. They suggested that pheromone lability might have prevented the anticipated increase of pheromone in the medium.

RESPONSE TO PHEROMONE

Mechanism of Pheromone Reception

Direct studies on nematode pheromone receptors are scant, and the few reports which are available deal exclusively with the male sense organs. Samoiloff et al. (1973) subjected males of *P. silusiae* to laser microbeam irradiation of the spicules, germinal gonad, or nerve ring. Later observations of the migratory responses of the treated individuals when they were placed in female pheromone gradients implicated the tips of the spicules as the pheromone receptors (Samoiloff et al., 1973). The lack of oriented male response when only one spicule was irradiated suggested that orientation to female pheromone required comparison of the chemical concentration at the receptors on both spicules. Such a chemoreceptive capacity of the spicules seems reasonable, since the spicules of many nematode species are innervated

(McLaren, 1976). However, intuitively it seems strange that the chemoreceptors controlling anterior-directed orientation should be located on the spicules at the posterior of the body. In contrast, Ward (1973) suggested that chemotaxis of the nematode *Caenorhabditis elegans* required comparison of chemical concentrations by the anterior sensory receptors. Croll (1977) postulated that a cephalic nerve fiber found in male *C. elegans* but absent in hermaphroditic worms is a likely candidate for pheromone reception. However, no sex pheromones are reported for *C. elegans*. Thus, the precise location of pheromone receptors remains unconfirmed. Perhaps anterior chemoreceptors control orientation toward a pheromone source from a distance and posteriorly located receptors control short-range copulatory reactions. Future studies using microirradiation techniques offer a technique for resolution of the receptor sites.

Nematodes apparently possess chemoreceptors with a high degree of pheromone specificity. This assumption is based in part on the high degree of specificity that is exhibited by male nematodes of a number of species when exposed to pheromone from their own females as opposed to females of closely related species or strains (Chin and Taylor, 1969; Green and Plumb, 1970; Cheng and Samoiloff, 1971; Balakanich and Samoiloff, 1974; Roberts and Thorson, 1977b). Also, Dusenbery (1975) recently reported that *C. elegans* exhibited stereospecificity for D- and L-tryptophan, a type of specificity now being found to occur in a variety of insect pheromones (Silverstein, 1977).

Sensory adaptation, a common feature of many animal receptors, has been demonstrated several times with regard to the reception of female sex pheromone information by male nematodes. Green (1966) and Greet et al. (1968) suggested that adaptation of the chemoreceptors caused by concentrated female *Heterodera* pheromone disrupted male orientation to the pheromone. Balakanich and Samoiloff (1974) postulated that differential rates of sensory receptor adaptation at high pheromone concentration to the different pheromone chemicals released from females of different strains of *Panagrellus* could cause the strain-specific attraction that occurs in this species.

Bone and Shorey (1977a) reported that 0.5- to 4-hr preexposures to female pheromone diminished the subsequent pheromone responsiveness of male *N. brasiliensis* with a total response inhibition occurring at the highest tested pheromone concentration. This inhibition probably represents some unknown interaction between sensory adaptation and central nervous system habituation. However, the responsiveness of the males remained low even after a 2-hr recovery from preexposure to the pheromone, probably indicating a prolonged period of habituation after the sensory neurons have recovered from adaptation.

Attraction of Members of the Opposite Sex

As indicated earlier, essentially all the nematode sex pheromone papers published to date deal with the attraction of members of one sex to pheromone-releasing members of the opposite sex prior to mating. We speculate that further behavioral research will reveal various close-range premating chemical interactions between the male and female. For example, pheromones released from a member of either sex might cause the other sex to position its body and external genitalia appropriately to enable coupling.

In all species for which pheromone-stimulated locomotion studies have been conducted (with the exception of the parthenogenetic *Meloidogyne*, in which no pheromone attraction has been found (De A. Santos, 1972), males are stimulated to approach a female pheromone source. In addition, in a number of free-living and animal-parasitic nematode species, females have been seen to approach male pheromone sources (Table 1). This female-to-male attraction might be even more general, and perhaps has not been adequately looked for in some of the species for which it is not reported. Although female-to-male attraction has not been reported for plant-parasitic species, the only such species studied are from the genus *Heterodera*, in which the mature females are immobile.

Effect of Physiological Factors on Pheromone Responsiveness

All detailed studies of the effect of physiological factors on pheromone responsiveness have been conducted in situations in which the responders were male nematodes that were tested for attractancy to female pheromone. These studies have been involved with the effects of age, time of day, and mating history on male responses.

Effect of Age. As was discussed earlier with respect to release of pheromone from females, the onset of pheromone responsiveness by the males coincides with the time of the final larval molt in *R. pellio* (Somers et al., 1977b), *N. brasiliensis* (Bone et al., 1977a), and *P. teres* (Jones, 1966). In *P. silusiae*, on the other hand, males are reported to become responsive while fourth stage larvae (Cheng and Samoiloff, 1971). At any rate, it seems that the system for sexual communication in both pheromone-releasing females and responding males are operative at or shortly before the time of sexual maturity. In both *N. brasiliensis* and *R. pellio*, male responsiveness is at its highest level in young adults and tends to decrease with increasing age. This might be correlated with increasing sexual senility in both species and with immune reactions from the rodent host in the case of *N. brasiliensis* (Bone et al., 1977a; Roberts and Thorson, 1977a).

Effect of Time of Day. Males of *R. pellio* have as strong a tendency to move toward a female pheromone source during the night as they do during

the day (Somers et al., 1977b). Similarly, males and females of *N. brasiliensis* were not found to vary in their responsiveness to pheromone released from the opposite sex at various test times throughout the 24-hr cycle (Bone et al., 1977a). Thus, as was noted earlier with respect to release of pheromone into the environment, responsiveness to pheromone has not been demonstrated to vary with the 24-hr cycle for any nematode species.

Effect of Mating. Only one report specifically addresses the question as to whether prior mating affects nematode pheromone responsiveness. Bone and Shorey (1978) obtained virgin adult males and females of *N. brasiliensis* after producing single-sex infections by inoculation of larval males or females into mice. Mated males and females were obtained from mice that had contained mixed-sex nematode infections. On the average, with both the males and females, mated individuals were as responsive to pheromone released from the opposite sex as were virgin individuals.

Communication with Members of the Same Sex

Few studies have been conducted to determine whether nematodes influence the locomotion of other members of the same sex, in the absence of the opposite sex. The more complex situation where members of one sex might influence other members of the same sex when the nematodes are also exposed to members of the opposite sex will be dealt with in the next section.

Interactions between members of the same sex were suggested first by Bonner and Etges (1967), who found that males of *Trichinella spiralis* moved away in response to pheromone released from other males. However, groups of males of this species often move en masse when responding to female pheromone, and Bonner and Etges suggested that a positive thigmotaxis might supersede the tendency for repellency among males. Somers et al. (1977b) also found a tendency toward a pheromonally mediated male-to-male and female-to-female repellency.

In contrast to the above suggestions of repellency, Stringfellow (1974) reported that males of *P. strongyloides* were attracted to other males, although the degree of male attractancy was not as high as it was when they were exposed to female pheromone. Green et al. (1970) had also found a slight male-to-male attraction to *H. rostochiensis.* In addition, both sexes of *N. brasiliensis* exhibit a tendency to be attracted to high concentrations of pheromone obtained from other members of the same sex.

The biological function served by attraction or repellency among members of the same sex is not clear. However, mutual repellency might cause optimal spacing and dispersion in the environment, and perhaps optimal utilization of some environmental factor, such as food, could be brought about by mutual attractancy.

Response Inhibition and Complex Interactions

A number of investigators have placed groups of males in bioassay chambers and have measured their locomotory responses toward female pheromone sources. Although the grouped males have typically been found to make a positive response in this situation, they have sometimes been found to be less strongly attracted toward the pheromone source than were single males, when placed in the same situation. Alphey (1971) was the first to observe this phenomenon and proposed that thigmokinetic responses occurred among males of *N. brasiliensis* that were in physical contact with each other and interfered with their attraction to female pheromone sources. Salm and Fried (1973) found that groups of three to five males of *Camallanus* sp. required more than twice the time to move comparably to single males when in a female pheromone gradient; they also postulated that thigmokinetic reactions among the males were responsible for inhibition of male movement, although they recognized that short-range chemical communication among the males was also a possibility.

Bone and Shorey (1977b) and Bone et al. (1978) examined this inhibitory phenomenon further. They found that *N. brasiliensis* males had a reduced tendency to migrate toward a female pheromone source when as few as two males were placed together in the response portion of a bioassay chamber. Also, the presence of males with single migratory males, but isolated from direct contact with them, in the response portion of the chamber inhibited the attraction response of the migrating males toward the female pheromone source. Even the exposure of males to the presence of other males only prior to their being placed as single responders in the bioassay chamber inhibited their subsequent approach toward the female pheromone source. These experiments clearly indicate a chemical male-to-male inhibiting message.

Our knowledge of this inhibitory effect may be confounded by another reaction that may be occurring simultaneously; present evidence indicates that females of *N. brasiliensis* may be stimulated to emit more male-attractive pheromone when they sense a critical concentration of pheromone released by the males in their nearby environment (Bone et al., 1978). The functional significance of this proposed pheromonal feedback system, in which males release a pheromone which apparently influences female pheromone release, is not clear. Perhaps some females are completely unattractive to males unless they sense the presence of nearby males. Green et al. (1970) indicated that some females of *Heterodera* spp. were unattractive to males initially, but were later highly attractive after continued exposure.

PHEROMONE BIOASSAYS

Essentially all nematode pheromone bioassays have been based on the

movement of responding individuals through an aqueous or semiaqueous medium in response to a pheromone gradient established in that medium. A plethora of basically similar bioassay apparatuses has been developed by the various investigators in response to their specialized bioassay needs.

The bioassay media have mainly consisted of saline solutions for animal-parasitic nematodes or agar for plant-parasitic and free-living species. In their development of a bioassay medium for *R. pellio*, Somers et al. (1977b) used the bacterial "film" that is the normal medium within which nematodes of this species develop and reproduce. A similar approach was employed by Roche (1966) and Gimenez and Roche (1972) for two zooparasitic species. This use of a natural medium has been rare in nematode bioassay technique and is probably a highly advantageous procedure, if one assumes that the most natural responses will probably occur in the most natural medium.

Pheromone sources in nematode bioassays have often consisted of specified numbers of living worms, confined in one portion of the bioassay apparatus behind a permeable barrier such as filter paper, cellophane, or dialysis tubing. Other pheromone sources have consisted of the chemical secretions that had previously been released by the nematodes and that were incorporated in agar blocks, saline solution, bacterial "film", or absorbed on filter paper. Finally, some investigators have used macerated or excised portions of nematodes as pheromone sources (Anya, 1976; Bone et al., 1978).

Pheromone researchers have sometimes used 50 or more female worms to contribute enough pheromone for a single bioassay (Bonner and Etges, 1967; Alphey, 1971; Bone et al., 1977b). We feel that the lack of highly sensitive bioassays has been partly responsible for the superficiality of most nematode pheromone work published to date. Most current bioassay techniques require large numbers of pheromone-producing nematodes and lengthy time periods for the establishment of pheromone gradients in the assay apparatuses and for the bioassay responders to move appropriately within the gradients. Major emphasis should be placed on the development of rapid and sensitive bioassay techniques. Then, if the various ecological and physiological regulatory parameters, such as the correct reproductive age and mating status of the tested worms are taken into account, it seems evident that bioassays based on the amount of pheromone released by only one "donor" worm should be possible.

Ideally, the development of a bioassay follows the acquisition of detailed knowledge of the natural pheromone-communication behavior of the organism. This natural behavior is observed in various ways in the laboratory, and reproducible responses such as activation, movement toward the pheromone source, or copulatory behavior are selected as reproducible bioassay indicators. A given bioassay may be based on one or more of these indicators

and, as is now being found with the insects, the pheromone chemicals that cause one of the responses will not necessarily be the same as those that cause another of the responses.

The testing of more than one concentration of pheromone in bioassays is mandatory. Through such testing, dosage–response curves can be constructed and the relative biological activity of different tested pheromone solutions can be calculated. Also, because excessive or subthreshold pheromone concentration may result in a low bioassay response, a series of concentrations must be examined to determine that the tested part of the bioassay curve is that part in which increasing concentrations cause increased responses (Bone et al., 1978).

CHEMICAL NATURE OF THE PHEROMONES

Our knowledge of the chemical nature of nematode sex pheromones is virtually nonexistent. No nematode pheromones have been identified, except for the proposal of Stringfellow (1974) that the [OH] ion functions as a pheromone in *Pelodera strongyloides*. Although pH gradients might indeed be involved in stimulation of some nematode behaviors, it would seem reasonable that premating behavior must rely on more specific stimuli. We make this assumption partly on the basis of the specificity of pheromone reactions, discussed in the following section, which would seem to be based on a wide variety of chemicals functioning as nematode sex pheromones.

Greet (1964), in the first report of nematode sex pheromones, showed the *Panagrolaimus rigidus* female pheromone chemical(s) to be soluble in aqueous agar. This aqueous solubility has been confirmed for all investigated nematode species through direct or indirect experimental design.

A volatile component also has been implicated for premating attraction in *H. rostochiensis* and *H. schachtii* (Greet et al., 1968). The aqueous *Heterodera* pheromone components appeared to be stable to ultraviolet light, drying, and moderate heat (Greet et al., 1968). Also, Green and Plumb (1970) reported that female pheromone from several *Heterodera* sp. was stable in agar for up to one month at 5°C in sealed containers. However, Green (1966) had also reported that the pheromone was labile in agar at 20°C and little biological activity occurred one day after pheromone-source females were removed from the agar or died. He proposed that such pheromone lability might intensify orientation to actively releasing pheromone sources and reduce permeation of the environment with false gradients which would lead to sensory adaptation or disorientation of the responding sex. Similarly, Somers et al. (1977b) maintained female *R. pellio* for varying periods in bacterial "film" on agar plates and found no significant increase in phero-

mone in the film following 2-, 6-, or 24-hr periods. They suggested pheromone lability as a possible explanation for this lack of pheromone buildup.

Balakanich and Samoiloff (1974) have contributed the most refined investigations to date into the chemical aspects of nematode sex pheromones. Differential solubility indicated that the female sex pheromone of a *Panagrellus redivivus* strain complex was present in water-soluble and ether–water interface fractions (Balakanich and Samoiloff, 1974). Column chromatography of these fractions revealed at least two main pheromone components of different molecular weights which cause aggregation of males in a strain-specific manner.

PHEROMONAL SPECIFICITY AND REPRODUCTIVE ISOLATION

A few studies have been conducted to examine the specificity of male behavioral responses to pheromone released from females of their own versus closely related species. These studies already indicate a high degree of species specificity, which further suggests that the closely related species studied may be releasing pheromones that are qualitatively different. Thus, Chin and Taylor (1969) demonstrated that the *Cylindrocorpus longistoma* female pheromone did not cause the approach of males of *C. curzii* and vice versa, although these pheromones did cause the approach of conspecific males. However, Roberts and Thorson (1977b) found that interspecific pairing between living males and females of certain zooparasitic species, such as *Nippostrongylus brasiliensis* and *Nematospiroides dubius*, or *T. spiralis* occurred readily, although their experiments did not eliminate tactile stimuli among the communicating nematodes.

The most complete study of pheromone specificity in nematodes was conducted with ten species of *Heterodera* (Table 1) (Green and Plumb, 1970). Differing patterns of intraspecific movement of males to female pheromone suggested at least three subgeneric groups of the *Heterodera* species. Green and Plumb postulated that six different pheromone components, which they called alpha, beta, gamma, delta, epsilon, and zeta, may be present among those species to explain the varying degrees of interspecific specificity observed.

The work of Balakanich and Samoiloff (1974) indicates that pheromonal specificity may also occur at the strain level. They found varying degrees of specificity among males and females of several strains of *P. redivivus*. In their pheromone crossing studies between males and females of different strains, they found differing optimal concentrations which elicited the highest degree of male movement toward the pheromone sources. Based on these findings, they proposed that the patterns of interstrain differences observed might

promote inbreeding (within a strain) at low population densities and outbreeding at high densities.

FUTURE PROSPECTS

Pheromones are the only known communication means through which males and females of bisexual species of nematodes are caused to move toward each other prior to mating. We suspect that further research will indicate that pheromones (not necessarily the same ones that brought the sexes together) will also be found to act as stimuli, together with tactile stimuli, for various close-range precopulatory and copulatory reactions. That this aspect has received essentially no study points out a very important consideration: despite the almost 40 research publications on nematode pheromones to date, extremely little attention has been directed toward studies of the natural pheromone behavior of the animals. Rather, most "behavioral" studies have been at the level of putting nematodes into an artificial aqueous medium and determining how many millimeters they have moved from their release point after a stated number of hours. Much of this lack of natural behavioral information arises through difficulties presently encountered in trying to conduct pheromone-communication experiments in natural environments. Yet, considerable need exists for more knowledge concerning the natural behavior. For example, as indicated earlier, such knowledge will provide the basis for the development of rapid, sensitive, and meaningful bioassays.

Our degree of sophistication of understanding of nematode sex pheromone systems is about on a level with the degree of sophistication that entomologists had concerning insect pheromone systems 20 years ago. After recognizing in a number of instances that pheromone communication is an essential component of the premating behavior of a number of pest insect species, many entomologists are now directing their research toward environmentally safe insect-control strategies based on manipulation of the communication systems (Birch, 1974; Shorey and McKelvey, 1977). One promising strategy, called "disruption of pheromone communication," involves the permeation of the pests' environment with a synthetic pheromone that is identical to the pheromone released by the living insects prior to mating (Rothschild, 1975; Arn et al., 1976; Beroza, 1976; Gaston et al., 1977). With the synthetic pheromone odor being everywhere in the environment, the sex that normally responds to the pheromone is rendered incapable of locating the pheromone-releasing sex.

Intuitively, it seems reasonable that if certain species rely on pheromones for premating communication, then the natural system can be disrupted (like

the insect systems mentioned above) and pest control can be obtained. Preliminary research with *N. brasiliensis* has already indicated, for example, that males may be rendered incapable of orienting to a gradient of pheromone emanating from living females for up to 2 hr after a prior exposure to an environment permeated with the pheromone (Bone and Shorey, 1977a). Thus, although a number of intermediate experimental goals will have to be challenged, including detailed studies of the natural pheromone communication systems and chemical characterizations of the pheromones, it seems likely that nematode pheromone research will ultimately also be directed toward practical goals.

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SHORT-CHAIN ALIPHATIC ACIDS IN THE INTERDIGITAL GLAND SECRETION OF REINDEER (*Rangifer tarandus* L.), AND THEIR DISCRIMINATION BY REINDEER

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Abstract—Interdigital gland secretion from reindeer (*Rangifer tarandus* L.) was analyzed by thin-layer chromatography and gas chromatography. The short-chain acid fraction consisted of acetic, propionic, isobutyric, *n*-butyric, isovaleric, 2-methylbutyric, *n*-valeric, isocaproic, and *n*-caproic acids. The short-chain acids were produced by sterol esters when hydrolyzed in the gland—probably by microorganisms. Triglycerides present did not contain any short-chain acids. By testing isovaleric acid and isobutyric acid applied on small filter papers placed in a pen and measuring the number of sniffings on and towards the samples, we elicited good response at 1 ng application compared with the blanks, while pivalic acid gave no response under the same conditions.

Key Words—reindeer, skin glands, interdigital gland, fatty acids, sterol esters.

INTRODUCTION

Odor-producing skin glands and their role in communication between higher animals have recently been discussed in several zoological and ethological articles. However, only a few chemical investigations have been made. A survey of this field has been made by Mykytowycz (1970) and Albone (1977).

Reindeer (*Rangifer tarandus* L.) and caribou (*Rangifer tarandus groen.*

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landicus L.) possess four large skin glands—the antorbital, tarsal, interdigital- and caudal glands—the last-mentioned having recently been discovered in reindeer (Müller-Schwarze et al., 1977b).

The interdigital glands are located in the dorsal interdigital skin. The gland in the hindfoot forms a glandular sac (Figure 1), while the glandular epithelium in the forefoot is much less developed and does not form a specialized sac. The interdigital gland histology in caribou was described by Quay (1955). We previously reported the composition of volatile compounds in tarsal gland secretion as a mixture of C_7 to C_{10} aldehydes and some fatty alcohols (Andersson et al., 1975). We also gave a preliminary report on the chemical constitution of interdigital gland secretion (Sokolov et al., 1974).

This article reports the result of an investigation on nonvolatile compounds and volatile fatty acids in the interdigital gland secretion of male and female reindeer and of tests on the behavioral significance of some main compounds in an effort to establish their behavioral activity.

METHODS AND MATERIALS

Interdigital, antorbital, and tarsal glands from both sexes—adults and calves—were collected in the spring and autumn. Control samples of the

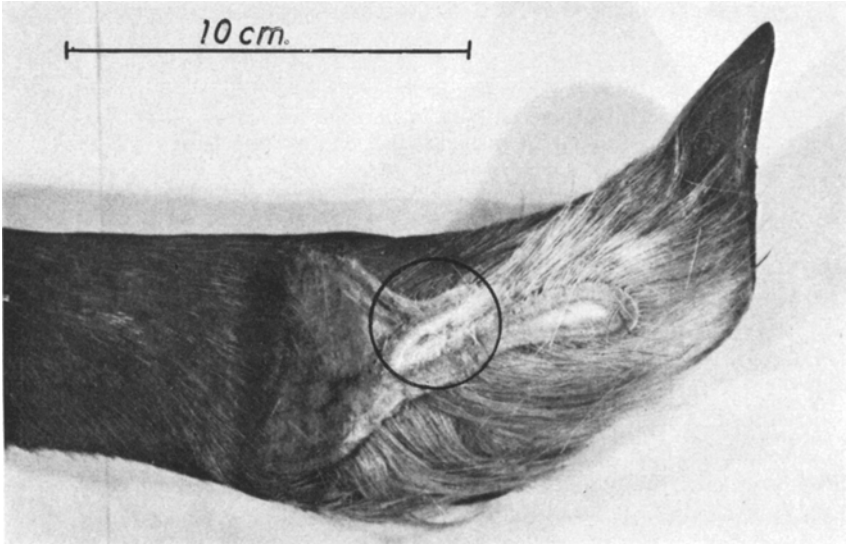

FIG. 1. Cross-section of a reindeer hindfoot interdigital gland. The glandular sac has been encircled.

skin from a region lacking specialized skin glands (the back) were taken on the same occasion. The material was collected and further treated under the conditions stated by Andersson et al. (1975). The glands were cut into pieces and extracted with dichloromethane (Merck) or diethyl ether (Mallinckrodt), and evaporated to a small volume. On evaporation, each interdigital gland yielded up to 50 mg of a lipid with a characteristic sharp odor. Dichloromethane extracts from different glands were applied on alumina sheets with a 0.25-mm silica gel layer (DC Alufolien, Silicagel, Merck), and eluted with *n*-hexane–diethyl ether (7:3). Detection was effected by spraying with 5% *p*-methoxybenzaldehyde in concentrated sulfuric acid, followed by gentle heating of the plate. Gas chromatographic investigations of the non-volatile compounds were made by alkaline hydrolysis or transesterification.

Hydrolysis. The dichloromethane extract from one interdigital gland was evaporated to dryness in a stream of nitrogen; 10 ml methanol and 5 pellets of sodium hydroxide were then added and the mixture was boiled under reflux for 2 hr. After cooling, 5 ml of the methanolic solution was removed, washed with ether, dried ($MgSO_4$) and evaporated. To this residue, 1 ml pyridine solution of trimethylchlorosilane (Trisil) was added and the trimethylsilyl ethers of the alcohols were further analyzed by GC (alternative 1 below).

The residual extract from the hydrolysis was evaporated to dryness and 2 M H_2SO_4 was slowly added, with cooling, until pH 2 was reached. The aqueous layer was extracted with ether and the ethereal solution was dried (Na_2SO_4) and gently evaporated in a stream of nitrogen. The residue was dissolved in 0.5 ml diethyl ether and tested for short-chain fatty acids by GC on columns (alternatives 2 and 4 below).

Transesterification. The secretion from one gland, approximately 50 mg, was boiled with 4 ml 0.5 M methanolic sodium hydroxide for 5 min. After cooling, 5 ml of 12.5% (w/v) BF_3 solution in methanol was added. The mixture was boiled under reflux for 2 min, cooled, and transferred into a separatory funnel with 20 ml saturated aqueous sodium chloride. The methyl esters were extracted with 3×10 ml *n*-hexane, and the hexane layer was dried ($MgSO_4$) and evaporated. The residue was dissolved in 1 ml dichloromethane and tested by GC (alternative 1 below).

Free Acids. One interdigital gland was cut into pieces and extracted with 2×25 ml 10% aqueous sodium bicarbonate. After acidification to pH 1 with concentrated HCl, the solution was extracted with 4×20 ml ether. The ethereal solution was dried ($MgSO_4$) and evaporated. Ether (0.5 ml) was added and the free short-chain fatty acids were analyzed by GC (alternative 4 below).

Gas Chromatographic System. Pye-Unicam model 64 gas chromatograph equipped with a flame-ionization detector (FID), and glass columns (2.5 m, ID 0.4 cm) were filled with:

1. 3% OV- 17 on acid-washed, dimethyldichlorosilane-treated Chromosorb W (100–120 mesh) for long-chain methyl esters and trimethylsilyl ethers of alcohols. The carrier gas was nitrogen at a flow rate of 40 ml/min. Temperature programed from 130–to 270° at 4°/min.
2. 10% Reoplex 400 on acid-washed, base-washed, dichlorosilane-treated Chromosorb W (80–100 mesh), for short-chain carboxylic acids (C_2–C_6). Column temperature 100° isothermal condition.
3. 5% Carbowax M on Diatomite C (100–120 mesh). Column temperature 10 min, 50°C; 50–200 at 10°/min; for analysis of methyl ester of short-chain (C_2–C_5) acids.
4. 10% behenic acid on acid-washed, dimethyldichlorosilane-treated Chromosorb W (100–120), isothermally at 100°C for the analysis of short-chain acids. This column resolved 2-methylbutyric acid from 3-methylbutyric acid.

For the GC-MS system, a Pye model 84 gas chromatograph in combination with a LKB 9000 mass spectrometer was used. (For description, see Andersson et al., 1975.)

Behavior Tests. Four adult male reindeer (*Rangifer tarandus* L.) of the forest variety were used as test animals, one at a time. In an experimental pen, 24 metal holders were set into the ground, being arranged in a hexagon with six arms. Filter papers, 25 mm in diameter, were placed on the holders 5 cm above the ground and used as scent carriers. The holders, 65 cm apart, were divided into six test units, each comprising four holders with filter paper (Figure 2). New filter papers were used in each test. The test substances—isovaleric, isobutyric, and pivalic acids—dissolved in dicloromethane, were applied with a syringe, so as always to obtain 1 ng on the filter papers. In each test, two randomly chosen test units were applied with either isovaleric or isobutyric acid, two randomly chosen test units were applied with pivalic acid, and two were used as blanks applied with the same amount of solvent (CH_2Cl_2) as with the test acids above. The tests lasted for 15 min unless they had to be terminated earlier, owing either to some disturbance or to inactivity on the part of the animal. Only reactions such as sniffing directly at a test paper or sniffing or winding towards it (ie, within 30 cm) were noted as positive reactions.

RESULTS

Chemistry. As reported in earlier experiments, TLC gave bands from the cholesterol and lanosterol esters of fatty acids, triglycerides, one unidentified compound or group of compounds, free fatty acids, and free lanosterol and

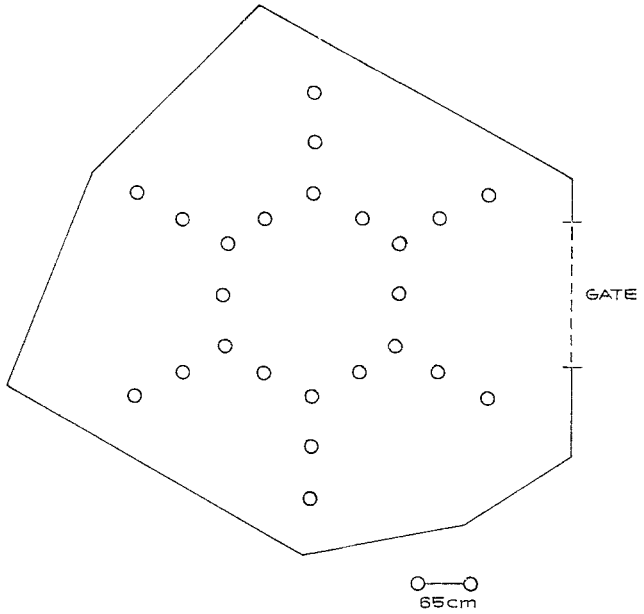

FIG. 2. Test pen. Circles indicate the filter papers used as scent carriers. The pen was surrounded by 2-m-high burlap.

cholesterol (Sokolov et al., 1974). Identification was made by R_f values and IR spectroscopy. The compounds also gave special color reactions by the TLC evaluation methods described above (Figure 3). Very little difference was seen in TLC between material from different individuals or seasons (Figure 3). However, the tarsal gland secretion differs from the others by having a tailing spot just above that of lanosterol. The most dominant spots in Figure 3 are the sterol esters (with highest *Rf* value), which constitute approx. 60% of the secretion. For the composition, see Sokolov et al. (1974).

By investigation of the sterols as trimethylsilyl ethers in GC-MS, it was possible to confirm the existence of cholesterol and lanosterol as well as of 24,25-dihydro-Δ^8-lanosterol, which had spectra identical with reference spectra (Eneroth et al., 1969). The hydrogenated lanosterol has a spot in TLC which is close to that of lanosterol.

In GC, peaks were observed corresponding to the most common short-chain fatty acids. The distribution of free acids and the acids given by hydrolysis of the secretion is presented in Table 1. The values given for acetic acid, and to some extent also for propionic acid, are uncertain (too low) owing to evaporation losses. The total amount of short-chain acids varied between 0.1 and 1 μg/gland in alkaline extraction of the secretion (free acids). After

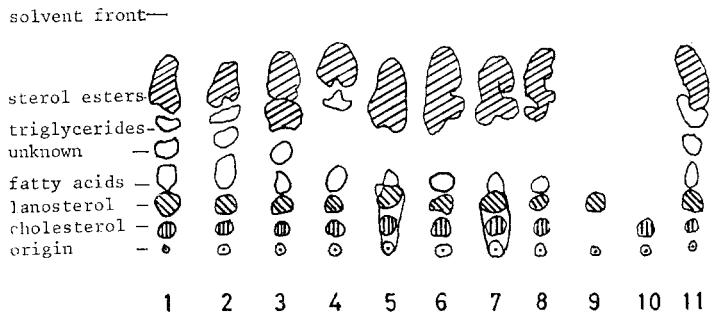

FIG. 3. TLC of skin gland secretion from reindeer (eluted in *n*-hexane–diethyl ether, 7:3). (1) Interdigital (♂♂, taken in September); (2) control from other nonglandular area (the back); (3, 4, and 5) interdigital, antorbital, and tarsal, respectively, from one adult animal (♂); (6, 7, and 8) interdigital, tarsal, and antorbital, respectively, taken from one calf (♂); (9) lanosterol; (10) cholesterol; (11) identical with (1) above. Numbers (3) through (8) were taken at the same slaughter in March. *Color reactions:* ⊘ deep red–violet, strong color; ○ weak violet, the spots from fatty acids—pink; ⊗ sharp red, strong color; ◍ deep blue–violet, strong color.

hydrolysis, the amount of short-chain acids increased about 10 times compared with the amount of free acids.

When the sterol esters described above were separated by preparative TLC and hydrolyzed, the same short acids as given in Table 1 were detected. There were no signals of short acids when the hydrolysis experiment with the triglycerides from preparative TLC was repeated. No signals were obtained from unsaturated acids as was tentatively proposed in the first presentation (Sokolov et al., 1974). GC indicated no difference in the qualitative content of short-chain acids between (adult) male and female material taken at the same slaughter in September. Investigation of the seasonal variation of short-chain acids was not made.

By investigating long-chain acids, peaks were obtained from the common fatty acids as methyl esters (C_{12}–C_{20}). Methyl palmitate was used as internal standard. By testing antorbital and tarsal secretion in the same way, it was possible to see some minor differences between these secretions and the secretion from the interdigital gland. This will be further investigated (Sokolov et al., 1977).

We also investigated a control sample from the skin, taken from a region lacking specialized skin glands (the back). In this extract it was possible to identify the acids mentioned above, but at a concentration much lower than in the interdigital gland secretion. On repeating the hydrolysis

TABLE 1. VALUES AS PERCENTAGES OF ALL C_2–C_6 ACIDS

Content of short acids (C_2–C_6)[a]	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	2-Methylbutyric acid	*n*-Valeric acid	Isocaproic acid	Caproic acid
Free acids in the secretion	2.1	4.2	22.1	Trace	31.6	8.4	Trace	31.6	Trace
Acids after hydrolysis	6.6	4.9	18.1	8.2	21.4	13.4	0.7	26.7	Trace

[a] Error in measurement ±1%.

without any gland extract (blank), no signals from short-chain acids were obtained in GC.

Behavior Tests. Reindeer have never been seen performing "excitation jumps" like the caribou (Pruitt, 1960) and wild reindeer (*Rangifer tarandus fennicus*, Lönnb.) (T. Helle, personal communication). This behavior has been assumed to be linked with the interdigital glands. The only reaction to the secretion we noted in the animal is the habitual sniffing on and towards their own interdigital glands on both forefeet and hindfeet.

The tests reported here were performed in order to test individual compounds in the gland secretion. As Table 1 shows, isobutyric acid and isovaleric acid are two of the short-chain acids most abundantly found in the gland secretion. The behavior responses to these substances were compared with those to blanks and pivalic acid. The latter, an isomer of isovaleric acid, is not found in the gland secretion.

Before the tests with the substances mentioned above were performed, a suitable amount of acid for use in the tests was determined (Table 2A). In tests with 10 ng or more, it was obvious that the test animals noticed the test substance, isovaleric acid, as soon as they entered the pen—manifested by a high frequency of sniffing and winding. Since this was undesirable for testing selective animal behavior, these amounts were regarded as being too high, even though the animals clearly reacted to the substances. The animals showed a clear response to 1 ng of the substance but did not react to amounts of 0.1 ng acid, and thus 1 ng was considered to be a suitable amount.

In the tests, 1 ng of either isovaleric acid (20 tests) or isobutyric acid (27 tests) was tested with pivalic acid and blanks. The other acids in the secretion were not tested.

Evaluation of the animal tests showed that it was obvious that the animals sniffed significantly more often on or towards isovaleric and isobutyric acids than on or towards pivalic acid and blanks (Table 2B). The number of sniffing reactions to pivalic acid is comparable with that to blanks—that is, the animals showed no interest in this substance

DISCUSSION

The interdigital gland secretion from reindeer has previously been submitted to behavior tests (Müller-Schwarze et al., 1977a). The behavior role of the gland is still not clear, but one probable function of the secretion is that of laying trails on the ground. The amount of test substance (1 ng) is clearly perceptible on application. After 15 min, the smell is barely detectable by the human nose, and the amounts used were close to the lower limit that could elict a response from an animal in our tests.

TABLE 2. NUMBER OF RESPONSES FROM 4 ADULT MALE REINDEER (*Rangifer tarandus* L.) TO ISOVALERIC ACID OF DIFFERENT AMOUNTS AND TO BLANKS (A), AND TO TEST ACID, PIVALIC ACID, AND BLANKS (B)

A

Amount (ng)	N	Acid	Blanks	
100–10 n	11	9	0	$P < 0.05$
1 n	14	15	5	$P < 0.05$
0.1 n	14	1	0	N.S.

B

	N	Test acid (1 ng)	Pivalic acid (1 ng)	Blanks	
Isovaleric acid	20	14	1	2	$P < 0.05$
Isobutyric acid	27	14	2	2	$P < 0.05$

[a] Acid was applied randomly to 2 out of 6 test units each comprising 4 scent carriers. n = number of tests. Probability was determined from χ^2 values.

Most of the noted responses occurred within the first few minutes of the tests when the animals were reconnoitering the pen. More than one response was seldom noted, giving the impression that the animal entered the pen, searched around and, having once responded to the smell, obviously paid no attention to it again. The animals responded to both carboxylic acids tested. This does not exclude the possibility that other components listed in Table 1 may also contribute to the appropriate scent. In black-tailed deer, four substances were found to elict behavior reactions (Müller-Schwarze, 1969).

In reindeer it is obvious that the animals show interest in at least two fatty acids and that they can discriminate between two of the acids found in the gland and a compound, viz., pivalic acid, not found in nature.

Isovaleric acid has also been found to be a dominant compound in the subauricular scent of the male pronghorn (Müller-Schwarze et al., 1974). The short-chain acids listed in Table 1 were also reported by Albone et al. (1974) as major constituents of the anal gland secretion of the red fox.

The assumption that the volatile fatty acids can result from microbial decomposition of nonvolatile compounds (in our case sterol esters) leads to the hypothesis that the trail could have a long-term effect, since the esters are slowly decomposed by the microorganisms.

As Table 1 shows, a difference between the distribution of free acids and acids given by hydrolysis is evident, although not marked. This experi-

ment was performed in order to see if the microflora would selectively hydrolyze the secretion, compared with a hydrolysis carried out in the laboratory according to the description above. In this case, the microflora seem to hydrolyze almost all the esters with short acids present in the secretion. However, no microbiological tests have been made to confirm this theory.

An analogous hypothesis on the presence of decomposing microorganisms was put forward by Albone et al. (1974), where the different types of microorganisms in the anal gland secretion of the red fox were also present.

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SEX PHEROMONE OF THE TEA TORTRIX MOTH (*Homona coffearia* NEITNER[1])

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Abstract—Three compounds were isolated from female abdominal tips of the tea tortrix, *Homona coffearia* Neitner (Lepidoptera: Tortricidae) and identified as 1-dodecanol, 1-dodecyl acetate, and (*E*)-9-dodecen-1-yl acetate by chemical tests and mass spectrometry. These were the only appreciably active compounds detected in female gland extracts by male electroantennography of gas chromatographic collections. Synthetic (*E*)-9-dodecenyl acetate was shown to be active in attracting male moths in the field, and this activity was greatly increased by addition of dodecyl acetate and dodecanol. A very active lure for monitoring consists of 1 mg (*E*)-9-dodecenyl acetate, 3 mg dodecanol, and 1 mg dodecyl acetate in a polyethylene cap.

Key Words—Pheromone, tea tortrix, *Homona coffearia*, 1-dodecanol, 1-dodecyl acetate, (*E*)-9-dodecen-1-yl acetate.

INTRODUCTION

The tea tortrix (*Homona coffearia* Neitner) is a serious pest of tea in southeast Asia. The females of this species have been shown (Sivapalan and Vitarana, 1975) to produce a potent pheromone to attract males. Female pheromone production reaches a peak on the fourth day of adult life and then declines. Sivapalan and Vitarana showed that use of virgin females as bait in sticky traps was a valuable tool for predicting larval damage on tea. We undertook the identification of the pheromone of the tea tortrix to facilitate population monitoring of this destructive insect.

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METHODS AND MATERIALS

Insect Rearing and Collection

The insects used were reared in the laboratory in Sri Lanka on an artificial diet (Sivapalan et al., 1977) consisting essentially of minced tea leaves supplemented with brewers' yeast, with the addition of agar and fungistats.

Emerged females were maintained for three days by feeding with 5% sucrose + 2% honey solution (1:1), since maximum pheromone production occurred at this time (Sivapalan and Vitarana, 1975). Abdominal tips (last 3 segments) were removed between 23:00 hr and midnight, dropped into chilled CH_2Cl_2, ground in a tissue homogenizer, and filtered. Filtrates were pooled and stored under refrigeration. Solvent was removed from the extract on a rotary evaporator at 30° before shipment. A total of 500 abdominal tips sufficed for this identification.

Male pupae and the female extract were shipped by air from Sri Lanka to New York City. After agricultural quarantine clearance, they were forwarded to Geneva, New York. Total travel time was 2–4 days, and many of the pupae did not survive the trip. Pupae and emerged males were maintained at 25°C under a 16:8 photoperiod and the adults were provided with 5% sucrose solution as a food source.

Chemical and Physical Determinations and Assays

The gas chromatographs were equipped with flame ionization detectors and glass columns. Preliminary separations were carried out on a 2.5-m×8-mm column packed with dimethyl silicone (5% SE-30 on 60/80 mesh Gas Chrom Z). Other columns used were 2 m×4 mm of cyclohexanedimethanol succinate (CHDMS, 3% on 100/120 Gas Chrom Q), dimethyl silicone (OV-1, 3% on 100/120 Gas Chrom Q), Carbowax 20M (3% on 100/120 Chromosorb W) and a 2-m×2-mm column of methyl (cyanoethyl) silicone (XF-1150, 10% on 100/120 Chromosorb W).

There was some duplication of GLC columns in our laboratory, so absolute retention times were not identical for different phases of this work. In all cases, however, appropriate standards were run immediately prior to any injections of natural material.

Electroantennograms (EAG) were carried out by our standard method (Roelofs and Comeau, 1971, Roelofs et al., 1971, Roelofs, 1977) and a normalized response profile was prepared by the method of Hill et al. (1974).

For microchemical tests, the capillary tubes containing EAG-active fractions from GLC collections were rinsed into appropriate containers and solvent was carefully evaporated in a slow stream of N_2. For treatments

with Br_2/CCl_4 and acetyl chloride (AcCl) the reaction vessel was a micro-test-tube prepared from 5-mm OD Pyrex tubing by heating a 4- to 5-cm length of tubing in the middle and drawing it out carefully to give two tubes with conical bottoms. The reagent (either 5% Br_2/CCl_4 or neat AcCl, 2–3 drops) was added and allowed to react for approx. 5 min in the dark, excess reagent was evaporated in a stream of N_2, and the residue taken up in 5–10 μl of CS_2 for injection on the GC and collection of 1-min fractions for EAG analysis.

Microsaponifications were carried out by rinsing the GLC-collection capillaries into a 4-ml vial, evaporating the solvent, adding approx. 10 drops of 10% KOH/MeOH, sealing the vial with a Teflon-lined screw cap, and allowing the reaction to proceed at 40–50° for an hour or so. Approximately 500 μl of distilled water was then added, and the product was extracted by adding 1 ml of ether, shaking the resealed vial vigorously, and drawing off the ether layer with a disposable pipet. The organic phases from three such successive extractions were combined and dried by filtration through a 2-mm layer of anhydrous $MgSO_4$ in a disposable pipet and evaporated under N_2. The saponification product was then dissolved in a small amount of CS_2 and injected onto the GLC for subsequent collection and EAG analysis.

The procedure for microozonolysis was generally that of Beroza and Bierl (1966, 1967), but the apparatus was modified for use with smaller quantities of substrate. Gases (O_2 and N_2) were introduced from the sides of the ozone generator and controlled with fine needle valves. The center electrode to which high voltage was applied was introduced from the top of the apparatus. The ozone was conducted into the micro-test-tube holding the substrate in approx. 10 μl of CS_2 through a 27-gauge syringe needle attached to a Luer fitting on the bottom of the ozone generator. Oxygen flow was adjusted to give a bubbling rate just too fast to count, and the high-voltage vacuum tester was applied for 30–45 sec. The blue color of excess ozone in the solution was used to indicate the endpoint. With this apparatus, we could routinely ozonize a few hundred nanograms of material and on occasion were successful with only 40–50 ng. In cases where determination of trace amounts of double-bond isomers was essential, larger amounts (2–3 μg) could be easily used (e.g. Miller et al., 1976).

Mass spectra were run on a Finnegan GLC–mass spectrometer under computer control. The column was 2 m$\times$4 mm packed with 3% SE-30. Chemical ionization was used with methane as reagent gas.

Laboratory and Field Bioassays

(*E*)-9-dodecen-l-yl acetate (*E*9-12:Ac) was obtained from Farchan Division, Storey Chemical Co.; it was >99% pure by GLC and showed no

(<0.5%) *Z* isomer. Chemicals for field tests were placed in silicone vial stoppers (5×9 mm, A. H. Thomas Co.) or, for some of the treatments in test 3, polyethylene caps (OS-6 natural polyethylene closures, Scientific Products). The caps for each treatment were wrapped with aluminum foil and shipped by air to Sri Lanka for field testing during 1976 and 1977. A set of blank septa was included. Traps were galvanized sheet-metal cylinders 18 cm wide and 46 cm long, with sticky-coated paper liners. Virgin females (3 days old, 1/trap) were held in small cylindrical wire mesh cages (4 cm wide ×5 cm long) suspended in the traps (Sivapalan and Vitarana, 1975). They were replaced every 48 hr when the traps were checked.

Treatments were set up as randomized complete blocks and the traps (at 10-m intervals) were rerandomized at 48-hr intervals. Trap data were transformed to $\sqrt{n+1}$ and submitted to an analysis of variance by Duncan's new multiple-range test. Means in the tables are males/trap per 48-hr interval.

RESULTS

Collection of crude female gland extract from the SE-30 column at 185° gave a small area of EAG activity (0.6 mV above background) at 5–6 min, suggesting a 12-carbon alcohol, and a larger amount of EAG activity in the 12-carbon acetate region at 7–8 min (1.0 mV above background) and 8–9 min (2.4 mV above background). The retention time for dodecyl acetate (12:Ac) was 8.85 min.

The 7- to 8-min and 8- to 9-min fractions were combined and injected onto a CHDMS column at 140° and the effluent collected in 1-min fractions. EAG analysis of the fractions showed a broad area of activity from 11 to 15 min (12:Ac at 11.6 min, *E*9–12:Ac at 13.5 min), indicating that more than one EAG-active compound was involved.

Treatment of the main active area (7–9 min from SE-30 at 185°) with Br_2 in CCl_4 and collection from OV-1 at 160° eliminated almost all the EAG activity. Treatment with acetyl chloride (AcCl) had no affect on the activity, whereas saponification (KOH/MeOH) destroyed the 7- to 9-minute activity, and gave a new EAG-active area at 4–5 min (1-dodecanol (12:OH) at 4.75 min; 12:Ac at 8.3 min) corresponding to an alcohol. This fraction, when treated with AcCl, had its activity restored in the 7- to 9-min area, confirming that the major EAG activity is associated with one or more 12-carbon acetates.

Structural Identification of Active Components

Alcohol Area. The early EAG-active area from the SE-30 column showed

only one peak in the 12-carbon alcohol region, at the retention time of 12:OH (9.1 min at 150° vs. 9.1 min for an authentic sample) when injected onto a CHDMS column. Material that had been collected from SE-30 and then CHDMS also showed identical retention times to those of the standard on OV-1 (4.65 min at 150°) and XF-1150 (6.6 min at 150°). Treatment of the alcohol with AcCl gave an acetate with retention times identical to those of 12:Ac on XF-1150 (8.95 min at 140°) and Carbowax (6.45 min at 150°).

The alcohol peak was inert to ozone. Ozonized material had the same retention time (within experimental error) as the starting compound on OV-1 and CHDMS columns. The chemical ionization mass spectrum of the alcohol, collected from OV-1 and then CHDMS was the same as that of 1-dodecanol, with a base peak at *m/e* 85, a small peak for $(M+1)^+-H_2O$ at *m/e* 169, and a typical alkane breakdown pattern, confirming the identity of the alcohol with 1-dodecanol.

Acetate Area. Two peaks (I and II) were observed in polar columns. Collection of these peaks separately and reinjection on various polar and nonpolar columns (Table 1) gave retention times corresponding to 12:Ac and *E*9–12:Ac in all cases (differences found were all within experimental error).

Acetate I was inert to ozone and had a mass spectrum essentially identical to that of dodecyl acetate. Both showed $(M+1)^+$–HOAc at *m/e* 169. All major peaks were coincident, and minor differences in relative intensity were attributable to instrumental variations.

Ozonolysis showed that acetate II was a Δ^9 compound. The retention times of the ozonolysis product and those of 9-oxononyl acetate from ozonolysis of authentic *E*9–12:Ac were coincident (5.55 min vs. 5.6 min on OV-1 at 150°, difference within experimental error, and 19.7 min on CHDMS at 150°).

The mass spectrum of acetate II showed $(M+1)^+$–HOAc at *m/e* 167, confirming the presence of one double bond and the same fragmentation pattern as the standard *E*9–12:Ac.

TABLE 1. RETENTION TIMES OF TEA TORTRIX ACETATES

Column (temperature)	Time (min)			
	Acetate I	12:Ac	Acetate II	*E*9–12:Ac
OV-1 (150°)	6.95	6.95	8.10	8.10
CHDMS (150°)	8.45	8.45	9.50	9.45
XF-1150 (130°)	7.95	7.95	9.00	9.05
Carbowax 20M (150°)	6.50	6.50	7.50	7.50

Acetate II on XF-1150 showed the presence of 2% of a compound with the correct retention properties for (*Z*)-9-dodecenyl acetate (Z9–12:Ac). No further confirmation of its identity was possible because of the small quantity available.

Collection of both alcohol and acetate fractions in the same tube and subsequent injection onto a Carbowax column (which was chosen because it separates both alcohol and acetates from each other and from early extraneous material) gave the following composition: alcohol (49%), acetate I (15%), and acetate II (36%).

A small amount of EAG activity (approx. 0.2 mV above background) occurred at approx. 2× the retention time of 12:Ac on CHDMS. For such a long retention time, the compound would have to be a conjugated diene acetate (if indeed it were a 12-carbon acetate at all). The material, which showed a visible peak on GLC, was collected, but we were unable to obtain a mass spectrum because of the small amount available. Since the EAG activity and the quantity available were so small, this compound was not investigated further.

EAG Results

The EAG data confirmed the results given above. Standardized EAG responses of a series of monounsaturated 12-, 14-, and 16-carbon acetates and alcohols showed that the greatest responses were elicited by the 12-carbon acetate series. In the 12-carbon acetate series, the best antennal response (normalized to *Z*5–12:Ac=10.0) was to *Z*9–12:Ac (27.0), then to *E*6 (21.7), *E*9 (20.0), *E*5 (17.0), *Z*8 (16.4), and *E*10 (16.3). All other responses were below 15. Good responses on these antennae were from 3 to 4 mV.

Field Trapping Results

Problems with contamination during the 1976 flight made it impossible to do more than determine that *E*9–12:Ac was attractive to male tea tortrix moths in the field and that mixtures of components seemed more active than *E*9–12:Ac alone.

During the 1977 season, greater precautions were taken to avoid contamination (changing sticky papers in traps at each sampling, rotating entire trap assemblies instead of just treatment holders, etc.)

Test I (Table 2) showed that 300 μg of *E*9–12:Ac was as attractive as virgin females, but that the attractancy could be greatly increased by addition of either 12:Ac or 12:OH.

Two of the best treatments in the second test (Table 3) contained all

TABLE 2. FIELD ATTRACTION OF MALE TEA TORTRIX (TEST 1)[a]

Treatment[b] (μg)			
*E*9–12:Ac	12:OH	12:Ac	$\bar{X}$ ♂/trap[c]
300		300	54.36 a
300	3000		55.00 a
1000	1000		36.32 b
300	1000		31.93 bc
300	300		24.75 cd
300		150	19.64 d
300			7.75 e
	♀		3.36 ef
	Blank		0.32 f
	300		0.21 f

[a] Run April 4–18, 1977, Fernlands Estate, Punduluoya, Sri Lanka; 4 replicates sampled and rerandomized 6 times.
[b] In rubber septa.
[c] Male catches followed by the same letter are not significantly different at the 5% level.

TABLE 3. FIELD ATTRACTION OF MALE TEA TORTRIX (TEST 2)[a]

Treatment[b](μg)			
*E*9–12:Ac	12:OH	12:Ac	$\bar{X}$ ♂/trap[c]
1000	3000	1000	32.11 a
300	3000	300	27.67 a
1000	1000		27.61 ab
1000	3000		21.17 bc
1000	10000		14.06 cd
300	3000		13.56 cd
	♀		7.89 de
300 (2% *Z*)	3000		4.24 ef
1000			3.00 ef
300 (5% *Z*)	3000		1.78 f
	Blank		0.72 f

[a] Run May 7–21, 1977, Fernlands Estate, Punduluoya, Sri Lanka. Three replicates, sampled and rerandomized 6 times at 2-day intervals.
[b] In rubber septa.
[c] Treatments followed by the same letter are not significantly different at the 5% level.

TABLE 4. FIELD ATTRACTION OF MALE TEA TORTRIX (TEST 3)[a]

Treatment (mg)				
*E*9–12:Ac	12:OH	12:Ac	Carrier	$\bar{X}$ ♂/trap[b]
1.0	3.0	1.0	Polyethylene	44.25 a
1.0	3.0	1.0	Rubber	33.00 b
1.0		3.0	Polyethylene	31.95 b
1.0	3.0	3.0	Rubber	29.75 b
1.0	3.0	3.0	Polyethylene	25.80 bc
1.0		1.0	Polyethylene	22.40 bc
1.0		1.0	Rubber	19.50 c
1.0		3.0	Rubber	10.15 d
	♀			5.40 de
1.0		10.0	Rubber	4.55 e
	Blank			1.80 e

[a] Run July 10–18, 1977, St. Leonards Group, Halgranoya, Sri Lanka. Five replicates, sampled and rerandomized 4 times at 2-day intervals.
[b] Means followed by the same letter are not significantly different at the 5% level.

three components, whereas the treatment of 1 mg *E*9–12:Ac was not significantly more attractive than blank traps. Furthermore, addition of 2% and 5% of the *Z* isomer decreased catches significantly.

The best treatment in the third series (Table 4) had all three components in a polyethylene cap. We have no specific data on longevity of this bait, but the polyethylene treatment in this series showed no apparent deterioration in attractiveness after 10 days in the field, while some of the earlier formulations seemed to decrease in effectiveness with time. In any case, it is apparent that a bait of 1 mg (*E*)-9-dodecen-l-yl acetate + 3 mg l-dodecanol + 1 mg l-dodecyl acetate provides a very active lure for field monitoring of the tea tortrix. These compounds have been chemically characterized from female gland extract and are all behaviorally active, and so all three are sex pheromone components of the tea tortrix moth.

The phermone structures are consistent with the generality that pheromone structures of Olethreutinae species tend to be 12-carbon chain compounds (Roelofs and Comeau, 1971). The *E*9–12:Ac and the 12:Ac have been found to be pheromone components of other species, but the alcohol, 12:OH, has not previously been reported as a sex phermone component. The *E*9–12:Ac compound is a pheromone component for another olethreutid species, the European pine shoot moth, *Rhyacionia buoliana* (Smith et al., 1974), and a noctuid species, the red bollworm, *Diparopsis castanea* (Nesbitt et al., 1973), whereas the 12:Ac compound is a pheromone component for

some tortricid species, the redbanded leafroller moth, *Argyrotaenia velutinana* (Roelofs et al., 1975), and the fruit tree leafroller moth, *Archips argyrospilus* (Roelofs et al., 1974).

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EVIDENCE FOR THE CONTROLLED RELEASE OF A CRUSTACEAN SEX PHEROMONE[1]

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Abstract—Evidence for the controlled release of a crustacean sex pheromone: mature male *Portunus sanguinolentus* (Herbst) did not give the characteristic display described by Ryan (1966) to water from a container with a carried or artificially restrained premolt female. However, the sex pheromone was still present in the urine of the restrained female, suggesting that she inhibits the emission of the sex pheromone by regulating her urine flow.

Key Words—Sex pheromone, crustacean, *Portunus sanguinolentus*.

INTRODUCTION

In many brachyuran crustaceans, the male recognizes the premolt female, and grasps and carries her beneath his abdomen until ecdysis. Recognition of the premolt female involves modifications in the female's behavior (Chidester, 1911; Broekhuysen, 1937) and a release of a chemical signal, the female sex pheromone (Hay, 1905; Verrill, 1908; Burkenroad, 1947; Carlisle and Knowles, 1959; Knudsen, 1964; Snow and Neilsen, 1966; Edwards, 1966; Ryan, 1966; Kittredge, Terry, and Takahashi, 1971; Eales, 1974). Ecdysis is then followed by copulation.

When mature *Portunus sanguinolentus* (Herbst) males are exposed to the female sex pheromone, a characteristic behavior display is elicited. The male begins a searching behavior which climaxes in a display during which

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he assumes a specific posture: chelipeds extended, revealing the ox-blood coloration along the chelae, he rises on the tips of the dactyls to maximum height above the substrate, raises the swimming legs, and moves about (Ryan, 1966).

Ryan (1966) found that premolt female crabs (in the last immature or first or second mature instar) in stage D_3 (intermolt stages according to Passano, 1960) began to release the sex pheromone 7–8 days prior to ecdysis. Release of the sex pheromone was associated with the release of urine (Ryan, 1966). When the nephropore was blocked with paraffin, test males would not display; but when the paraffin was removed, the display could once again be obtained.

Unpaired mature male individuals of *P. sanguinolentus* do not display when a premolt female is carried by another male. The lack of a display in unpaired males suggests either a visual or chemical inhibition (masking) of the display or a control of the pheromone's release. The results described in this paper indicate the latter as a possible explanation of this observation.

METHODS AND MATERIALS

Individual specimens of *Portunus sanguinolentus* were collected at various sites around the island of Oahu, Hawaii. Crabs were maintained for up to three months in a recirculating seawater system. The stages of maturity and molting cycle were determined using the criteria established by Passano (1960) and Ryan (1965). A premolt female crab was recognized by a combination of the following criteria: cessation of eating, bluish pigmented abdomen with a white border, or being carried by a male.

Urine was collected from crabs in drawn capillaries (Kimax capillary tubes 0.8–1.2 mm ID $\times$ 100 mm). The opercular flap over the nephropore was lifted with a bent stainless-steel dissecting needle. The outflow of urine from the nephropore was drawn into the capillary by capillary action. Urine samples were blown into 1/4-dram vials and quickly frozen on dry ice. The samples were tested by resuspending them in seawater and pipetting the solutions into a holding tank with a mature male crab. Test males were then observed for one minute for display behavior.

To determine the presence of the pheromone released by the premolt female crab into the water, the female crab was placed into reservoir R (Figure 1). At this time, clamp C (Figure 1) was closed and water from R flowed out of the apparatus via the drain pipe into the outlet (O). This water did not contaminate the water present in trough TT (Figure 1).

The female crab in R was either free (unrestrained), carried by a male, or held by the long anterolateral spines by means of beaker tongs which had

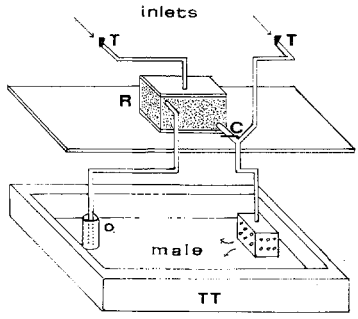

FIG. 1. Apparatus used for testing display response of male *P. sanguinolentus* to substances produced by females being held by males or other physical restraint. A pair (male holding a premolt female), a premolt female held with beaker tongs, or premolt female was placed in reservoir R. Assay of the water flowing through any of the three situations was made by observing the response of the test male. C = clamp, O = outlet, R = reservoir, T = tap, TT = test trough.

Styrofoam blocks epoxyed to its tips. In the latter two cases, the crabs were considered to be restrained. A mature male crab, designated the test male, was placed into the trough TT (Figure 1). After a 1-hr adaptation period, water from R was allowed to flow into TT by opening clamp C for 15 min. During this period, the male was observed for a display. Then C was clamped shut again and the female was either restrained or released. After 1 hr, C was again opened, and the test male was given another 15-min trial period.

The presence of a female pheromone-masking substance was tested 2 hr after a 70-μl urine sample had been collected from the female. First the water from reservoir R containing the unrestrained female was tested; then the female was artificially held (beaker tongs) while the water was again tested. After 15 min the urine sample was pipetted into reservoir R with the restrained female, and the test male was again observed. The display behavior of mature male crabs was utilized as the bioassay for establishing the presence of the sex pheromone.

RESULTS

Exposed to a total of 33 urine samples from premolt and 33 from intermolt females, none of the males exhibited a display to urine from intermolt females, but all responded with a display to urine from premolt females. Three samples of immature premolt female urine and two samples of premolt male urine failed to elicit a display.

Only one out of 21 test males displayed when water flowed past a restrained female, and 20 of the 21 males displayed when water flowed past the unrestrained female (Table 1). These results show that visual cues or a male "masking factor" were not required to explain the absence of the display.

The artificially restrained premolt female apparently does not release a masking substance since the test male displayed only when the female was unrestrained or 30 sec after the previously collected urine sample was released into the reservoir with the restrained female.

Urine samples collected from premolt female crabs after they were restrained for 1, 2, or 3 hr all initiated display behavior of the male when tested, suggesting that the restrained female withholds her urine rather than stopping pheromone production.

DISCUSSION

The problem of pair formation is a very real one, even in rather dense populations. Certain specific signals are required to find a mate that is receptive. Since individuals of *P. sanquinolentus* were collected at depths

TABLE 1. BIOASSAY FOR PHEROMONE PRODUCED BY FEMALE *P. sanguinolentus* AS INDICATED BY NUMBER OF DISPLAYS OF TEST MALES TO WATER FROM RESERVOIR R CONTAINING EITHER RESTRAINED (NATURAL—HELD BY A MALE; ARTIFICIAL—HELD WITH BEAKER TONGS) OR UNRESTRAINED FEMALE

Female	Number of males tested	Number of males responding to test water from: Restrained females	Number of males responding to test water from: Unrestrained females
1	3	Natural—0	2
2	5	Natural—0	5
3	4	Natural—0	4
4	3	Natural—0	3
5	3	Artificial—1	3
6	3	Artificial—0	3
Total 6	21	1	20

ranging from 5 cm on sand bars down to a depth of 229 m, the importance of visual cues seems minimal. At all locations, premolt females were found, suggesting that breeding does occur at all of these depths. While visual signals may be of importance in well-lit, shallow water, their value becomes questionable at depths of 107 m and more.

Ryan (1966) was the first to present evidence for the presence of a chemical signal, a sex pheromone, released by the premolt female *P. sanguinolentus*. That earlier investigators were unable to observe any chemical sex recognition in crabs can most probably be explained by their use of soft females. Broekhuysen (1937) suggested that male *Carcinides maenas L.* carry females for a few days prior to molting because the females are sluggish at this stage. In *P. sanguinolentus*, my behavioral experiments revealed, however, that males do not show their characteristic sex display when water flowing past a recently molted female reaches them, but that they always do so with water flowing past a premolt female. Tactile stimuli can also be of importance, as was observed when a soft female was placed in a container with a male. Copulation would take place immediately after bodily contact between the crabs was established. Normally, however, sex recognition occurred prior to molt, and this important point was missed by the earlier investigators.

The selection of an easily recognizable and specific response to the sex pheromone was very important for the bioassay. This had to be a response which could not be confused with responses to other stimuli. The movements of the antennulae could easily be confused with normal movements elicited by other stimuli such as food substances. Random movement of the male was not affected by premolt females, but the display ("dance") was triggered only by premolt female crabs. Schöne (1961) states that sexual and agonistic (fighting) activities appear to be closely related in brachyuran crabs. The display posture of a male *Portunus sanguinolentus* indeed seems to be basically similar to the fighting stance (Skolnick, 1965), but it varies in position of appendages and degree of activity of the male.

The behavioral display was never observed except when mature male *P. sanguinolentus* were exposed to (1) premolt female crabs, (2) water which had been flowing past premolt female crabs, or (3) urine from premolt female crabs; and it was consequently considered specific and used as the bioassay.

When a pair (male carrying a female) is formed, the female is able to inhibit the output of the sex pheromone apparently by controlling the release of her urine, but the possibility of her releasing a masking factor cannot a priori be completely discounted since such factors have been found to occur in insects. A male masking factor was shown to occur in *Tenebiro molitor* L. (mealworm beetle); one of its male pheromones acts as an antiaphrodisiac, inhibiting the response of other males to the female scents (Happ, 1969).

Female *Dendroctonus pseudotsugae* Hopk. (Douglas fir beetles) produce a separate factor which masks the female aggregation pheromone (Rudinsky, 1969). The observation that in *P. sanguinolentus* the test male responds after the urine sample of restrained females is introduced could possibly be explained by the amount of masking factor present being insufficient. In these experiments factors like vision and the effect of male masking substances were eliminated because a test male did not display when exposed to water in which restrained females were kept. Controlled urine flow is suggested when the sex pheromone is found to be in the female's urine after several hours of restraint.

Very little is known about the control of urine flow in crustaceans. Maluf (1941) considers the flow of urine in crayfish to be a passive process controlled, if at all, by contraction of the body musculature. Muscular contraction results in an increase of internal hydrostatic pressure above that of the surrounding medium causing the opercular flap to open. When a female *P. sanguinolentus* is carried, there is a reduction in her movements and thus perhaps in the contraction of her body musculature. There are also changes in the amount of urine produced; a maximum (of urine) can be obtained from intermolt crabs whereas no urine can be collected 12 hr prior to ecdysis. Ono and Kamemoto (1969) also found a reduced urine production in premolt crayfish.

The reduced motor activity and urine production of carried premolt females tends to eliminate any unnecessary chemical cues to male crabs in the vicinity. However, until the female is carried, there is an intermittent release of the sex pheromone (in the urine) which increases the chance of pair formation.

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RESISTANCE OF WESTERN WHITE PINE TO FEEDING AND OVIPOSITION BY *Pissodes strobi* PECK[1] in WESTERN CANADA[2]

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Abstract—Forced and choice feeding and oviposition experiments were conducted by caging overwintered *Pissodes strobi* on lateral branches or leaders of sympatric Engelmann spruce and/or western white pine trees in Manning Provincial Park, British Columbia, or on transplanted, potted trees in Burnaby, British Columbia. Whereas female *P. strobi* preferred to feed on 1-year-old Engelmann spruce lateral branches in both the forced- and choice-feeding experiments, male weevils failed to discriminate between this host and western white pine. Forced-feeding experiments that utilized 1-year-old leaders demonstrated that western white pine is an acceptable host for *P. strobi* feeding, but that the native host, Engelmann spruce, is preferred under choice conditions. In all experiments, female weevils oviposited only on Engelmann spruce. These results indicate that the releasing stimulus for feeding is present in the bark of both conifer species and suggests that a separate releasing stimulus for oviposition is absent from western white pine.

Key words—*Pissodes strobi*, weevil, host selection, phytophagy, oviposition, chemoreception.

INTRODUCTION

Western white pine, *Pinus monticola* Dougl., is less susceptible to attack by the white pine weevil, *Pissodes strobi* Peck, than is eastern white pine, *Pinus*

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strobus L. (Wright and Gabriel, 1959). Although *P. monticola* is attacked in plantations in eastern North America (Plank and Gerhold, 1965), this conifer is apparently not susceptible to *P. strobi* attack across its native range in western North America. In British Columbia, the preferred hosts of the white pine weevil are Engelmann spruce, *Picea engelmannii* Parry, and Sitka spruce, *Picea sitchensis* (Bong.) Carr (VanderSar et al., 1977). Both spruce species, however, are sympatric with western white pine (Fowells, 1965).

Plank and Gerhold (1965) examined the feeding behavior of *P. strobi* that were offered a choice of cut leaders of eastern and western white pines. No significant difference was observed in the numbers of feeding punctures produced on each host species, even though eastern white pine was more susceptible to weeviling under natural field conditions. In feeding-preference studies on living trees, *P. strobi* produced significantly more feeding punctures on western white pine leaders than on those of eastern white pine (Soles et al., 1970). In addition, the oviposition behavior of overwintered weevils did not appear to be adversely affected on western white pine leaders as might be anticipated on the basis of field observations. Adult mortality during the test interval, and the numbers of *P. strobi* progeny that emerged from leaders of both eastern and western white pines did not differ significantly (Soles et al., 1970).

These results suggest that western white pine is an acceptable host for *P. strobi.* However, since *P. strobi* did not become established on *P. monticola* following its hypothesized westward dispersal across North America (VanderSar et al., 1977), it is of interest to compare the host selection behavior of a western *P. strobi* population on its preferred host and sympatric western white pine.

My objectives were to examine the feeding and oviposition behavior of overwintered *P. strobi* adults caged on living lateral branches or leaders of Engelmann spruce and western white pine trees under both forced and choice experimental conditions.

METHODS AND MATERIALS

Overwintered *P. strobi* were collected on May 6, 1977, from Engelmann spruce regeneration in Kootenay National Park, British Columbia. The majority of weevils were found feeding or copulating on the upper living bole of brood hosts that retained the dead 1976 leader. In the laboratory, the weevils were maintained at 2–4°C on fresh lateral branch sections of *P. engelmannii.*

Forced feeding and forced oviposition experiments during May and

June, 1977, utilized living trees in Manning Provincial Park, 190 km east of Vancouver, British Columbia. Within the Park, naturally regenerating Engelmann spruce and western white pine are sympatric at elevations above 1000 m. Unlike previous host selection experiments that relied on excised host material (VanderSar and Borden, 1977a), the use of living trees permitted more accurate assessment of host acceptance and suitability for *P. strobi* maintenance and reproduction. The experimental trees were 1.8–3.6 m high and grew in close proximity on a recently burned, south-facing slope.

The forced-feeding experiment comprised 9 replicates for each sex on each host species. In each replicate, a single weevil of either sex was caged in a 10-cm-long nylon-mesh sleeve on a living 1-year-old lateral branch of either an Engelmann spruce or western white pine tree. After a 10-day test interval (May 13–23), each caged branch was cut, and weevil mortality ascertained. In the laboratory, the numbers of feeding punctures produced by each weevil on each host lateral branch were counted. The data were examined using standard *t* tests.

In a 15-replicate, combined forced-feeding and oviposition experiment, a single male and female *P. strobi* were caged in a nylon-mesh bag securely placed over the living 1-year-old leader of either an Engelmann spruce or western white pine tree. The inclusion of males ensured that female *P. strobi* were mated. After a 47-day test interval (May 13–June 29), each caged host leader was cut and weevil survival on each host species noted. The number of feeding punctures produced by each weevil pair and the number of oviposition punctures produced by each female were counted. Each leader was then placed cut-end in water inside a screened cage to permit further development and natural emergence of weevil progeny. The feeding puncture data were analyzed using standard *t* tests.

Choice-feeding and oviposition experiments utilized live trees that were transplanted into large fiber pots and moved to Simon Fraser University. Ten 1.5 to 2.4-m-high Engelmann spruce, grown from certified seed, were transplanted from a commercial nursery on April 9, 1977. All 10 trees flushed readily. As western white pine was not commercially available (owing to susceptibility to *Cronartium ribicola* J.C. Fisch, ex. Rab.), 15 healthy trees 1.5–2.7 m high were transplanted from Manning Provincial Park on April 29, 1977. Experiments using transplanted trees were delayed until the buds of 10 of these had flushed. In two-choice experiments, an Engelmann spruce and western white pine of approximately equal height were paired in each of 10 replicates.

In the choice-feeding experiment, a single weevil of either sex was caged inside a 30-cm nylon-mesh sleeve that contained a living 1-year-old lateral branch of both Engelmann spruce and western white pine, one at each end of the bridging sleeve. Each weevil was released in the center of

the sleeve, between the two host lateral branches. After an 8-day test interval (May 30–June 6), both lateral branches of each host pair were cut and the sleeve examined for weevil mortality. The numbers of feeding punctures produced in the bark of each host lateral branch were counted and the results evaluated using *t* tests.

In the choice feeding and oviposition experiment, one male and one female *P. strobi* were released in the center of an inverted, U-shaped nylon-mesh sleeve fitted over the living 1-year-old leaders of Engelmann spruce and western white pine. After a 43-day test interval (June 2–July 14), both host leaders in each of 10 replicates were cut and removed to the laboratory. The number of both feeding and oviposition punctures produced in the bark of each leader were counted, and the feeding puncture data analyzed using *t* tests. Each leader was placed cut-end in water inside a screened cage to permit further development and natural emergence of weevil progeny.

RESULTS

In the forced-feeding experiment that utilized 1-year-old host lateral branches, female *P. strobi* produced significantly more feeding punctures on Engelmann spruce than on western white pine, whereas males produced approximately equal numbers of feeding cavities on each of the two hosts (Table 1). In addition, a single female weevil produced two oviposition punctures on an Engelmann spruce lateral branch. Although oviposition in the bark of host lateral branches was unexpected (VanderSar and Borden, 1977b), examination of these punctures confirmed the presence of eggs. In the experiment that offered *P. strobi* a choice of 1-year-old host laterals neither sex discriminated between the two host species (Table 1). In two replicates, however, female *P. strobi* again produced three and seven oviposition punctures, respectively, on Engelmann spruce lateral branches. Each of these punctures contained one or more eggs.

When not offered a choice of hosts, *P. strobi* pairs produced comparable numbers of feeding punctures in the bark of both Engelmann spruce and western white pine leaders (Table 1). When presented a choice, however, the weevils produced significantly more feeding punctures in the bark of Engelmann spruce than western white pine leaders.

In both the forced and choice oviposition experiments on living 1-year-old leaders, female *P. strobi* produced oviposition punctures in the bark of only Engelmann spruce (Table 1). No oviposition punctures were detected in the bark of western white pine leaders, nor did new-generation adults emerge from these leaders. In the forced oviposition experiment, weevil survival over the 47-day test period was identical on the two host species.

TABLE 1. FEEDING AND OVIPOSITION PUNCTURES PRODUCED BY OVERWINTERED *Pissodes strobi* IN FORCED AND CHOICE EXPERIMENTS UTILIZING ENGELMANN SPRUCE AND WESTERN WHITE PINE HOSTS

Experiment	No. replicates	Duration days	Sex	Mean no. feeding punctures		Mean no. oviposition punctures	
				P. monticola	*P. engelmannii*[a]	*P. monticola*	*P. engelmannii*[b]
Forced feeding on 1-yr-old laterals	9	10	♀	2.8	10.1 *	0.0	0.2 (1)
			♂	3.3	3.1 n.s.	—	—
Choice feeding on 1-yr-old laterals	10	8	♀	3.7	7.4 n.s.	0.0	1.0 (2)
			♂	5.2	5.3 n.s.	—	—
Forced feeding and oviposition on 1-yr-old leaders	15	47	♀♂	48.8	54.2 n.s.	0.0	10.1 (6)
Choice feeding and oviposition on 1-yr-old leaders	10	43	♀♂	10.8	31.7 *	0.0	8.1 (5)

[a] * = significant difference between paired means (t test, $P < 0.025$). n.s. = difference between means not significant.
[b] Numbers in parentheses denote number of trees in which oviposition punctures were excavated.

From August 3 to 18, a total of 105 *P. strobi* (53♀♀ and 52♂♂) emerged from the original egg population deposited in 151 oviposition punctures on six attacked Engelmann spruce leaders. During August 19–September 8, a total of 30 *P. strobi* (15♀♀ and 15♂♂) emerged from three of five Engelmann spruce leaders that bore oviposition punctures following termination of the choice experiment. Weevil broods did not become established in the remaining two Engelmann spruce leaders.

DISCUSSION

In both the forced- and choice-feeding experiments that utilized 1-year-old lateral branches, male *P. strobi* clearly failed to discriminate between the sympatric conifer species, and accepted both Engelmann spruce and western white pine as hosts suitable for feeding. Female weevils, however, demonstrated a feeding preference for Engelmann spruce in both experiments, although this margin of preference was not significant in the choice experiment. Thus western white pine is apparently partially resistant to feeding by *P. strobi.* The divergent results in feeding behavior between the sexes are consistent with previous observations that suggested female *P. strobi* are more sensitive than males to both feeding deterrents and stimulants present in nonhost and host materials (VanderSar and Borden, 1977a). In the forced-feeding and oviposition experiment that utilized 1-year-old leaders, the results clearly demonstrate that both conifer species are acceptable hosts for maintenance feeding, even though a preference is expressed by *P. strobi* for its natural host, Engelmann spruce, under choice conditions (Table 1).

The most interesting result of this study was the failure of female *P. strobi* to oviposit in the bark of western white pine leaders. Weevils oviposited in Engelmann spruce leaders as well as 1-year-old lateral branches, even though the latter represent a marginal microenvironment for oviposition (VanderSar and Borden, 1977b). Apparently, olfactory or gustatory cues that promote oviposition in Engelmann spruce bark are absent in the bark of western white pine. The results also suggest that chemical compounds that act as feeding stimulants do not release oviposition behavior in *P. strobi.* Alternatively, feeding and oviposition behaviors could be a function of the concentration of appropriate chemical stimulants.

The acceptability of western white pine for oviposition by *P. strobi* in eastern North America (Soles et al., 1970) does not agree with its rejection by a western weevil population reared from Engelmann spruce (Table 1). This discrepancy may reflect conditioning of the western weevil populations to members of the genus *Picea* as hosts over many generations. To disperse west of the natural range of eastern white pine, *P. strobi* likely adapted its

host selection behavior to white spruce, *Picea glauca* (Moench) Voss, which is closely related to its natural hosts, Sitka and Engelmann spruce, in western Canada (Roche, 1969). Although a feeding preference for the hypothesized ancestral host, *P. strobus*, is retained (VanderSar et al., 1977), the weevil apparently has not adapted to western white pine, even though the vigorous leaders of this conifer represent a potential breeding site exploited by no other insect species.

In conclusion, the results of this study point out both the variability and flexibility that is inherent in the host selection behavior of *P. strobi* populations. Cognizance of this fact should guide programs that are designed to breed resistant tree varieties.

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BEHAVIORAL RESPONSES OF THE FEMALE EASTERN SPRUCE BUDWORM *Choristoneura fumiferana* (LEPIDOPTERA, TORTRICIDAE) TO THE SEX PHEROMONE OF HER OWN SPECIES

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Abstract—Female eastern spruce budworm moths respond to the synthetic sex pheromone of their own species (a mixture of *cis*- and *trans*-11-tetradecenal) by walking, antennal grooming, flexation of the body, extension of the ovipositors, and oviposition. The sex pheromone is perceived by receptors on the antennae. Electroantennogram responses from the female are approximately two-thirds the amplitude of those obtained from males.

Key words—Spruce budworm, *Choristoneura fumiferana*, pheromone, female behavior, electroantennogram, dispersal.

INTRODUCTION

The role of sex pheromones in premating behavior is well documented in over a hundred species of Lepidoptera (Tamaki, 1977). In most of these cases the males are attracted to the females, the pheromone emitters. A few reports are available indicating that in some species females approach from a distance to males, the latter then being the emitting sex (Röller et al., 1968; Dahm et al., 1971). However, a number of other reports indicate that male-produced sex pheromones, operating at close range, may act as "aphrodisiacs" (Shorey et al., 1968; Grant 1974). Thus, it is accepted generally that sex pheromones are perceived by the opposite sex and result in various behaviors which precede mating.

Mitchell et al. (1972) reported female *Trichoplusia ni* were attracted to

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traps baited with synthetic sex pheromone and Birch (1977) reported similar responses to traps baited with virgin females of *T. ni.* Electrophysiological evidence for moths perceiving their own pheromone has been reported for males of *Pseudaletia unipuncta* (Grant, 1971) and for females of *Trichoplusia ni* (Grant, 1970). With the exception of the trapping studies cited above, studies of the behavioral responses to the pheromone by the emitting sex have not been explored in the Lepidoptera.

METHODS AND MATERIALS

Choristoneura fumiferana were reared on synthetic diet (McMorran, 1965) as described by Grisdale (1970). The sexes were isolated as pupae and female moths were collected every day at midnight and housed in glass jars until experimentation. All rearings were carried out at 21°±1°C and 55% relative humidity. Pupae and adults were entrained to a light–dark cycle of 17:7 hr with the scotophase from 14:00 to 21:00 hr. All bioassays were carried out with the 3-day-old virgin females at 2.5–4.5 hr before the onset of scotophase, a period during which the moths were actively calling. The synthetic sex pheromone, a 97:3 mixture of *trans-* and *cis*-11-tetradecenal (Sanders and Weatherstone, 1976), was obtained from Chemical Samples Co., Columbus, Ohio. Purity of the pheromone was determined by GLC analysis to be 98%.

Moths were removed from holding cages and placed individually in 250-ml Erlenmeyer flasks about 2 hr prior to testing. To prevent contamination of the room and the other flasks by pheromone, assays were carried out one at a time with one moth per flask in the fume hood. The pheromone was dissolved in pentane. Pheromone cartridges consisted of 2-cm×2-cm squares of pheromone impregnated Whatman No. 1 filter papers from which the pentane had been evaporated. These were folded and placed in the barrels of glass cartridges. To analyze the behavior of a moth, charcoal-filtered compressed air at a flow rate of 1.5 liter/min was passed through a cartridge held about 2.5 cm from the insect and directed toward the antennae. The moth's behavior was observed during an initial 20-sec exposure to the pheromone-laden airstream, a 20-sec gap during which the airstream was removed, another 20-sec exposure to pheromone and a final 20-sec period during which the airstream was again removed. Fifteen insects were used at each pheromone concentration. The experiment was replicated three times. Cartridges were used for seven or eight insects and then replaced. Cartridges containing filter paper which had been treated with pentane were used as controls. The following five categories of reactions were scored on every insect and recorded separately: walking, flexing of the body without the

extrusion of the ovipositor, extrusion of the ovipositor with or without body flection, ovipositional behavior, and antennal grooming. Statistical analysis was done using the Kendall rank correlation coefficient method.

RESULTS

A general description of different types of female responses to pheromone and their degree of occurrence follows. As seen in Figure 1, it is evident that the walking response resulted more commonly than any other type of behavior. On exposure to pheromone a responsive insect, which was otherwise quiescent, started walking, immediately or within a few seconds following exposure, covering several centimeters before stopping. Walking often resumed once the pheromone source was removed at which time the insects exhibited the other types of behavioral reactions. In controls, 8 of 45 moths showed a walking response.

Downward flection of the body and protrusion of ovipositors were the next most frequently observed responses. Some insects reacted by instantaneously stretching their abdomen and protruding their ovipositors once or twice, while others showed extreme bending of the abdomen but without ovipositor protrusion. However, the latter insects eventually extended the ovipositors, apparently with considerable force, and in that process a drop of liquid was extruded. Such drops were noticed on several occasions. In controls only 2 moths exhibited body flection and none protruded the ovipositor.

The above behavior was frequently observed among females that were calling prior to pheromone application. Such calling females reacted by abruptly retracting their ovipositors, but within seconds typically extruded them further outside the body than they were at the time of calling. Some exhibited a characteristic behavior typified by slight projection of the last abdominal segments, movement of the tip of the abdomen more or less in a rotating fashion, and pulsated extrusion of the ovipositors in and out a few times. Other calling females, on exposure to pheromone, extruded their ovipositors further outside without the initial retraction. This characteristic behavior was also noticed among females that were not previously calling, but less frequently than in calling females. In controls no such response was noticeable.

When the pheromone was applied to females while they were ovipositing, some responded by suddenly bending their abdomen and increasing abdominal pulsations. On two occasions it was noted that they forced out, within seconds of exposure to the pheromone, a mass of eggs which otherwise were being laid singly. No such response was seen in controls. Statistical

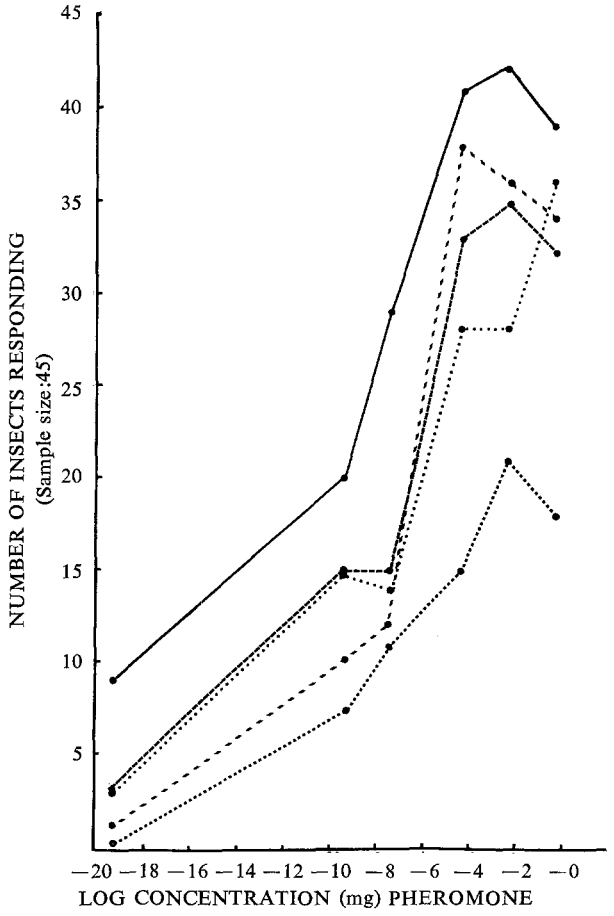

FIG. 1. Response of 3-day-old female budworm moths to different concentrations of synthetic pheromone. Categories of behavioral responses: antennal grooming (···); flection of the body without protrusion of ovipositors (----); protrusion of ovipositors with or without body flexion (- - -); typical ovi-positional behavior (- - -); walking response (——). Actual concentration of pheromone used: 5×10^{-20}; 5×10^{-10}; 5×10^{-8}; 5×10^{-5}; 5×10^{-3}; 5×10^{-1} mg. Data are the combination of the results from three replicate experiments containing 15 insects each. It should be noted that a concentration of 5×10^{-20} mg represents a virtually blank cartridge.

analysis of the behavioral responses indicated a positive correlation between the number of insects responding in each category and the concentration of pheromone delivered to them.

Antennal grooming was especially noticeable at high pheromone concentrations. In controls only 2 moths of 45 showed this type of behavior.

Preliminary electroantennogram (EAG) studies based on 8 females indicated that a 0.8 mV response was obtained to 10 μg of pheromone and that there was little increase in EAG amplitude when the concentration was increased to 100 μg. The amplitude of the EAG response of the female was approximately 2/3 that of similarly tested males. Further EAG studies are presently being carried out and will be reported elsewhere.

DISCUSSION

Although it has been established that the sex pheromone released by female eastern spruce budworms is perceived by males (Albert et al., 1970, 1974; Sanders, 1971), resulting in a sequence of sexual behaviors leading to mating (Sanders, 1971), it was not known whether or not a female could perceive or respond to the sex pheromone of her own species. Results of this behavioral study supplemented with results from preliminary EAG tests demonstrate that the females are indeed capable of perceiving the sex pheromone of their own species and that they respond differently to it than do males.

The intensity of the female behavioral response is a function of concentration, as illustrated in Figure 1. All five categories of response which were measured increased with increasing concentration. However, it appears that the response level is independent of pheromone concentration above 5×10^{-5} mg, which may indicate an adaptation of the sensory system as is reported in the males of this species (Seabrook, 1977) and other lepidopterans (Bartell and Shorey, 1969; Farkas et al., 1975). Also evident in the response curves is a rapid increase in female responsiveness in the concentration range 5×10^{-8} mg to 5×10^{-5} mg.

The females appear to have different threshold levels above which the various behavioral patterns become evident. These differing thresholds correspond to different pheromone concentrations. Pheromone concentration may be related to the density of field populations. It is suspected that above certain population densities, and thus certain pheromone concentrations, activities like premature oviposition and dispersal may be inevitable. A quantification of the amount of pheromone in the forest at different population densities may help to clarify these possibilities.

Analyzing the different kinds of behaviors that were recorded, one

might be tempted to label some of the responses as a calling response. Even though the gradual projection of abdominal segments, plus body flection and protrusion of the ovipositor are common in calling (Sanders, 1972) and in oviposition, the pulsing in and out movement of the ovipositor and movement of the abdominal tip in a sideways fashion are typical of ovipositing females and are not observed in calling females. Moreover, the females that were already calling also showed the typical ovipositional behaviors when exposed to the external pheromone source. Therefore, all the responses other than walking and antennal grooming may be considered as pheromone-induced ovipositional behaviors.

Perception of its own pheromone by the female spruce budworm may have important implications, especially as the pheromone appears to act as an oviposition stimulant. Greenbank (1973) reported that spruce budworm females do not make dispersal flights until they lay a portion of their egg complement and that dispersal occurs mainly from densely populated areas. In high population densities, one would expect a high pheromone concentration in the air. Female moths may be capable of using pheromone concentration to assess population density and thus be stimulated by the pheromone to lay a portion of their eggs and disperse.

Mitchell et al. (1972) have noticed that female *Trichoplusia ni* are captured in traps baited with synthetic sex pheromone and suggested that this behavior may be an artifact due to the impurities found in the pheromone sample. However, in one of our preliminary experiments, ovipositional behaviors were observed when air was passed from a flask that had housed only females for a few days, suggesting that the results we observed were not due to artifacts.

Birch (1977) reported attraction of females of *Trichoplusia ni* to virgin females and suggested that the pheromone may bring about aggregation, thus increasing local chances of mating. However, in the light of our experiments with eastern spruce budworm, it is suggested that the sex pheromone of this species may act as an ovipositional stimulant and consequently as a possible dispersal trigger. Investigations with virgin and mated females are underway to confirm these possibilities and will be reported elsewhere.

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GEOMETRICAL AND POSITIONAL ISOMERIZATION OF ALKENYL ACETATES PRODUCED BY HYDROGENATION OF ALKYNYL ACETATES OVER PALLADIUM METAL CATALYSTS[1]

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Abstract—Alkenyl acetates containing both geometrical isomers can be produced by a single controlled catalytic hydrogenation of the corresponding alkynyl acetate. The hydrogenation is capable of yielding formulations containing up to 60% trans isomer; the reduction is attended by positional isomerization in both geometrical isomers.

Key Words—Pheromone, trans isomer, cis isomer, alkenyl acetate, alkynyl acetate, hydrogenation, isomerization.

INTRODUCTION

Alkenol acetates for insect pheromone research are commonly synthesized by reduction of alkynol acetates (Warthen, 1968; Jacobson, 1972; Henrick, 1977). Hydrogenation of alkynes to alkenes using modified palladium catalysts on various supports have been reported by many workers (Augustine, 1965). The variation in the ratios of the geometrical isomers in the products is the most striking feature of these reports. It has been proposed that the amount of catalyst, the catalytic support, the rate of stirring, and additives such as quinoline and triethylamine have an effect on the ratio of the trans to cis isomers in the product (Rylander, 1967). We have determined that by far the overriding factor effecting isomerization is the amount of time the cis isomer remains over the catalyst. Under specific conditions, this type of

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hydrogenation is capable of yielding up to 60% trans isomer, and the isomerization is attended by bond migration. This report is intended to alert those using this method for the synthesis of cis olefins to the conditions that can produced geometrical and positional isomers.

METHODS AND MATERIALS

The acetylenic acetates used as starting materials were synthesized by a general route similar to that reported by Warthen (1968). A microhydrogenator, operated at atmospheric pressure, was used to reduce the alkynes to the alkenes and for their subsequent isomerization from the cis isomer to the trans isomer. The system contained a total gas volume of 20 ml; 5 ml of which was calibrated and could be read to 0.01 ml. A sidearm in the flask was closed with a rubber septum to facilitate sampling without interrupting the reduction. Solvents used were not prehydrogenated.

The products of reduction were quantitatively analyzed by gas chromatography using the internal standard method. A Hewlett-Packard gas chromatograph, model 5710A, with a flame ionization detector, equipped with 12-ft $\times \frac{1}{8}$-in. OD stainless-steel column packed with 15% SP 2340 on 100–120 mesh Chromosorb P AW–DMCS[2], helium flow 10 ml/min; hydrogen flow 20 ml/min; injector and detector temperatures were maintained at 250°C. The oven temperature was 225°C. The products of hydrogenation eluted from this column in the following order: alkyl acetate, *trans*-alkenyl acetate, *cis*-alkenyl acetate, and alkynyl acetate.

To the hydrogenation flask containing 4 ml of ethyl alcohol was added: 22.4 mg of 8-dodecynyl acetate, 2 drops of 4% quinoline in ethyl alcohol, 12 mg of tetradecyl acetate as an internal standard, and 1.5 mg of 5% palladium catalyst on $BaSO_4$.[3] The apparatus was assembled and flushed with hydrogen. A Teflon-coated magnetic stirrer to mix the reactants was started, and the progress of the reduction and subsequent isomerization was monitored every few minutes. The isomerization of *cis*-8-dodecenyl acetate and *trans*-8-dodecenyl acetate followed closely the foregoing procedure. The results are reported in Tables 1, 2, and 3, respectively.

In another experiment, a number of 100-mg samples of 6-tetradecynyl acetate in 10 ml of ethyl alcohol, containing 2 drops of 4% quinoline and 5 mg of 5% palladium on $BaSO_4$, were reduced to determine if bond migration had occurred. One reaction was terminated before reduction was complete; the others were allowed to progress until various amounts of the trans isomer had been produced. The cis and trans isomers were resolved by

[2] Supelco, Inc., Supelco Park, Bellefonte, Pennsylvania.
[3] Engelhard Industries Inc., 113 Astar Street, Newark, New Jersey.

TABLE 1. REDUCTION OF 8-DODECYNYL ACETATE[a]

Time (min)	Distribution of product (%)				
	Alkyne	Cis	Trans	Saturated	Total
15	46.0	31.5	2.4	0.2	80.1
25	0	72.4	9.5	1.7	83.6
35	0	59.0	21.6	6.4	87.0
45	0	42.6	32.4	8.2	83.2
55	0	32.0	33.0	9.0	74.0
65	0	28.6	38.1	10.6	77.3
75	0	26.5	42.6	12.3	81.3
90	0	20.4	42.0	14.3	76.7
105	0	16.8	44.0	16.1	76.9

[a] Hydrogenation performed at atmospheric pressure using 22.4 mg of 8-dodenyl acetate; 2 drops of 4% quinoline in ethyl alcohol; 1.5 mg of palladium catalyst on $BaSO^b$; internal standard 12 mg of tetradecyl acetate.

argentation liquid chromatography at atmospheric pressure (Houx et al., 1974) and checked for purity by gas chromatography. The double-bond position was located by ozonolysis in ethyl acetate, using a method similar to that reported by Beroza and Bierl (1967), and ozonolysis products were analyzed by gas chromatography. Standard acetoxyaldehydes and *n*-aldehydes were prepared by the oxidations of their corresponding alcohols with pyridinium chlorochromate (Corey and Suggs, 1975). A 3-ft $\times \frac{1}{8}$-in. OD stainless-steel column packed with 5% SE 30 on AW–DMCS Chrom W 80-

TABLE 2. ISOMERIZATION AND REDUCTION OF *cis*-8-DODECENYL ACETATE

Time (min)	Distribution of product (%)			
	Cis	Trans	Saturated	Total
5	81.0	0.3	0	81.4
15	76.0	5.3	2.6	83.9
25	59.1	12.4	2.0	73.5
35	40.0	27.6	8.3	75.9
60	22.6	34.7	13.5	70.8
75	18.6	33.8	15.0	67.8
90	16.0	41.4	19.1	76.5
105	20.0	43.5	21.8	85.3

TABLE 3. ISOMERIZATION AND REDUCTION OF *trans*-8-DODECENYL ACETATE

Time (min)	Distribution of product (%)			Total
	Cis	Trans	Saturated	
15	0	97.0	5.8	102.8
30	5.2	70.0	17.3	92.5
45	5.2	64.0	22.6	91.8
60	5.0	57.0	27.6	89.6
75	5.1	57.8	33.5	86.4
90	4.4	51.4	32.2	88.0
105	4.4	50.5	35.1	90.0
120	4.4	50.0	34.7	89.1
135	4.4	48.2	35.5	88.1

100 mesh was used to determine the percent distribution of the *n*-aldehydes; a 6-ft $\times \frac{1}{8}$-in. OD stainless-steel column packed with 8%, 1,3-propanediol succinate on AW Chrom W 60–80 mesh at 190° gave the percent distribution of acetoxy aldehydes. In both columns the helium flow was 40 ml/min; hydrogen flow 28 ml/min; injector and detector temperatures were maintained at 250°C. The results are reported in Table 4.

RESULTS AND DISCUSSION

The data obtained from the hydrogenation of 8-dodecynyl acetate, *cis*-8-dodecenyl acetate, and *trans*-8-dodecenyl acetate are presented in Tables 1, 2, and 3, respectively. They are representative of a number of experiments and show the following route is operating: acetylene→*cis*-olefin$\leftrightarrows$*trans*-olefin→saturated. In each experiment four compounds were assayed for: alkyl acetate, *trans*-alkenyl acetate, *cis*-alkenyl acetate, and alkynyl acetate. When the starting compound was *trans*-alkenyl acetate, the totaled components of the assay were 94%. When *cis*-alkenyl acetate or alkynyl acetate were the starting compound, the totaled components of the assay were 74% and 78%, respectively. The results suggest that either the *cis*-alkene or -alkyne, or perhaps both are bound to the catalyst and therefore are not available for assay. They also support a proposal (Dobson et al., 1961) that initially formed cis isomer stereomutates to the trans isomer on the catalyst surface.

Table 4 gives the distribution of trans and cis isomers obtained from a series of reductions in which 6-tetradecynyl acetate was the starting compound and each succeeding reduction was allowed to attain an increased

TABLE 4. GEOMETRICAL AND POSITIONAL ISOMERIZATION DURING REDUCTION OF 6-TETRADECYNYL ACETATE

Distribution		Time	Ozonized	Distribution of aldehydes (%)					
				n-aldehyde			acetoxy aldehyde		
isomer	%	(min)	(mg)	C_7	C_8	C_9	C_5	C_6	C_7
saturated	0								
trans	0	20							
cis	84		1	1	99	0	1	99	0
yne	16								
saturated	1								
trans	10	35	1	9	77	14	6	76	28
cis	89		1	3	95	2	0	100	0
saturated	1								
trans	33	70	1	14	60	26	25	62	13
cis	66		1	1	91	8	0	100	0
saturated	8								
trans	61	100	1	12	53	35	28	58	14
cis	31		1	4	94	2	5	90	5

level of isomerization; it also gives the percent distribution of aldehydes produced after ozonolysis of the separated *trans*- and *cis*-alkenyl acetates. 6-Tetradecynyl acetate was chosen for this reduction because the aldehydes produced by ozonolysis of the alkenyl acetates have gas chromatographic retention times that are very different from the solvent peak. The reproducibility of the analytical method used to determine the aldehydes was 10%. The data show double-bond migration to the adjacent carbon–carbon position and suggest the migration is proportional to the amount of isomerization: in the *trans* isomer up to 35%, and in the *cis* isomer to 5%.

Reductions performed in aprotic solvents such as hexane and cyclohexane, and using 5% palladium supported on either charcoal or calcium carbonate[4] gave essentially the same results. Lindlar's palladium catalyst[5] used at similar catalyst: substrate ratios produced olefins containing only small amounts of trans isomer. Frequently at low catalyst ratios no trans olefin was detectable at the point of disappearance of the substrate alkyne. However, by using abnormally high catalyst ratios (1:1 to 10:1) we were able to observe cis–trans isomerization following the reduction stage. In the

[4] Matheson Coleman and Bell, Norwood (Cincinnati) Ohio.
[5] Fluka, A.G., Buchs, S.G.

most extreme case the trans content reached 42%; the trans isomers were positionally isomerized just as with other palladium catalysts. While the rate of stirring and additives such as quinoline and triethylamine affected the rate of reduction, they did not appreciably alter the isomerization. Increasing the proportion of catalyst also increased the reaction rate. This indicates that the catalyst surface is the probable site of the isomerization reaction.

The foregoing results have important implications in the field of insect sex pheromone synthesis, for correct cis–trans and positional isomers are fundamental requirements for biological activity and to species specificity of action (Roelofs and Cardé, 1974; Silverstein and Young, 1976). Moreover, many lepidopterous sex pheromones involve alkenyl acetates whose chemical synthesis includes a catalytic hydrogenation step. Table 4 shows that in reductions where no trans has been produced the cis product will be free of positional isomers. Where the trans isomer is formed it will contain a substantial percentage of positional isomerism although the companion cis product will contain only a small amount of positional isomers. The presence of undetected positional isomers in a supposedly pure compound could confer spurious pheromone activity or might cause inhibition of genuine behavior responses. It is imperative that both the geometrical and the positional isomeric purity of chemical compounds produced by catalytic reduction be ascertained before they are used for insect behavior studies.

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HETEROCYCLIC COMPOUNDS AS RELEASERS OF THE FRIGHT REACTION IN THE GIANT DANIO *Danio malabaricus* (JERDON) (CYPRINIDAE, OSTARIOPHYSI, PISCES)

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Abstract—Fifty-nine pteridine, purine, and pyrimidine derivatives were tested with schools of the giant danio *Danio malabaricus* (Jerdon). The fright reaction was elicited by three pteridine derivatives: 2,6-diamino-4-oxodihydropteridine, isoxanthopterin, and 6-acetonylisoxanthopterin. A minor effect could not be excluded for three purine derivatives: I-5-MP, IDP, and ITP.

Key Words—Fish, *Danio malabaricus* (Jerdon), fright reaction, pheromone, heterocyclic compounds.

INTRODUCTION

The fright reaction of the European minnow *Phoxinus phoxinus* (L.) and other Cypriniformes is elicited by an alarm substance deriving from the fish's skin (von Frisch, 1941; recent reviews: Pfeiffer, 1974, 1977). Experiments with isoxanthopterin had shown that this substance may elicit the fright reaction in the giant danio *Danio malabaricus* (Jerdon), although it is not identical with the genuine alarm substance (Pfeiffer and Lemke, 1973; Pfeiffer, 1975). During the investigation on the isolation of the alarm substance from fish skin several heterocyclic compounds were tested with respect to their biological activity.

METHODS AND MATERIALS

Schools of the giant danio *Danio malabaricus* (Jerdon) were used, and

consisted of 6–8 juveniles, each measuring 30–50 mm. The fish were raised at 24°C and daily fed with dry food (Tetramin, Tetra-Werke, Melle, West Germany). Light period lasted from 7 AM to 6 PM. The health of the fish was daily controlled, and only healthy fish were tested. The fish were conditioned to the experimental aquarium prior to testing until they no longer fled when a person approached the tank but remained near the feeding corner at the surface in expectation of food. Such conditioning time was usually about one to two weeks. Since fish may rapidly adapt to substances that elicit the fright reaction, most schools were tested only once with an effective substance. If fish were tested repeatedly, a minimum interval of four weeks elapsed between two successive experiments. The experiments were performed in 150 aquaria ($40 \times 20 \times 20$ cm) containing 20 liters of water. All aquaria were filtered (Billi-filters, Tetra-Werke), and heated with 25-W thermostat heaters (Fa. Jäger, West Germany). Each tank contained moss and rods of *Monstera* or *Syndapsus*, plants which eliminated nitrate and nitrite from the water and also served as hiding places for the fish. After each test with a fright reaction the aquarium was carefully cleaned and filled with tap water from Lake Constance. Both the tanks and the substances tested were selected at random.

The behavior of the fish was observed for 5 min after the substance under investigation had been introduced into the tank. An active test substance induced a fright reaction in 5–30 sec. Fish which had assembled at the feeding corner close to the water surface seemed terrified and fled towards the bottom in confusion; they then retreated. Confidence returned after intervals ranging from minutes to several hours. The intensity of the fright reaction varied and three different situations were distinguished. Such stages were evaluated arbitrarily, and ranged from the most intense reaction where all fish were suddenly frightened and hastily fled, to a scarcely visible intimidation.

Positive Reaction. Most intense reaction with sudden fright and rapid swimming towards the bottom. The fish may rapidly swim around the tank or stay motionless close to the bottom.

Questionable Reaction. Only slightly frightened or somewhat confused; swimming to the middle depth of the aquarium where the school may crowd, but no retreating to the bottom and usually soon quieting down. Sometimes intimidated and less confident at the feeding corner with some crowding together, but the reaction soon disappears.

Negative Reaction. No reaction whatever.

One compound was tested simultaneously on many tanks. With each substance generally 5–12 tests were performed with as many schools. Isoxanthopterin, xanthopterin, inosine, and cAMP were tested for 2–4 days in order to confirm their effects; different schools were used. If the fish did not

react to a compound, their ability to react was tested the following day using skin extract from the European minnow, *Phoxinus phoxinus* (L.). Schools that did not react to the skin extract were thereby eliminated. Solutions were prepared from 1 mg substance and 100 ml solvent (0.01 N HCl or 0.01 N NaOH). Five ml of solution equivalent to 0.05 mg of substance were introduced into the aquarium within a period of 10–15 sec. Experiments with the solvent or with extracts from minnow skin were performed as controls. All the experiments were made by one person only without prior knowledge of the substance under investigation.

RESULTS

Positive results were obtained by a number of substances. However, whereas some substances elicited the fright reaction regularly, others induced it only exceptionally; three groups of compounds were distinguished:

Highly Effective Substances. A compound was considered highly effective if more than 50% of the tests performed produced a positive reaction, and less than 20% a negative reaction.

Ineffective Substances. A compound was considered ineffective if less than 20% of the tests performed produced a positive reaction and more than 50% a negative reaction.

Possibly Weakly Effective Substances. For one group of compounds closely related to one another a minor effectiveness could not be excluded since approx. 25% of the tests produced a positive reaction, and only 30–50% a negative reaction. A relatively high percentage of the tests with these substances induced questionable reactions.

Three of the pteridine derivatives tested elicited the fright reaction: 2,6-diamino-4-oxodihydropteridine (58% positive, 33% questionable tests), isoxanthopterin (56% positive, 28% questionable tests), and 6-acetonyl-isoxanthopterin (100% positive tests). In contrast, xanthopterin and 7-acetonylxanthopterin were ineffective, as were the other pteridines tested (Table 1).

A minor effect of purine derivatives could not be excluded for I-5-MP, IDP, and ITP: 23–26% of the experiments with these substances elicited the fright reaction, and 21–48% of the tests gave questionable reactions in addition. The other purine derivatives tested were ineffective, as were all the pyrimidine derivatives. All ribonucleoside 2′,3′-cyclic phosphates and 3′,5′-cyclic phosphates were ineffective, including A-3,5-MP and I-3,5-MP (Table 2).

Some substances elicited the fright reaction in less than 20% of the tests, and included pterorhodin, ekapterin, C-3,5-MP, hypoxanthine, and inosine (Tables 1, 2). These compounds were considered ineffective.

TABLE 1. RESULTS OF FRIGHT REACTION TESTS WITH PTERIDINE DERIVATIVES AND CONTROLS

Substance	Positive[a]	Questionable[a]	Negative[a]	Date
4-Hydroxypteridine	0	0	9	7/27/73
4-Hydroxy-6,7-dimethylpteridine	0	0	7	7/26/73
Lumazine (2,4-dihydroxypteridine)	0	0	9	7/27/73
4-Hydroxy-2-mercaptopteridine	0	0	9	7/27/73
8-Ribityl-6,7-dimethyl-lumazine	0	0	9	7/27/73
Pterin (2-amino-4-hydroxypteridine)	0	0	7	7/26/73
2,6-Diamino-4-oxodihydropteridine	7	4	1	1/22/73
L-Monapterin	0	0	7	1/22/73
Pterin-6-carboxylic acid	0	0	7	7/26/73
Pterin-6-acetic acid	0	0	5	1/3/73
Biopterin	0	1	10	10/11/73
D-Neopterin	0	0	5	1/5/73
6,7-Dimethylpterin	0	0	5	1/15/73
7-Methylpterin	0	0	6	1/15/73
Pterin-7-carboxylic acid	0	0	7	7/26/73
Leucopterin	0	0	5	1/3/73
Folic acid	0	0	10	5/30/73
Xanthopterin (6-hydroxypterin)	0	0	12	1/3/73 and 12/12/73

7,8-Dihydroxyxanthopterin	0	0	5	1/15/73
Chrysopterin	0	0	5	1/15/73
7-Acetonylxanthopterin	0	2	8	1/22/73
Ekapterin	1	1	4	1/5/73
Erythropterin	0	0	5	1/5/73
Lepidopterin	0	1	5	1/5/73
Pterorhodin	3	1	13	10/11/73
Isoxanthopterin (7-hydroxypterin)				
1	12	6	0	6/15/72
2	4	0	0	7/14/72
3	2	1	4	11/3/72
4	4	4	2	1/3/73
total	22	11	6	
Isoxanthopterin-6-carboxylic acid	0	0	5	1/3/73
6-Acetonylisoxanthopterin	9	0	0	1/22/73
Controls				
Solvent (0.01 N NaOH or 0.01 N HCl)	0	0	25	3/5/15 and 1/22/73
Skin extract from *Phoxinus*	110	6	4	

[a] See methods section for criteria of positive reaction.

TABLE 2. RESULTS OF FRIGHT REACTION TESTS WITH PURINE AND PYRIMIDINE DERIVATIVES

Substance	Positive[a]	Questionable[a]	Negative[a]	Date
Adenine	0	0	8	5/24/73
Guanine	0	0	8	5/24/73
Hypoxanthine	3	9	23	5/12/75
Adenosine	0	0	10	5/4/73
Guanosine	0	0	6	5/4/73
Inosine				
1	0	0	6	5/4/73
2	1	3	16	5/12/73
Total	1	3	22	
Uridine	0	0	6	5/4/73
Cytidine	0	0	6	5/4/73
2′-Deoxythymidine	0	0	7	5/4/73
Adenosine-5′-monophosphate (A-5-MP)	0	0	8	5/24/73
Adenosine-5′-triphosphate (ATP)	0	0	8	5/24/73
Guanosine-5′-triphosphate (GTP)	0	3	7	5/30/73
Inosine-5′-monophosphate (I-5-MP)	7	14	8	5/27/75
Inosine-5′-diphosphate (IDP)	6	6	14	5/27/75
Inosine-5′-triphosphate (ITP)	10	8	20	5/27/75
Cytidine-5′-monophosphate (C-5-MP)	0	0	8	5/24/73

Cytidine-5′-triphosphate (CTP)	0	0	8	5/24/73
2′-Deoxythymidine-5′-monophosphate (dT-5-MP)	0	4	6	5/30/73
2′-Deoxythymidine-5′-diphosphate (dTDP)	0	3	8	5/30/73
Adenosine-3′,5′-monophosphoric acid, cyclic (A-3,5-MP)				
1	0	0	7	10/18/73
2	0	0	7	12/12/73
3	0	3	12	5/24/74
Total	0	3	26	
2′-Deoxyadenosine-3′,5′-monophosphate, cyclic (dA-3,5-MP)	0	0	6	12/11/73
Guanosine-3′,5′-monophosphate, cyclic (G-3,5-MP)	0	0	6	12/11/73
Inosine-3′,5′-monophosphoric acid, cyclic (I-3,5-MP)	0	0	6	12/11/73
Xanthosine-3′,5′-monophosphate, cyclic (X-3,5-MP)	0	0	7	12/12/73
Cytidine-3′,5′-monophosphate, cyclic (C-3,5-MP)	1	2	4	10/18/73
Uridine-3′,5′-monophosphate, cyclic (U-3,5-MP)	0	0	7	10/18/73
2′-Deoxythymidine-3′,5′-monophosphate, cyclic (dT-3,5-MP)	0	1	6	10/18/73
Adenosine-2′,3′-monophosphoric acid, cyclic (A-2,3-MP)	0	0	7	12/12/73
Guanosine-2′,3′-monophosphate, cyclic (G-2,3-MP)	0	0	7	12/12/73
Cytidine-2′,3′-monophosphoric acid, cyclic (C-2,3-MP)	0	0	6	12/11/73
Uridine-2′,3′-monophosphate, cyclic (U-2,3-MP)	0	0	7	12/12/73

[a] See methods section for criteria of positive reaction.

In total, 59 pteridine, purine, and pyrimidine derivatives were tested in some 750 experiments from June 15, 1972 to May 27, 1975 (Tables 1, 2). None of the heterocyclic compounds tested was as effective as the skin extract from *Phoxinus*, with the possible exception of 6-acetonylisoxanthopterin. Whereas skin extract was effective in most experiments, the solvent was always ineffective (Table 1).

DISCUSSION

Whereas isoxanthopterin and especially 6-acetonylisoxanthopterin elicited the fright reaction in the giant danio (*Danio malabaricus*), their isomers xanthopterin and 7-acetonylxanthopterin were ineffective. The following results are in accord with these observations: (1) 6-Acetonylisoxanthopterin in the European minnow (*Phoxinus phoxinus*) produced bradycardia in contrast to 7-acetonylxanthopterin and xanthopterin which did not have this effect (Pfeiffer and Lamour, 1976). (2) Isoxanthopterin and 6-acetonylisoxanthopterin had a strong effect on the central nervous excitation measured quantitatively using the dorsal light response in the unilaterally illuminated black tetra (*Gymnocorymbus ternetzi*). An enhanced optical alertness shown by an increase of the fishes' inclination towards the light was produced with these substances, whereas both xanthopterin and 7-acetonylxanthopterin were ineffective (Pfeiffer and Riegelbauer, 1978).

Both isoxanthopterin and 6-acetonylisoxanthopterin elicit the fright reaction, produce bradycardia, and cause a strong change of the central state in contrast to their biologically ineffective isomers xanthopterin and 7-acetonylxanthopterin.

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SELECTED CHEMICAL CONSTITUENTS AND DEER BROWSING PREFERENCE OF DOUGLAS FIR

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Abstract—Douglas fir foliage from eight full-sib F_1 families was analyzed for chlorogenic acid and essential oils and ranked according to browsing preference of penned black-tailed deer. Foliage was obtained during the dormant season, and tissues for both the preference test and chemical analyses were collected from the same trees. Deer ranked the different families, and chlorogenic acid content was correlated with preference order. Families varied significantly in yield and composition of essential oils, but differences were not related to preference. Results may have application in selections for Douglas fir resistant to browsing.

Key Words—Browsing preference, essential oils, chlorogenic acid, Douglas fir, *Pseudotsuga menziesii*, deer (black-tailed), *Odocoileus hemionus columbianus*.

INTRODUCTION

Past studies have shown that browsing animals express within species variation in preferences for forest plants (Radwan, 1974). Recently, differences in browsing preference by black-tailed deer (*Odocoileus hemionus columbianus*) have been demonstrated among foliage of different Douglas fir (*Pseudotsuga menziesii*) genotypes (Dimock et al., 1976). Investigation of chemical factors affecting such differential responses would contribute to a better understanding of plant–animal relationships and could be useful in devising new methods to reduce browsing damage to trees.

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In recent studies with Douglas fir, we reported that greater susceptibility to deer browsing was associated with higher concentrations of chlorogenic acid (Radwan, 1972) or with lower levels of volatile terpenes emitted from the essential oils present in the foliage (Radwan and Ellis, 1975). Likewise, others have suggested that apparent deer preference for the young, new growth over old needles of Douglas fir was influenced by the composition of foliar essential oils (Longhurst et al., 1968; Maarse and Kepner, 1970). None of the previous studies, however, considered the oils and chlorogenic acid simultaneously to determine which assay provides the better correlation with deer preference.

This paper presents results of an investigation of the chlorogenic acid content and the level and composition of essential oils of Douglas fir foliage in relation to deer preference. The study involved trees of eight different families. Foliage for both chemical analyses and preference tests was collected from the same trees of each family.

METHODS AND MATERIALS

Plant Material

Test trees were from eight different full-sib F_1 families obtained by controlled matings among nine different grafted clones (eight different female parents and one pollen parent). Clones originated from different trees growing at middle elevations within one township on the northwestern part of Washington's Olympic Peninsula (Dimock et al., 1976). Families were randomly replicated with other trees of the same age class in one plantation at the Olympic National Forest's Dennie Ahl Seed Orchard in western Washington. At time of sampling in 1975, the trees were 10 years old.

Sampling

All samples were collected in the winter to minimize changes in chemical composition during sampling and handling and to prevent desiccation during the animal tests. Experience has also shown that winter is the best season for preference testing because deer do not readily feed on Douglas fir when natural vegetation in the pen becomes available during the growing season.

In January, composite foliage samples were collected in mid-morning from each family. Each sample was taken from 10 trees selected at random from available replicates, and the same trees were used for both deer-preference testing and chemical analyses. Samples were obtained from current year's growth cut from all sides of the trees at a height of about 1 m.

For the deer preference test, one sample per family was collected by cutting 10 branch terminals of about 30 cm in length from each tree and placing cut ends immediately in water to prevent desiccation during transport to the deer pen. For chemical analyses, three 75-g samples were collected from the 10 trees of each family. Each sample consisted of 5-cm tips of secondary laterals, and samples were individually sealed in precooled jars and brought to the laboratory in a portable cooler.

Deer-Preference Test

Deer preference was ranked in a 1-hectare enclosure located near the laboratory and maintained by the U.S. Fish and Wildlife Service. During the trial, the enclosure contained some natural forage, a constant supply of commercially prepared pelleted ration, and 5 black-tailed deer randomly chosen from a semitame herd of 20 animals captured from western Washington and maintained in Olympia for preference testing. Test material, 100 branch terminal cuttings from each family, was exposed in a randomized block design (Dodge et al., 1967) to voluntary feeding by the deer. Families were replicated in 10 blocks, and cuttings were tamped in the moist ground to uniform 24-cm height at 1- × 1-m spacing within blocks. Inspections for any feeding were made daily, and the day of first browsing was recorded for each cutting until all were browsed. Relative preference was ranked according to the mean number of days until all cuttings within each family were browsed (Dimock, 1971).

Chemical Analyses

In the laboratory, fresh foliage of each composite sample was chopped into small pieces. Subsamples were taken for moisture determination at 65°C, chlorogenic acid (CGA) extraction, and isolation of essential oils.

CGA was extracted (Soxhlet) with 80% methanol after tissue was defatted with petroleum ether (30–60°C). Extracts were purified, and CGA was separated by two-dimensional paper chromatography and quantified spectrophotometrically at 328 nm (Radwan, 1972, 1975). Average CGA concentrations in the tissues were calculated based on three replicates and three chromatograms per replicate.

Essential oils were isolated by blending tissue in minimum amounts of distilled water followed by steam distillation for 4 hr in a Clevenger-type apparatus (Clevenger, 1928) and collection of the oils in *n*-heptane. The oil solutions were diluted to a common volume with heptane after adding *n*-tetradecane as internal standard. They were then transferred to airtight vials equipped with Teflon septa, and stored at —15°C until analyzed by gas–liquid chromatography (GLC).

Volumes of oil solutions for GLC analyses were 0.3 μl each. Separations were carried out with a gas chromatograph equipped with flame ionization detectors and two open tubular, stainless-steel columns. Columns were 61 m $\times$ 0.05 cm (ID) coated with a mixture of 95% Carbowax 20M plus 5% Igepal CO-880. Operating conditions were: injection port, 250°C; detector, 250°C; column, isothermal at 70°C for the first 3 min, programed to 150°C at 2°C/min, and held at 150°C for 8 min; and N_2, H_2, and air flows of 4, 25, and 250 ml/min, respectively. Resolved peaks were identified by comparing unknowns' relative retention times on two columns [Carbowax 20M and SF-96(50)] and their infrared spectra with those of known compounds and by peak enrichment. Compounds were quantified by measuring peak areas with an electronic integrator. Average yields per gram tissue, as well as percent composition of the oils, were calculated based on three replicates and two injections per replicate.

Statistical Analyses

Data were subjected to analysis of variance after arc-sine transformation, if necessary, and means were separated according to Tukey's test as required (Snedecor, 1961). Correlation coefficients (r) between days to complete browsing and values of chlorogenic acid and components and yields of the oils were also calculated to estimate effects of these chemical variables on browsing preference (Snedecor, 1961).

RESULTS AND DISCUSSION

Deer Preference

Browsing of test cuttings began almost immediately but was interrupted by heavy snowfall which covered the cuttings for 1 week at the beginning of the test. After snowmelt, browsing resumed and continued at different rates for 25 days when all cuttings within the enclosure had been browsed and the test was concluded. From the start, day-to-day tabulations showed no large differences in preferential browsing among the eight families. Most cuttings were first browsed between the 8th and 18th day after exposure. By test end, mean days required for complete browsing were as follows:

Family	4×8	11×8	13×8	16×8	18×8	19×8	22×8	28×8
Mean days	13.7	14.7	13.7	14.2	13.6	13.7	14.5	14.4

These data provided a ranking among the families to which the deer were exposed, a result agreeing with findings of earlier studies (Dimock et al., 1976) in which deer preference was influenced by Douglas fir genotype. In general, the deer favored families 4×8, 13×8, 18×8, and 19×8 over other

genotypes. In addition, 18×8 and 11×8 were, respectively, the highest and lowest ranked families. Family differences, however, were small at best, with maximum separation in mean exposure periods to complete browsing of only 1.1 days. Such small differences, we believe, were expected since test families were all bred from the same pollen parent and because heavy concentration of deer in the restricted pen area provided heavy feeding pressure.

Moisture Content

Foliage varied in moisture content between 54.17 and 55.55% with no significant ($P \leq 0.05$) differences among families. Results were used to calculate levels of other chemical constituents on dry-weight basis.

Chlorogenic Acid Content

Measurable amounts of CGA were found in all tissues examined (Figure 1). In addition, mean CGA levels varied significantly ($P \leq 0.05$) among families, corroborating earlier findings with respect to genotype influence on CGA content in Douglas fir foliage (Radwan, 1975).

According to CGA values, test families ranked as follows: $19 \times 8 > 4 \times 8 > 18 \times 8 > 16 \times 8 > 13 \times 8 > 22 \times 8 > 28 \times 8 > 11 \times 8$. This is not the same ranking as that obtained in the deer preference test. However, the results show that a general inverse relationship exists between the two sets of data. More importantly, the correlation coefficient (r) between the CGA concentrations and exposure periods to complete browsing was —0.755, significant at the 0.05 level. Clearly, this result establishes a positive relationship between CGA content and deer browsing preference. The same relationship was noted earlier in a study with foliage from four different clones of Douglas fir (Radwan, 1972). In addition, our results are in full agreement with those obtained recently with Douglas fir and mule deer (*Odocoileus hemionus hemionus*) in Canada by Tucker et al. (1976).

Yield and Composition of Essential Oils

Total yield of essential oils as well as yields of compounds in the monoterpene hydrocarbon and oxygenated monoterpene regions varied significantly ($P \leq 0.05$) among families (Table 1). In each case, however, correlation coefficients (r) between yields and days to complete browsing were not significant ($P = 0.05$). The results, therefore, do not support data of an earlier study (Radwan and Ellis, 1975) which showed that it was possible to distinguish between Douglas fir clones of different susceptibilities to deer browsing on the basis of total volatile terpenes emitted by foliage. This

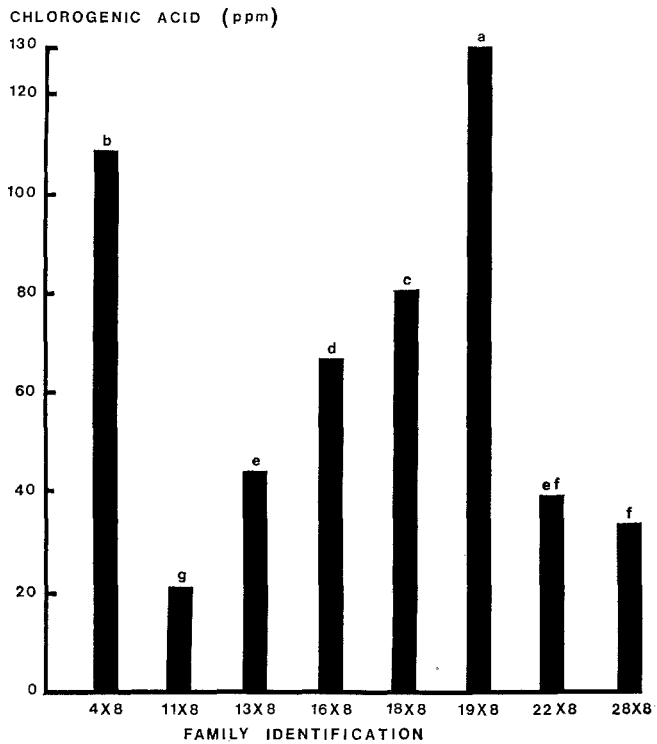

FIG 1. Concentration of chlorogenic acid in foliage of different families of Douglas fir. (Each bar is the mean of three composite samples, and means not superposed by a common letter differ significantly at the 5% level.)

discrepancy is understandable, however, since terpene analyses were performed on steam-distilled oil in the present study and on headspace vapor in the earlier investigation.

Oils of the different families contained over 40 compounds each, but many were consistently present in small or trace amounts. Compounds identified were similar to those found earlier in foliar oils of Douglas fir (Maarse and Kepner, 1970; Radwan and Ellis, 1975); and as expected, the oils were predominantly (79–85%) composed of monoterpene hydrocarbons. Most abundant components present in the oil were α- and β-pinene, terpinolene, sabinene, 3-carene, and δ- and α-terpinene in the monoterpene hydrocarbon region, and terpinen-4-ol, α-terpineol, and citronellyl and geranyl acetate in the oxygenated monoterpene region.

There were quantitative and not qualitative differences in composition

TABLE 1. COMPOSITION AND YIELD OF FOLIAR ESSENTIAL OILS OF DOUGLAS FIR[a]

Component composition (%)	Family identification								
	4×8	11×8	13×8	16×8	18×8	19×8	22×8	28×8	SS
Monoterpene hydrocarbon region									
α-Pinene	12.95	13.34	12.83	12.61	12.68	14.62	12.13	12.84	**
Camphene	0.49	0.46	0.43	0.49	0.44	0.51	0.39	0.48	**
β-Pinene	32.25	31.50	29.25	31.51	31.47	36.76	25.30	29.93	**
Sabinene	8.07	8.13	8.89	8.09	9.30	7.21	9.05	7.41	N.S.
Δ-3-Carene	2.90	7.28	6.17	3.97	4.44	4.04	5.11	4.87	**
Myrcene	1.96	2.26	1.97	1.98	1.93	2.08	2.14	1.80	N.S.
α-Phellandrene	0.33	0.33	0.35	0.31	0.31	0.26	0.38	0.36	*
α-Terpinene	2.68	2.30	2.67	2.45	2.56	1.90	2.97	2.87	**
Limonene	1.83	2.40	2.10	2.15	1.64	2.56	2.48	1.96	**
β-Phellandrene	1.50	1.63	1.73	1.49	1.51	1.61	1.56	1.59	**
2-Hexenal	0.60	0.69	0.87	0.45	0.56	0.38	0.44	0.73	**
Ethyl caproate	0.20	0.31	0.09	0.04	0.12	0.05	0.06	0.08	**
δ-Terpinen	4.52	3.86	4.67	4.20	4.33	3.42	4.94	4.76	**
p-Cymene	0.09	0.09	0.11	0.10	0.08	0.07	0.10	0.11	N.S.
Terpinolene	10.67	10.01	11.14	10.07	11.10	8.44	12.07	11.24	**
Oxygenated monoterpene hydrocarbon region									
Citronellal	0.21	0.16	0.16	0.26	0.18	0.28	0.19	0.19	**
Linalool	0.12	0.11	0.15	0.17	0.09	0.11	0.10	0.08	**
Unknown 21	0.26	0.22	0.34	0.28	0.28	0.18	0.28	0.32	**
Unknown 24	0.17	0.18	0.16	0.24	0.22	0.25	0.23	0.22	**
Terpinen-4-ol	7.77	5.99	6.91	7.11	7.10	5.25	8.18	7.67	**
Unknown 28	0.15	0.14	0.16	0.16	0.22	0.14	0.19	0.18	*
Citronellyl acetate	2.95	2.18	1.93	3.10	2.53	2.49	3.71	3.03	**
Unknown 31	0.27	0.31	0.28	0.43	0.44	0.36	0.33	0.35	N.S.
α-Terpineol	3.50	2.59	2.71	3.12	2.26	2.31	2.62	3.22	N.S.
Unknown 34	0.54	0.53	0.44	0.26	0.52	0.62	0.72	0.24	**
Geranyl acetate	2.13	2.19	2.46	3.69	2.47	3.01	3.18	2.26	**
Other unknowns	0.90	0.80	1.04	1.25	1.22	1.10	1.15	1.22	
Yield (area × 10^7)									
Monoterpene hydrocarbon region	76.24	69.21	68.66	63.46	75.72	72.62	70.58	69.61	*
Oxygenated monoterpene region	17.84	12.60	13.82	15.96	16.10	13.92	18.62	16.30	**
Total yield	94.08	81.81	82.48	79.42	91.82	86.54	89.20	85.91	*

[a] Values are means of three composite samples from 10 trees each. Statistical significance (SS) indicated by **, $P \leq 0.01$; *, $P \leq 0.05$; N.S., not significant. Area in arbitrary units determined by electronic integrator and calculated per gram dry tissue.

among oils of the different families. Thus, families differed significantly ($P \leq 0.01$–0.05) in concentrations of all oil components except sabinene, myrcene, *p*-cymene, unknown 31, and α-terpineol. All correlation coefficients (r) between concentrations of individual compounds and days to complete browsing were, however, nonsignificant ($P \leq 0.05$). Differences in the oils' makeup, therefore, were not related to observed variations in deer preference among families. This result is in contrast with earlier observations (Oh et al., 1967; Longhurst et al., 1968) suggesting that palatibility of plants, including Douglas fir, was influenced by composition of their essential oils.

CONCLUSIONS

Our data did not reveal any consistent relationship between deer browsing and yield or composition of essential oils. More importantly, results substantiate earlier findings with Douglas fir foliage with respect to influence of genotype on deer browsing preference (Dimock et al., 1976), genotypic differences of chlorogenic acid, and correlation between chlorogenic acid content and browsing preference (Radwan, 1972, 1975). Importance of these results lies in their possible application to reduce deer damage to Douglas fir by selecting planting or breeding stock resistant to browsing based upon chlorogenic acid content. Such screening procedure, we believe, would be less costly and more rapid than selection methods based on testing with live animals.

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THRESHOLD HYPOTHESIS FOR PHEROMONE PERCEPTION

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Abstract—Field results have shown that male moths of some species are not always trapped by the ratio of pheromone components produced by the female moths. For cases involving a binary mixture of geometrical or positional isomers, this phenomenon may be explained by use of a threshold diagram in which the isomer ratio in the mixture is plotted against release rate (concentration). In this diagram an "attraction area" is bounded by the threshold for flight activation over the full range of binary mixtures and by the threshold for alteration of in-flight behavior (disorientation) by each pheromone component. A low release rate of the natural mixture may fall within this "attraction area," or a high release rate of mixtures in certain other ratios may fall within this area and would be expected to attract male moths. This hypothesis has been used to explain heretofore anomalous trapping data with some moth species, and it can be useful in future studies on defining pheromone blends, species specificity, and potentially disruptive mixtures for insect control.

Key Words—Sex pheromone, bioassays, redbanded leafroller, *Argyrotaenia velutinana*, omnivorous leafroller, *Platynota stultana*, pink bollworm, *Pectinophora gossypiella*, behavioral thresholds, disruption, sex attractant trapping.

INTRODUCTION

Sex pheromones of many lepidopteran species are composed of two or more pheromone components. In many cases, a combination of geometrical (Table 1) or positional isomers is used. The female moths produce a specific ratio of these components and the males usually are attracted in the field to

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TABLE 1. EXAMPLES OF GEOMETRICAL ISOMERS USED AS PHEROMONE COMPONENTS IN SOME TORTRICID SPECIES[a]

Species	*Z*11-/*E*11-14:Ac[b]	Reference
Choristoneura rosaceana	97:3	Hill and Roelofs, unpubl.
Argyrotaenia velutinana	91:9	Roelofs et al., 1975
Archips argyrospilus	60:40	Roelofs et al., 1974
Archips podana	60:40	Persoons et al., 1974
Archips semiferanus	33:67	Miller et al., 1976
Archippus breviplicanus	30:70	Sugie et al., 1977
Platynota stultana	11:89	Hill and Roelofs, 1975

[a] Additional pheromone components are used by some of these species.
[b] (*Z*)-11-/(*E*)-11-tetradecenyl acetate; these ratios represent those found in female moth extracts.

lures emitting the components in that ratio. These compounds have similar vapor pressures and would be expected to evaporate at precise ratios throughout a range of temperatures.

It is not known how each species regulates the production of its own specific blend, but a study (Miller and Roelofs, 1978a) involving single-female-tip analyses of redbanded leafroller, *Argyrotaenia velutinana*, moths taken from two different generations in the field revealed that the quality control of the pheromone component ratio for this species is excellent. In this case each female produced a component ratio of approx. 91:9, and the absolute range from over 400 individuals varied only from 96:4 to 85:15. Another study (Miller and Roelofs, 1977) showed that the ratio did not vary with moth age, even though the pheromone titer increased sharply in the first 4 days following moth emergence.

The precise component ratio emitted by female moths should be the one that optimally elicits long-distance anemotactic flights by male moths (Roelofs and Cardé, 1977). A field trapping test with *A. velutinana* showed that males were captured best with component ratios of 94:6 to 90:10 (female produces approx. 91:9 ratio), and a test (Miller et al., 1976) with *Archips semiferanus* showed that males were captured significantly better with a 34:66 component ratio than with either 40:60 or 30:70 ratios (female produces approx. 33:67 ratio). In other cases, however, most males were captured with a broader range of ratios and, in some tests, with ratios quite different from that produced by the corresponding females. For example, the pink bollworm, *Pectinophora gossypiella*, was captured best by a 67:33 ratio of pheromone components, (*Z*,*Z*)/(*Z*,*E*)-7,11-hexadecadienyl acetates, in the early season, but by ratios between 40:60 and 70:30 in late season flights

(Flint et al., 1977) (Figure 1). Females were found to produce a 66:34 component ratio (Bierl et al., 1974), analogous to the early season trapping results. Field tests by Bierl et al. (1974), however, showed that the ratios 30:70 and 50:50 captured many more males than the 70:30 ratio (292, 641 and 41 males, respectively), which is the closest to the reported natural ratio.

Field trapping studies with the omnivorous leafroller moth, *Platynota stultana*, also gave anomalous results. The females were found to produce an

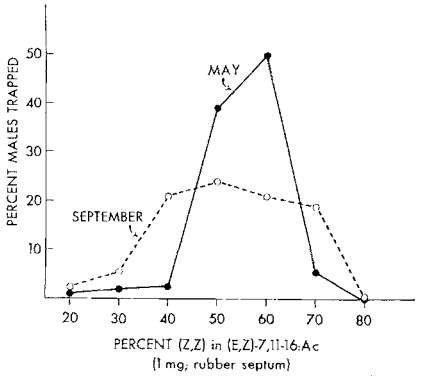

FIG. 1. Male pink bollworm catches in May and September with various binary mixtures of pheromone components. Data taken from Flint et al. (1977).

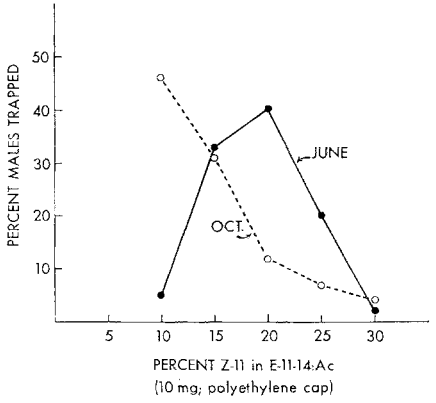

FIG. 2. Male omnivorous leafroller catches in June and October of 1973 with various binary mixtures of pheromone components.

11:89 ratio of pheromone components, *Z*11-/*E*11–14:Ac (Hill and Roelofs, 1975), but field trapping tests with various ratios were confusing (Baker et al., 1978). In 1973, the best trapping ratios in June were 15:85 and 20:80, whereas the same test repeated in October showed that the best ratio was 10:90 (Figure 2). A season-long test in 1974 also showed changes in the most attractive ratio, with a 5:95 ratio being best in March, and ratios of 15:85 to 30:70 being the most effective in May and July (Figure 3). Analyses of individual *P. stultana* taken from the field at various times of the season showed that the females consistently produced a component ratio of approx. 11:89. The curious change in component ratios for male attractancy through the season provided the impetus to develop a hypothesis that would explain this and other similar phenomena.

DISCUSSION

Activation Threshold

The redbanded leafroller moth, *Argyrotaenia velutinana*, females produce a 91:9 mixture of *Z*11-/*E*11–14:Ac pheromone components (Roelofs et al., 1975). In laboratory moth activation bioassays, this mixture elicits a response from a higher percentage of males than other blends at the 2-ng level (Figure 4) (Baker et al., 1976). These data and field-trapping data suggest that an increase in the amount of the *E*11 isomer is "inhibitory" to the male's response. However, further behavioral studies in the laboratory (Baker et

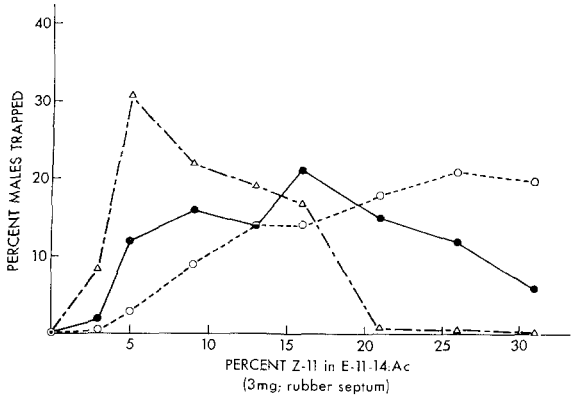

FIG. 3. Male omnivorous leafroller catches in March, July, and October of 1974 to various binary mixtures of pheromone components. Δ, March; ○, July; ●, October.

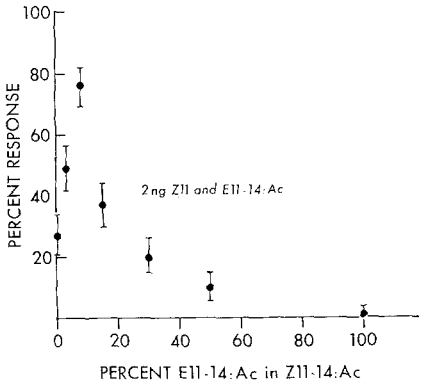

FIG. 4. Percentage activation response of redbanded leafroller males to binary mixtures of pheromone components. Data taken from Baker et al. (1976).

al., 1976) showed that pure Z11–14:Ac and a 70:30 blend of *Z*11/*E*11 elicit good activation responses if the dosage is tenfold higher than that of the natural blend and that pure *E*11–14:Ac elicits a good activation response if the dosage is increased 1000-fold (Figure 5). These results show that the moth activation response is exhibited if the concentration is above a certain threshold for the particular component mixture being tested. A suggested activation threshold curve for initial male *A. velutinana* moth activation during their receptive period is shown in Figure 6. The important point is that a low concentration of the pheromone components in the natural ratio activates the males, but other ratios also elicit responses if present in sufficiently high concentrations.

Alteration of In-Flight Behavior

It would appear from the flight activation threshold curve (Figure 6) alone that any binary mixture of pheromone components could attract males if the release rate were high enough. However, Wright (1964) predicted that pheromone concentrations up to a hundred thousand times higher than the threshold concentration of detection would fatigue or saturate the antennal receptors. Evidence that high pheromone concentrations can affect the male's responses was obtained from field-trapping studies in which male catches of various moth species were reduced at concentrations only 3–100 times higher than the optimum trap catch concentration (Roelofs and Cardé, 1977). An early example of this was reported for the cabbage looper moth, *Trichoplusia ni*. Male moths were trapped maximally with a release rate of approx.

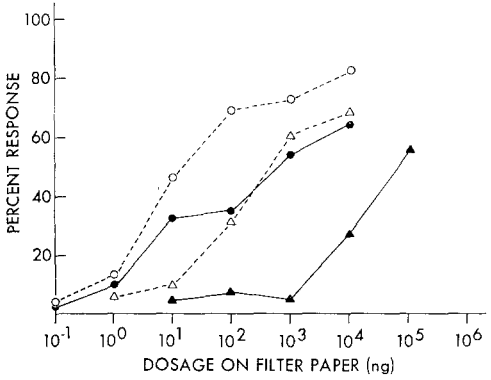

FIG. 5. Percentage wing-fanning response by redbanded leafroller males in box olfactometers to various concentrations of pheromone components and alone and in mixtures. Data taken from Baker et al. (1976). ●, TLC pure *Z* 11–14:Ac; ▲, TLC pure *E* 11–14:Ac; ○, 8% *E* 11–14:Ac in *Z* 11–14:Ac; △, 30% *E* 11–14:Ac in *Z* 11–14:Ac.

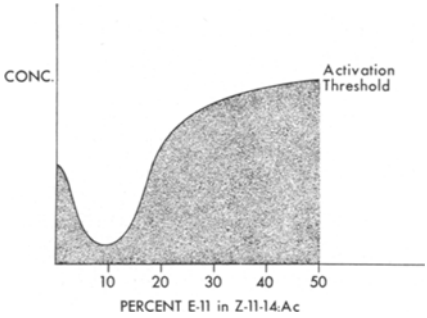

FIG. 6. A postulated threshold curve for male redbanded leafroller moth flight activation by binary mixtures of pheromone components. Activation would occur at concentrations above the shaded area with properly conditioned males.

60 μg/hr of pheromone, (*Z*)-7-dodecenyl acetate, but the trap catches were signicantly decreased with a three-fold increase in the pheromone release rate (Shorey et al., 1968). Later studies (Shorey et al., 1967; Marks, 1976) suggest that permeation of the atmosphere with pheromone concentrations of 10^2–10^3 times the male threshold response level can disrupt the male's long-distance anemotactic responses to an odor source. The exact *modus operandi* of this disruption is not understood, although it probably involves both sensory adaptation of the antennal receptors and habituation of the central nervous system. With the red bollworm, *Diparopsis castanea*, the activation threshold was estimated as 9.2 molecules pheromone/mm^3/sec over a 90-sec exposure period (5000 molecular impacts on the male antennae) (Marks, 1976). The pheromone release rate required for 95% disruption of *Diparopsis* mating from field-test data was estimated to be 1.5×10^4 molecules/mm^3/sec, which is 1.6×10^3 greater than the threshold concentration.

The alteration in the male's response to increased pheromone release rates places an upper limit on the pheromone concentration that can be used to attract males in the field. If this flight alteration (disorientation) threshold were reached at a constant increase in concentration relative to the activation threshold, the attractant concentration range for various binary mixtures would be visualized as in Figure 7. This diagram implies that for any mixture of pheromone components, there would be a certain concentration range that could attract the male moths. Sustained-flight-tunnel studies of in-flight behavior, however, indicated that further modifications had to be made in the thresholds for disorientation at the higher concentrations.

The gypsy moth, *Lymantria dispar*, pheromone (*cis*-7,8-epoxy-2-

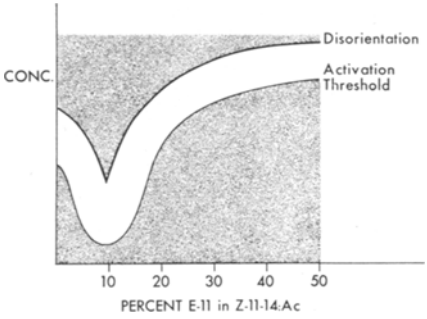

FIG. 7. A postulated disorientation curve added to the flight activation threshold curve of Figure 6. Trap catch would be reduced when release rates of the binary pheromone component mixtures are higher than the disorientation threshold line or lower than the activation threshold line.

methyloctadecane, disparlure) (Bierl et al., 1970) has been used in numerous laboratory and field studies since its identification, but recent availability of the (+) and (−) optical isomers of disparlure has permitted reinvestigations of the male gypsy moth's pheromone responses. In our studies (Miller and Roelofs, 1978b, c) a comparison was made between flight responses in a sustained-flight wind tunnel and trap catches in the field. Initially the results showed that traps hung in the tunnel with either (+)-disparlure or with racemic (±)-disparlure captured released males equally well, although field tests showed that the purest (+)-disparlure captured the most males and that racemic (±)-disparlure was not significantly more attractive than unbaited traps. This apparent discrepancy between field and flight tunnel data was resolved by using a moving floor in the tunnel to sustain moths in flight in the pheromone plume for prolonged periods. The duration of anemotactic flights in the tunnel was shown to be directly related to the optical purity of (+)-disparlure. Thus, males responding to 1 μg of (+)-disparlure flew 10 times longer and 20 times farther than males responding to 2 μg of racemic disparlure. The percent of males initiating anemotactic flights to 1 μg of (+) isomer with and without 1 μg of (−) isomer were equal, which indicated that the (−) isomer had little effect on flight activation at this concentration, although its effect in decreasing in-flight duration and distance was very pronounced. The in-flight alteration appears to be correlated with the amount of (−)-disparlure present, rather than to the threshold for moth activation.

Although the (−) isomer in the above mixtures probably is not a pheromone component for the gypsy moth, the results suggest that the in-flight behavior is more discriminating of pheromone composition and concentration changes than is moth activation. This implies that the disorientation threshold does not need to be directly related to the threshold for flight activation. The threshold for altering in-flight behavior with binary pheromone mixtures could well depend on the increasing amount of each pheromone component as one moves in either direction from the natural ratio in Figure 8. The slope of the line in either direction would depend on the relative effect of each component and, thus, the threshold lines in either direction from the natural blend could have different slopes. If the insect were particularly sensitive to increasing amounts of one component, then the disorientation threshold line in that direction would be more parallel to the abscissa than the threshold line for the other component.

Figure 8 would suggest that redbanded leafroller males would be activated to fly by concentrations of the two components that are above the activation threshold, but that the resulting in-flight behavior would be altered by concentrations in the shaded area above the disorientation threshold lines. Thus, one would not expect to get normal upwind anemotactic behavior

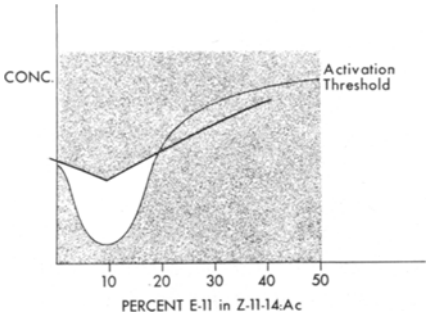

Fig. 8. A modification of Figure 7 in which the disorientation effect of higher concentrations of each pheromone component on in-flight behavior delineates the upper level for attracting male redbanded leafroller moths. Males are trapped by concentrations that fall within the unshaded "attraction area."

from any concentration of ratios containing 20% *E*11–14:Ac and higher. The "attraction area" shows that high concentrations of mixtures between approx. 2 and 17% *E*11–14:Ac should attract males as well as low concentrations of the natural blend containing 9% *E*11–14:Ac.

The disorientation threshold lines in Figure 8 were approximated with redbanded leafroller moth information from air permeation tests in the field (Roelofs et al., 1976) that showed the natural blend to be more disruptive than a 50:50 blend, and from studies conducted in a sustained-flight tunnel (Miller and Roelofs, unpublished). In the flight tunnel the preliminary data showed that a threefold increase in the number of emitters at the odor source for a mixture of components containing 8% *E* isomer (approximately the natural blend) dramatically affected flight behavior. The number of moths activated was not very different throughout the range of release rates, but moths responding to the higher release rates exhibited abnormal anemotactic flights, with the forward movement reduced at least by half and the net upwind distance traveled before leaving the plume decreased to at most 1/10 that of normal flights. Typically, an exaggerated anemotactic zig-zag pattern accounted for the reductions in upwind speed and net upwind distance traveled. This in-flight behavioral effect of the high release rates of the 8% *E* isomer mixture would represent the type of behavior expected for males responding to release rates that are far enough above the activation threshold to be above the disorientation threshold as well.

Indeed, further preliminary trials with a 30% *E* isomer mixture showed that higher release rates were required to activate the moths, but the in-flight

behavior at these release rates were similar to those at the high release rates of 8% *E* isomer mixture. This would be in accord with Figure 8, which shows that any release rate above the activation threshold also is above the threshold for altering in-flight behavior. All of the flight-tunnel mixtures contained the same amount of a third pheromone component, dodecyl acetate, which is necessary for good trap catches in field tests by apparently eliciting close-range landing behavior (Baker et al., 1976).

Field-trapping tests (Miller and Roelofs, unpublished) were also consistent with the above results. Results from a test involving a range of release rates from 1, 3, 10 and 30 caps per trap of mixtures containing 3%, 9%, 20% and 50% *E* isomer showed that only the 3% and 9% mixtures attracted significant numbers of males. Male catches with the two lowest release rates of 9% were significantly better than those with the three lowest of 3%, but the trap catch with the 30 caps at 3% was as good as the catches with 1 and 3 caps of 9%. Using Figure 8 as a guide it can be predicted that at low release rates only the 9% mixture would be above the activation threshold, whereas a higher release rate would fall in the "attraction area" for both 3% and 9% mixtures, but not for the 20% or 50% mixtures.

Applications of the Threshold Hypothesis

The anomalous data described above in the Introduction can be explained to a large degree by delineating an "attraction area" for each species. The flight activation threshold line would be drawn so that the lowest threshold corresponds to the naturally-occurring mixture. The threshold lines for alteration of in-flight behavior by each component are drawn to accommodate the existing data.

Omnivorous Leafroller. A possible "attraction area" for the omnivorous leafroller is illustrated in Figure 9. From this figure it can be predicted that low release rates (such as those possible in the cool spring and autumn months) of approx. 5–20% *Z* isomer would be attractive to male moths, whereas mixtures containing more than 20% *Z* isomer would not be active in stimulating males at those release rates. At high release rates (such as those possible in the hot summer months), the mixtures containing less than 15% *Z* isomer are above the disorientation threshold and no longer would be expected to attract males. However, the mixtures containing 15–30% *Z* isomer would now be releasing at rates that fall within the "attraction area", and these mixtures would be expected to attract males.

Pink Bollworm. The apparent increase in the component ratio range that attracts pink bollworm males in the summer (Figure 1) also could be explained by a threshold diagram (Figure 10). At low release rates, component ratios close to the naturally occurring ratio would be the most effective in

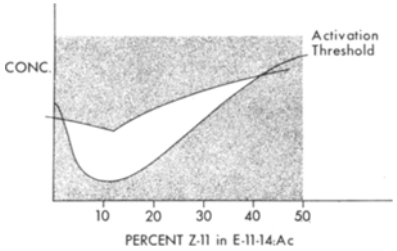

FIG. 9. A postulated "attraction area" for male omnivorous leafroller moths as described by flight alteration threshold lines for each pheromone component above and the flight activation threshold curve below.

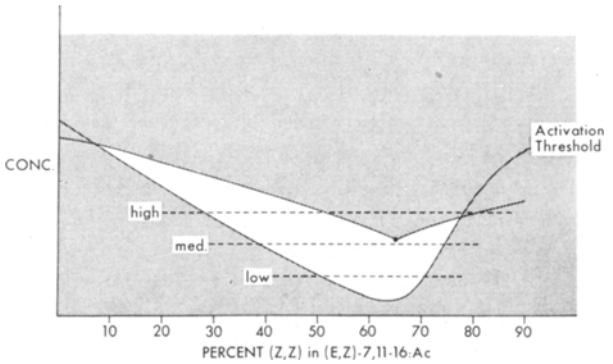

FIG. 10. A postulated "attraction area" for male bollworm moths. The dashed lines indicate several release rates that would give trap catches to explain variations in reported field trapping data.

attracting moths. At slightly higher release rates a larger section of the "attraction area" is intersected and ratios from approx. 40–70% (*Z*,*Z*) isomer would be expected to attract males. At high release rates, such as those from dental wicks (Bierl et al., 1974), the only ratios that are not above the disorientation threshold are those between 30 and 50% (*Z*,*Z*) isomer (Figure 10). This would explain the catches with 30% by some investigators and with 70% by other investigators. Clearly, field tests run for the purpose of defining the component ratio to which the males are most sensitive must be conducted at release rates approximating that used by the corresponding female moths.

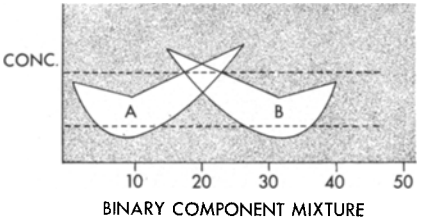

FIG. 11. Postulated "attraction areas" for two hypothetical species that utilize the same two pheromone components, but in different ratios. The dashed lines indicate where two release rates would intersect the "attraction areas."

Pheromone Specificity. An important implication of the threshold hypothesis is that specificity for a particular component ratio does not always mean that the insect is using that particular ratio. For example, if two species use the same two pheromone components but in different ratios, such as 90:10 and 70:30 (Figure 11), they could both be trapped very specifically by traps emitting a high rate of 80:20. In this case the conclusion would be made that there is no species specificity since they are both trapped specifically by the same blend of components. According to the threshold hypothesis, further studies with varying concentrations of 80:20 would be expected to reveal that both species are attracted best to the high release rates. A test with the various ratios at low release rates, however, should reveal that a low release rate of 90:10 attracts only species A, a low release rate of 70:30 attracts only species B, and that a low release rate of 80:20 attracts neither species. This would again point out the necessity of approximating the female moth release rate when conducting specificity studies.

Another implication of the "attraction areas", as seen in Figure 11 for two species, is that the amount of overlap, or reproductive isolation, can be regulated by various means. The release rate of the pheromone is important, but the shape of the "attraction area" can easily be affected by changes in flight activation threshold curves or by the altered flight effects of either component, thus changing the slope of the disorientation lines. Any of these changes could decrease the amount of overlap between the "attraction areas" of the two species and increase the degree of reproductive isolation for the two species. It may not be necessary to change the component ratios to effect better reproductive isolation.

Control Programs. For species that utilize a precise binary mixture of pheromone components to effect long-distance upwind anemotaxis, the threshold diagrams indicate that in-flight alterations occur at the lowest

concentration with the natural blend of components. Therefore, mating disruption for insect control by the air permeation technique would probably be affected by lower release rates more for the natural blend than for either component used alone. Generally, this has been found to be true, e.g., catches of redbanded leafroller males in monitoring traps were reduced 98%, 89% and 67% by air permeation from microencapsulated formulations of an approximate natural blend of *Z*11-/*E*11–14:Ac components (89:11), a 50:50 mixture, and pure *E*11–14:Ac, respectively (Roelofs et al., 1976). Further tests with the natural blend and the 50:50 mixture released from hollow fibers (Reissig and Roelofs, unpublished; Taschenberg and Roelofs, 1978) showed that the former was approx. 99% effective in disrupting male redbanded leafroller orientation at a rate of 5 mg/hr/hectare, whereas a rate of 15 mg/hr/hectare was required to get the same effect with the 50:50 mixture. Also, a 50:50 mixture of pink bollworm pheromone components, (*Z*,*Z*)- and (*Z*,*E*)-7,11–16:Ac, which approximates the natural blend, is approx. 100-fold more disruptive at low release rates than either component alone (Shorey et al., 1976).

In some situations in which it is not desirable to attract males into the disruption areas by using the natural blend of components, it might be advisable to use slightly higher release rates of a ratio that falls just outside of the "attraction area" on the threshold diagram, e.g. approx. a 20% mixture in Figure 8, a 3% mixture in Figure 9, and an 80% mixture in Figure 10.

Concluding Remarks

A hypothesis is presented that utilizes a threshold curve for flight activation as a lower limit, and threshold curves for altered in-flight responses for each component of a binary mixture as an upper limit, to circumscribe an "attraction area" for a particular species. Component ratios included in this area and released at rates that fall within the area should be effective in attracting males in the field. This hypothesis has been described only for pheromone systems that utilize a precise ratio of two positional or geometrical isomers. It is recognized that the simplicity of the hypothesis as presented would be complicated by additional pheromone components and by changing behavioral threshold levels from a variety of environmental inputs present at various times of the year. The shape of the "attraction area" would be altered if environmental inputs, e.g., temperature, markedly affect the flight activation threshold curve. The large daily changes in the activation threshold are not considered in this hypothesis because it deals only with the thresholds existing during the active portion of the circadian rhythm. The figures are drawn to show the lowest activation thresholds at the natural

pheromone blend ratio, although continuous changes in the shape of the threshold curve from the daily rhythmic shifts and various environmental inputs could produce a threshold minimum at times that does not coincide with the calculated pheromone blend for a species. The generalities of the hypothesis, however, should be useful in interpreting pheromone field-trapping results and in setting up experiments for defining pheromone blends, species specificity, and the best disruptive mixture for insect control.

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ATTRACTION OF *Carpophilus* Spp. (COLEOPTERA: NITIDULIDAE) TO VOLATILE COMPOUNDS PRESENT IN FIGS[1]

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Abstract—Various volatile compounds present in ripening figs were attractive to *Carpophilus hemipterus*, *C. mutilatus*, *C. freemani*, and *C. lugubris*. Field tests demonstrated that traps baited with a mixture of acetaldehyde, ethyl alcohol, and ethyl acetate caught more adult *C. hemipterus* than those baited with (1) other mixtures involving various combinations of 19 compounds, (2) with 16 single compounds, or (3) with fig paste.

Key Words—*Carpophilus hemipterus*, *C. mutilatus*, *C. freemani*, *C. lugubris*, Nitidulidae, fig, attractant.

INTRODUCTION

Commercially grown figs are infested by several *Carpophilus* species (Coleoptera: Nitidulidae) in California. Among these species are *C. hemipterus* (L.) (dried fruit beetle), *C. mutilatus* Erichson (confused sap beetle), *C. freemani* Dobson (Freeman sap beetle), and *C. lugubris* Murray (dusky sap beetle). The first three species are pests of major importance in figs (Smilanick and Ehler, 1976). Infestations by adults and larvae of these species render the fruit unfit for marketing. Fig paste, a mixture of dried figs, brewer's yeast, and water, has been used to trap adult insects in the field in order to monitor populations and attempt to reduce population density (Obenauf et al., 1976).

Our objectives were: (1) to determine the attractiveness of volatile compounds produced by ripe figs to *Carpophilus* species, and (2) to determine if

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an appropriate mixture of certain volatile compounds could be used in traps to replace fig paste in order to increase the efficiency of the trapping method.

METHODS AND MATERIALS

Three separate field tests were conducted near Winters, California, during September and October 1976. Traps baited with various treatments were deployed in an orchard of Black Mission figs. Compounds used as baits in the field test were selected from a list of the volatiles isolated from figs by Jennings (1977). All compounds tested in this study were obtained from commercial vendors and were of reagent quality.

The compounds were tested singly and in various combinations to assess their attractiveness to adults of *Carpophilus* species. The treatment mixture in each replication contained paraffin oil as a diluent and 0.4 ml of each compound tested. The mixtures were eluted in the field from 4-ml glass vials. Compounds used in treatments composed of more than one compound were mixed together rather than maintained separately. Treatments containing six or more compounds necessitated the use of up to four vials for field elutions. To prevent early volatilization of the compounds, vials containing the compound(s) were transported to the field in a chilled container and placed in the insect traps at the test site.

The attractiveness of volatile compounds to adults of *Carpophilus* species was compared with that of fig paste. The fig paste treatment contained 16 g of figs, 24 g of water, and 0.08 g of brewer's yeast. Fig paste was used rather than unaltered figs because (1) it could be standardized, (2) it was the substance for which a replacement was sought, and (3) the attractiveness of unaltered figs varies with physiological state. The relative attractiveness of successive physiological states of figs is currently under investigation.

The trap used to capture the beetles consisted of a one-pint Mason jar into which the vials of compounds were hung on wire loops. For the fig paste treatment, the paste was placed in a paper cup on the bottom of the jar. A plastic funnel was then fastened into the jar in an upright position. A cube (2.5 cm^3) of 18.6% dichlorovos (Vapona®, Shell Chemical Corp.) was placed in the jar to kill insects upon entering.

Traps were placed in the field in a systematic pattern. In the first field test, traps were randomly divided into five groups of 7–8 traps per group. Groups of traps were placed equidistantly on the ground, 1 m from the base of a fig tree. After 24 hr all *Carpophilus* species were removed, identified, and counted. The vial containing the treatment compound(s) was replaced with a fresh vial of the same treatment. The traps in each group were then rotated clockwise, one position around the tree. Captured specimens were again

removed after the second 24 hr. The replicates of each treatment were conducted on two consecutive days. For tests two and three, a similar procedure was followed, except that there were four groups of traps, each group containing one trap for every treatment. Thus, there were four replications of each treatment per day. These treatments were repeated on four consecutive days.

The first field test consisted of 19 treatments, each replicated four times. Compounds were tested individually in 17 treatments. A combination of three compounds (acetaldehyde, ethyl acetate, and ethyl alcohol) was tested in one treatment and a control of paraffin oil was also included in the test. Limonene, although not isolated from figs, was included among the single compounds screened because it has been isolated from citrus hosts of *Carpophilus* species (Schultz et al., 1964). Also, Jennings (personal communication, 1976) had preliminary data which indicated that limonene was present in figs. The three compounds, acetaldehyde, ethyl acetate, and ethyl alcohol, were chosen for the combination treatment due to their universal presence in fruits which are hosts of *Carpophilus* species.

The second field test consisted of six treatments, replicated 10–16 times. The treatments were comprised of various combinations of compounds as follows: (1) the three-compound combination screened in the first test, (2) fig paste, (3) 19 compounds, (4) 6 compounds, (5) 10 compounds and (6) a paraffin-oil control. The 19-compound treatment was a combination of all the volatile compounds of figs which could be obtained. The 6-compound combination consisted of the three compounds screened in test one and the three volatile butyrates. The 10-compound combination included these 6 compounds plus 4 volatile acetates. These combinations were selected in order to observe the effect of additional compounds on the activity of the original three compounds tested. Two of the compounds isolated by Jennings (1977), 2-ethyl-1,2-dihydrothiophene and ethyl-2-methyl butyrate, were not included due to unavailability. The 19-compound treatment was replicated 10 times, the 3-compound treatment 15 times, and all other treatments 16 times.

The third field test consisted of only those compounds from the 3-compound treatment: acetaldehyde, ethyl acetate, and ethyl alcohol. The treatments included the 3-compound combination, the three possible 2-compound combinations, and a paraffin-oil control. Each treatment was replicated 16 times.

The data for test 1 were analyzed using the Whitney-Mann U test for unpaired samples. The data for tests 2 and 3 were analyzed using the Wilcoxon signed-rank test for matched pairs. Pairs consisted of treatments located under the same tree on the same day, thereby minimizing the effects of location and daily variation.

RESULTS AND DISCUSSION

A total of 19 compounds were field assayed for their attractiveness to adults of *Carpophilus* species. Four species were attracted: *C. hemipterus*, *C. mutilatus*, *C. freemani*, and *C. lugubris*. The 3-compound mixture of acetaldehyde, ethyl acetate, and ethyl alcohol consistently attracted the greatest number of beetles.

Results for the first test indicate that the response of all four *Carpophilus* species was significantly greater to the 3-compound mixture than to any single compound (Table 1). There was no significant statistical difference among the single-compound treatments. However, acetaldehyde did not elicit the highest numerical response from *C. hemipterus* and *C. lugubris*. Similarly, ethyl acetate elicited the highest numerical response from *C. mutilatus* and *C. freemani*.

TABLE 1. RESPONSE OF *Carpophilus* SPECIES TO VOLATILE COMPOUNDS OF FIGS: TEST 1, WOLFSKILL FIELD STATION, SOLANO COUNTY, CALIFORNIA, 1976

Treatment	Mean number of adults attracted[a]			
	C. hemipterus	*C. mutilatus*	*C. freemani*	*C. lugubris*
Acetaldehyde	84.0 (2–211)	9.5 (0–33)	0.3 (0–1)	0.5 (0–1)
Ethyl acetate	1.5 (0–3)	14.5 (5–36)	1.8 (0–7)	1.0 (0–2)
Isoamyl acetate	0 (0–1)	0.5 (0–1)	0	0
Isobutyl acetate	1.3 (0–4)	2.8 (0–5)	0.5 (0–2)	0.5 (0–1)
Methyl acetate	0.8 (0–2)	3.5 (1–9)	0	0
2-Methylbutyl acetate	0	1.3 (0–4)	0.3 (0–1)	0
Propyl acetate	7.0 (0–25)	4.8 (0–8)	0	0
Ethyl butyrate	1.0 (0–1)	0	0	0
Methyl butyrate	0.8 (0–2)	1.3 (0–3)	0.3 (0–1)	0.3 (0–1)
Ethyl isobutyrate	1.0 (0–2)	0.5 (0–2)	1.3 (0–1)	0
Ethyl alcohol	0.8 (0–2)	1.3 (0–2)	0.3 (0–1)	0
Isobutyl alcohol	0	3.3 (1–6)	0.3 (0–1)	0.5 (0–1)
3-Hydroxy-2-butanone	2.3 (0–8)	1.5 (0–4)	0	0
Ethyl hexanoate	0	0	0	0
Ethyl propionate	2.8 (0–7)	0.5 (0–1)	0	0
Isobutyl isovalerate	0	1.3 (0–2)	0	0
Limonene	0.5 (0–1)	0	0	0
Control	0	0.3 (0–1)	0	0
Acetaldehyde, ethyl acetate, ethyl alcohol	909.5[b] (338–1552)	56.8[b] (30–81)	6.5[b] (1–17)	27[b] (7–73)

[a] Range in parentheses, N=4 in all treatments.

[b] For respective species the response to this treatment was significantly different from other treatments, using the Whitney-Mann U test, $P < 0.05$.

In the second test, the 19-, 10- and 6-compound treatments caught significantly fewer *C. hemipterus* than the 3-compound treatment (Table 2). No significant difference was found among the other species caught. Although these data suggest that one or more compounds may have a repellent or masking effect, other factors such as concentration effects, which were untested, may also be involved. Traps baited with the 3-compound mixture also captured more beetles than did the fig-paste treatment. The 3-compound treatment may have contained quantitatively more attractants or fewer inhibitors than the fig-paste treatment. However, the 3-compound chemical attractant out-competed the "natural bait." Therefore, there is potential for it as a bait for survey trapping in place of fig paste.

The 3-compound combination tested in the third field test was significantly more active for *C. hemipterus* than any 2-compound combination (Table 3). These tests suggest that the compounds are synergistic in their attractiveness, since the activity of the combination is far more than that of the three pairs combined.

To determine if an association existed between attractiveness of a

TABLE 2. RESPONSE OF *Carpophilus* SPECIES TO VOLATILE COMPOUNDS OF FIGS: TEST 2, WOLFSKILL FIELD STATION, SOLANO COUNTY, CALIFORNIA, 1976

Treatment	N	Mean number of adults attracted[a]			
		C. hemipterus[b]	*C. mutilatus*	*C. freemani*	*C. lugubris*
Acetaldehyde, ethyl acetate, ethyl alcohol	15	72.3 (3–412) a	0.2 (0–2)	0.6 (0–6)	0.5 (0–3)
Fig paste	15	28.9 (1–90) ab	0.8 (0–3)	0.7 (0–2)	0.4 (0–1)
19 compounds[c]	10	8.3 (2–22) bc	0.3 (0–3)	0	0.5 (0–2)
6 compounds[d]	16	4.4 (0–14) c	0.1 (0–1)	0.1 (0–1)	0.2 (0–2)
10 compounds[e]	16	2.0 (0–9) c	0.1 (0–2)	0.3 (0–1)	0.1 (0–2)
Control	16	0.1 (0–1) d	0	0.1 (0–2)	0

[a] Range in parentheses.

[b] The response of *C. hemipterus* was analyzed by a Wilcoxon signed rank test for matched pairs. Values followed by a common letter not significantly different, $P < 0.05$.

[c] Acetaldehyde, ethyl acetate, isoamyl acetate, isobutyl acetate, methyl acetate, 2-methyl butyl acetate, propyl acetate, ethyl butyrate, methyl butyrate, ethyl isobutyrate, ethyl alcohol, isobutyl alcohol, 3-hydroxy-2-butanone, ethyl hexanoate, ethyl propionate, ethyl valerate, isobutyl isovalerate, limonene, dimethyl acetal.

[d] Acetaldehyde, ethyl acetate, ethyl alcohol, ethyl butyrate, methyl butyrate, ethyl isobutyrate.

[e] Acetaldehyde, ethyl acetate, ethyl alcohol, ethyl butyrate, methyl butyrate, ethyl isobutyrate, isoamyl acetate, isobutyl acetate, 2-methylbutyl acetate, propyl acetate.

TABLE 3. RESPONSE OF *Carpophilus* SPECIES TO VOLATILE COMPOUNDS OF FIGS: TEST 3, WOLFSKILL FIELD STATION, SOLANO COUNTY, CALIFORNIA, 1976

Treatment	*N*	Mean number of adults attracted[a]			
		C. hemipterus[b]		*C. mutilatus*	*C. freemani*	*C. lugubris*
Acetaldehyde, ethyl acetate, ethyl alcohol	16	12.9 (1–70)	a	0.3 (0–1)	0.1 (0–1)	0.9 (0–3)
Acetaldehyde, ethyl acetate	16	3.3 (0–14)	b	0.4 (0–3)	0	0.6 (0–4)
Acetaldehyde, ethyl alcohol	16	1.7 (0–10)	bc	0.1 (0–1)	0.1 (0–1)	0.1 (0–2)
Ethyl acetate, ethyl alcohol	16	1.0 (0–13)	cd	0.1 (0–1)	0.1 (0–1)	0.1 (0–1)
Control	16	0.1 (0–1)	d	0	0	0

[a] Range in parentheses.
[b] The response of *C. hemipterus* was analyzed by a Wilcoxon signed rank test for matched pairs. Values followed by a common letter not significantly different, $P < 0.05$.

compound and its frequency in host fruits of *Carpophilus* species, a comparison was made between (1) the volatile compounds isolated from figs, and (2) those isolated from seven other host fruits of *Carpophilus* species [i.e., apple, (Kevei and Kozma, 1975; van Straten, 1977), citrus (Schultz et al., 1964; van Straten, 1977) grape (van Straten, 1977), cantaloupe (Yabumoto and Jennings, 1977), peach (Sevenants and Jennings, 1966; van Straten, 1977), pineapple (Dupaigne, 1970).] This comparison indicated that acetaldehyde, ethyl acetate, and ethyl alcohol are universally present in these host fruits. Several butyrates, acetates, isobutyl alcohol, and ethyl isopropionate were present in some fruits, but not universally so. The following compounds were present in figs but have not been reported from any of the above fruits: dimethyl acetal, 2-hydroxy-2-butanone, ethyl hexanoate, isobutyl isovalerate, ethyl valerate, ethyl-2-methyl butyrate, and 2-ethyl-1,2-dihydrothiophene. The first five of these compounds, which were included in our field tests, demonstrated no attractiveness to *Carpophilus* species.

To summarize, our field tests indicate that acetaldehyde, ethyl acetate, and ethyl alcohol are the principal attractants of *C. hemipterus* of 19 volatile compounds isolated from figs. These same three compounds are present in other hosts of *C. hemipterus*. Thus this polyphagous species appears to use a restricted number of olfactory stimuli to locate suitable hosts.

If these three active compounds are used as trap baits, the current survey techniques might be simplified and, in turn, other insect control measures

could be improved. Additional research concerning chemical attraction to *Carpophilus* species should include (1) testing ratios and quantities of the active compounds, (2) assessing their relative attractiveness compared to ripe figs, and (3) determining the sensitivity of the trapping method to field populations.

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Heliothis virescens: ATTRACTION OF MALES TO BLENDS OF (*Z*)-9-TETRADECEN-1-OL FORMATE AND (*Z*)-9-TETRADECENAL[1]

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Abstract—In field trapping experiments, 16:1 and 32:1 blends of (*Z*)-9-tetradecen-1-ol formate (*Z*-9-TDF) and (*Z*)-9-tetradecenal (*Z*-9-TDAL) caught as many *Heliothis virescens* (F.) as 3 virgin females and virelure, the synthetic pheromone of this species [a 16:1 mixture of (*Z*)-11-hexadecenal (*Z*-11-HDAL) and (*Z*)-9-tetradecenal]. *Z*-9-TDF and (*Z*)-7-dodecen-1-ol formate (*Z*-7-DDF) are structurally similar to *Z*-11-HDAL and *Z*-9-TDAL, respectively. The sensory input elicited by *Z*-9-TDF appears to substitute for the sensory input of *Z*-11-HDAL. In contrast, *Z*-7-DDF had no significant effect on catches of male *H. virescens* when used alone, in combination with either *Z*-11-HDAL or *Z*-9-TDF as a bait in traps, or as a disruptant of pheromone communication via permeation of the atmosphere. Furthermore, *Z*-9-TDF may be a more stable and economical attractant for *H. virescens* males than is *Z*-11-HDAL.

Key Words—Tobacco budworm, *Heliothis virescens* (F.), virelure, pheromone, sex attractant, (*Z*)-9-tetradecen-1-ol formate, (*Z*)-7-dodecen-1-ol formate, (*Z*)-11-hexadecenal, (*Z*)-9-tetradecenal.

INTRODUCTION

The sex pheromone (virelure) of the tobacco budworm, *Heliothis virescens*

[1] This paper reports the results of research only. Mention of a pesticide in this paper does not constitute a recommendation for use by the U.S. Department of Agriculture nor does it imply registration under FIFRA as amended. Also, mention of a commercial or proprietary product in this paper does not constitute a recommendation or an endorsement of that product by the USDA.

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(F.), has been isolated and identified as a 16:1 mixture of two compounds, (*Z*)-11-hexadecenal (*Z*-11-HDAL) and (*Z*)-9-tetradecenal (*Z*-9-TDAL) (Roelofs et al., 1974; Tumlinson et al., 1975). *Z*-11-HDAL also has been reported as a part of the pheromone for the corn earworm, *H. zea* (Boddie), although there is no evidence that *Z*-9-TDAL is involved (Roelofs et al., 1974). Priesner et al. (1975) presented electroantennogram (EAG) data that showed that in *H. zea*, (*Z*)-9-tetradecen-1-ol formate (*Z*-9-TDF) is approx. 30 times more effective than the reported pheromone constituent, *Z*-11-HDAL. Subsequently Mitchell et al. (1975, 1976) showed that *Z*-9-TDF, a chemical of nonbiological origin but structurally similar to *Z*-11-HDAL (Figure 1), effectively disrupted pheromone communication between male and female *H. zea* and male and female *H. virescens* in small field plots. In flight tests in a laboratory olfactometer, neither *Z*-9-TDF nor *Z*-11-HDAL attracted *H. virescens* males, but males did respond by flying upwind to virelure. However, when the males were preexposed to 25 mg of *Z*-9-TDF or *Z*-11-HDAL for 1 hr, they then would not fly upwind to the virelure source (Tingle and Mitchell, 1978). Therefore, from the similarity in structures and the disruptive nature of this chemical on pheromone communication, it appeared that the formate was acting as a pheromone mimic of *Z*-11-HDAL.

We report here the results of field experiments conducted at Gainesville, Florida, during the summer of 1977 to determine whether *Z*-9-TDF would substitute for *Z*-11-DHAL in the virelure mixture. Also, (*Z*)-7-dodecen-1-ol formate (*Z*-7-DDF), which is similar structurally to *Z*-9-TDAL (Figure 1), was evaluated as a pheromone communication disruptant for *H. virescens* males and as a substitute for *Z*-9-TDAL in mixtures with *Z*-11-HDAL and *Z*-9-TDF.

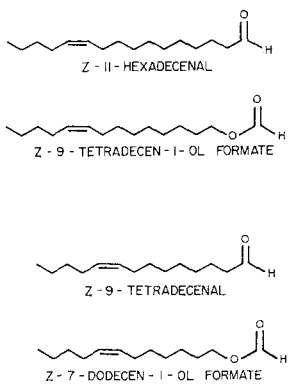

FIG. 1. Aldehydes and formates tested as attractants for *H. virescens* males.

METHODS AND MATERIALS

Previous research established that the individual components of virelure, *Z*-11-HDAL and *Z*-9-TDAL, are not attractive when used alone, but the mixture is highly attractive to *H. virescens* males when used in a ratio of 16:1 (Tumlinson et al., 1975). Therefore, *Z*-9-TDF or *Z*-7-DDF were substituted in the attractant mixture in the same ratio as *Z*-11-HDAL and *Z*-9-TDAL, respectively.

The following compounds and/or mixtures thereof (16:1 ratio) were evaluated as attractants for *H. virescens* males (test 1): *Z*-7-DDF; *Z*-9-TDF; *Z*-11-HDAL/*Z*-9-TDAL (virelure); *Z*-11-HDAL/*Z*-7-DDF; *Z*-9-TDF/*Z*-9-TDAL; and *Z*-9-TDF/*Z*-7-DDF. The *Z*-11-HDAL and *Z*-9-TDAL were obtained from Farchan Chemical Co., Willoughby, Ohio, and have been demonstrated to be active as disruptants and attractants in previous field tests. *Z*-9-TDF was used as obtained from Farchan Chemical Co. (at least 97% pure geometrically when analyzed by gas chromatography on Carbowax® 20M and UV-101 packed columns and on a 50-m glass capillary column coated with SP-2340) and in highly pure form (99+%; purified by liquid chromatography on silver nitrate-treated silica gel, toluene-mobile phase). *Z*-7-DDF was prepared in our laboratory by esterification of (*Z*)-7-dodecen-1-ol (99+% pure) with formic acid. Its mass and infrared spectra were consistent with those expected for *Z*-7-DDF, and its purity was greater than 99% when analyzed by gas chromatography on 24-m glass capillary columns coated with OV-101 and Carbowax 20M.

Attraction to Traps

The baits (25 mg total in 50 μl hexane) were dispensed from 1.25-ml polyethylene vials suspended inside cylindrical electrocutor grid traps (Mitchell et al., 1975). A small quantity of antioxidant (<0.5% by vol UOP 688, Universal Oil Products Co., East Rutherford, New Jersey) was mixed with each bait chemical before the chemical was placed in the vial. The traps in each test were placed next to a mature tobacco field at intervals of approx. 30 m. Three unmated *H. virescens* females (2 days old) were used as a control in each test. Bait females were replaced every 2–3 days. Captured insects were collected and counted daily, and the treatments were rotated. For statistical analysis, each collection was considered a replicate.

Atmospheric Permeation

The disruptive effect of *Z*-7-DDF on pheromone communication by *H. virescens* was assessed by determining whether males could locate calling

females placed at the center of an 81-m^2 plot of tobacco when the males were simultaneously exposed to an atmosphere permeated with this chemical. Sixteen polyethylene vials (1.25 ml) containing *Z*-7-DDF (25 mg each in 50 μl hexane) were arranged in a 4×4 checkerboard pattern (3 m apart) around an electrocutor grid trap baited with 3 unmated females. The vials were attached to a cotton string that was draped over the tops of the maturing tobacco plants. Insects captured in the treated and control plots (approx. 75 m apart) were counted daily, and the locations of the treatment and control were rotated every 2nd day. For statistical analysis, each of the 8 collections was considered a replicate.

RESULTS

Attraction to Traps

Z-7-DDF did not attract *H. virescens* males when used alone or in combination with either *Z*-11-HDAL or *Z*-9-TDF (Table 1). *Z*-9-TDF (97%) also failed to attract males when used separately, but when it was

TABLE 1. CAPTURE OF WILD *H. virescens* MALES WITH ELECTROCUTOR GRID TRAPS BAITED WITH DIFFERENT COMBINATIONS OF ALDEHYDES AND FORMATES (TEST 1)

Bait	Replicates	$\bar{X}$ no. captured/night ($\pm$SE)[a]
Z-11-HDAL + *Z*-9-TDAL[b]	7	12.0 ± 4.8 a
3 females	7	19.0 ± 5.7 a
Z-9-TDF + *Z*-9-TDAL[b]	11	9.6 ± 2.9 a
3 females	11	11.6 ± 4.3 a
Z-9-TDF + *Z*-7-DDF[b]	8	0 a
3 females	8	32.0 ± 10.5 b
Z-11-HDAL + *Z*-7-DDF[b]	4	0 a
3 females	4	11.0 ± 3.2 b
Z-9-TDF	5	0 a
3 females	5	7.0 ± 1.9 b
Z-7-DDF	4	0 a
3 females	4	8.5 ± 1.8 b

[a] Means in the same group followed by like letters are not significantly different at the 5% level (Student's *t* test).
[b] Chemicals were mixed in a 16:1 ratio.

mixed in a 16:1 ratio with the minor component of virelure, Z-9-TDAL, the mixture was as attractive to *H. virescens* males as 3 virgin females. Comparable results were obtained in traps baited with virelure and *H. virescens* females.

A 2nd test was conducted to determine whether the observed attraction of *H. virescens* males to the mixture containing Z-9-TDF was indeed due to the presence of this chemical or perhaps some impurity present in this particular batch of formate. Z-9-TDF (99+%) was therefore mixed (16:1 ratio) with Z-9-TDAL and tested as before. In test 2, there was no significant difference between the numbers of *H. virescens* males captured with the mixture containing the 99+% pure Z-9-TDF and the number attracted to the mixture containing 97% pure formate (Table 2). In addition, the 97% and 99+% pure formate mixtures were each as attractive to *H. virescens* males as virelure or 3 females.

In test 3, Z-9-TDF (99+%) was mixed with Z-9-TDAL in ratios of 1, 16, and 32:1 and compared with 3 females as trap baits. Again the test procedure was as previously described. Traps baited with females did not catch significantly more *H. virescens* males than the 16:1 and 32:1 mixtures of Z-9-TDF/Z-9-TDAL did (Table 2). However, the 1:1 mixture of Z-9-TDF/Z-9-TDAL captured significantly fewer moths than any of the other 3 treatments.

TABLE 2. MEAN CAPTURES OF NATIVE MALE *H. virescens* MOTHS IN ELECTROCUTOR GRID TRAPS BAITED WITH VIRELURE AND DIFFERENT RATIOS OF Z-9-TDF+Z-9-TDAL

Bait	Ratio	$\bar{X}$ no. males captured/night (±SE)[a]
Test 2 (12 *replicates*)		
Z-11-HDAL+Z-9-TDAL	16:1	3.9± 1.3 a
Z-9-TDF +Z-9-TDAL	16:1[b]	7.7± 1.8 a
Z-9-TDF +Z-9-TDAL	16:1[c]	7.8± 2.0 a
3 females		10.6± 3.2 a
Test 3 (4 *replicates*)		
Z-9-TDF +Z-9-TDAL	1:1[c]	2.5± 2.1 a
Z-9-TDF +Z-9-TDAL	16:1[c]	30.8± 7.4 b
Z-9-TDF +Z-9-TDAL	32:1[c]	24.3± 4.7 b
3 females		42.3±14.7 b

[a] Means in the same test followed by the same letter are not significantly different ($P = 0.05$, Duncan's multiple range test).
[b] The Z-9-TDF used in this mixture was approx. 97% pure.
[c] The (Z)-9-TDF used in these mixtures was 99+% pure.

Atmospheric Permeation

The female-baited trap located in the plot treated with *Z*-7-DDF captured a mean (±SE) of 13.1 ± 2.5 *H. virescens* males/night and the control trap captured an average of 14.6 ± 3.8; the difference was not significant at the 5% level by Student's *t* test. However, significantly more (5% level, Student's *t* test) *H. zea* moths were captured in the trap placed in the area and treated with *Z*-7-DDF ($\bar{X}$ ± SE = 9.1 ± 2.4/night) than in the control trap ($\bar{X}$ ± SE = 1.0 ± 0.5), although both traps had only females as bait.

DISCUSSION

In these tests, *Z*-9-TDF readily substituted for *Z*-11-HDAL in the virelure mixture. The 16:1 mixture of this formate and *Z*-9-TDAL was clearly as efficacious in capturing *H. virescens* males as the natural or synthetic pheromones were. However, the physiological basis for the observed behavioral responses to *Z*-9-TDF is unknown. Nevertheless, these and other results (Mitchell et al., 1975, 1976; Tingle and Mitchell, 1978) suggest that *Z*-9-TDF is indeed a mimic of *Z*-11-HDAL and apparently is perceived by the same antennal receptor sites as the sex pheromone.

Pheromone analogs evoke the same behavioral reactions as the pheromones in a number of moth species, but usually more analog than pheromone is needed to attract. For example, Shorey et al. (1976) showed that the analog (*Z*)-7-hexadecen-1-ol acetate was 100-fold less attractive to male *Pectinophora gossypiella* (Saunders) than the pheromone, (*Z*,*Z*)- and (*Z*,*E*)-7,11-hexadecadien-1-ol acetates in a 1:1 ratio. Likewise, Voerman et al. (1975) showed that (*Z*)-11-tetradecen-1-ol acetate (*Z*-11-TDA) could be replaced by (*Z*)-11-tridecen-1-ol acetate in the pheromones of *Adoxophyes orana* (Fischer von Röslerstamm) and *Clepsis spectrana* (Treitschke) (a 9:1 mixture of (*Z*)-9-tetradecen-1-ol acetate and *Z*-11-TDA), but only with a reduction in attractancy.

In contrast, Cardé and Roelofs (1977) showed that an 85:15 blend of two pheromone analogs, (*Z*)- and (*E*)-11-tridecen-1-ol acetates, produces a trap catch of *Argyrotaenia velutinana* (Walker) equivalent to that with the natural pheromone blend, a 92:8:150 mixture of (*Z*)- and (*E*)-11-tetradecen-1-ol and dodecyl acetates.

In view of the attractiveness of *Z*-9-TDF to male *H. virescens* and its ability to disrupt pheromone communication (Mitchell et al., 1975, 1976), it was surprising that *Z*-7-DDF had no significant effect on the attraction of this species when mixed with either *Z*-11-HDAL or *Z*-9-TDF or on

pheromone communication when evaporated around calling females. The apparent attraction of *H. zea* males to the trap baited with *H. virescens* females located in the area permeated with *Z*-7-DDF was unexpected, especially since there is little evidence of cross attraction in these species (Tingle et al., 1978). Indeed, when females of *H. zea* and *H. virescens* are confined in the same trap, captures of males of both species are greatly reduced (Haile et al., 1973). However, increased trap captures have been observed in several species where chemicals other than their pheromone were used to permeate the atmosphere. For example, Rothschild (1974) recorded increased trap catches of *Grapholitha molesta* (Busck) when an orchard atmosphere was permeated with dodecyl acetate. Mitchell et al. (1974) obtained a significant increase in the numbers of *Spodoptera frugiperda* (J. E. Smith) captured when (*Z*)-7-dodecen-1-ol acetate was evaporated around pheromone traps. Similarly, permeation of the atmosphere with (*Z*)-7-dodecen-1-ol increased the numbers of male *Trichoplusia ni* (Hübner) captured (McLaughlin et al., 1974).

As noted by Cardé and Roelofs (1977), attraction to traps is but one measure of the complex orientation and precopulatory behaviors caused by pheromones. The contrasting effects of *Z*-9-TDF and *Z*-7-DDF on the attractiveness of pheromone traps should warn us not to assume from structural similarity that insects will react in the same way to compounds as they do to the natural pheromone.

The use of *Z*-9-TDF as a component of the attractant mixture for *H. virescens* males should have significant practical value because the formate is more economical to synthesize and has a longer effective life in the field. However, Tumlinson et al. (1975) suggests that unspecified components remain to be characterized for the attractant pheromone of the species. It is conceivable that once these are fully characterized, their addition to the attractant blend could alter the effectiveness of *Z*-9-TDF.

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SEX PHEROMONE SCENT MARKING BY FEMALES OF *Pectinophora gossypiella* (LEPIDOPTERA: GELECHIIDAE)

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Abstract—Females of the pink bollworm moth, *Pectinophora gossypiella* (Saunders), scent marked the substrate to which they were clinging with the extended pheromone gland during nocturnal pheromone-releasing periods. Scent marks were deposited on both natural (cotton leaf) and atypical (glass) substrates. In both situations, the scent marks elicited attraction responses from conspecific males.

Key Words—Pheromone, scent marking, *Pectinophora gossypiella*, Gelechiidae, cotton.

INTRODUCTION

Scent marking is the deposition of a pheromone, allomone, or kairomone by an organism upon an object. Scent marking has been studied extensively in mammals (Thiessen et al., 1968; Johnson, 1973; Adams, 1976), and has been reported in some insects (Wood, 1970; Prokopy, 1972; Wilson, 1971; Bringer, 1973; Svensson and Bergström, 1977). Although moth chemical communication systems have been researched extensively, sex-pheromone scent marking has not previously been reported in lepidopterans. However, there have been reports that female moths of the pink bollworm, *Pectinophora gossypiella* (Saunders), sometimes contact with their extruded pheromone glands the substrate to which they are clinging (Leppla, 1972; Colwell et al.,

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1978). The observational study described here was conducted to substantiate these reports by showing that the areas on the substrate contacted by the glands do contain and release pheromone, as indicated by the attraction of male moths to those areas.

METHODS AND MATERIALS

P. gossypiella larvae were reared according to previously described procedures (Adkisson et al., 1960; Patana, 1969), and the sexes were separated as pupae. Adult moths were kept in wire-screen cages and were provided with 8% sucrose solution, as described by McLaughlin et al. (1972). The moths were maintained at 26 ± 2°C on a 14:10 light–dark photoperiod and were used for experimentation 2–5 days following emergence from pupae. The experimental light intensity was maintained at 0.4 lux, as measured with a Photovolt 200 C photometer.

Before the initiation of each replicate of the first series of experiments, two cotton leaves were picked from potted plants. The petiole of each leaf was placed in a separate 2-dram water-filled vial, and the leaf preparations were placed at opposite ends of a 20-cm-long × 6-cm-wide × 20-cm-high glass chamber. Then four virgin female moths were introduced through a hole into the chamber. When a female exhibited pheromone-releasing behavior (by extending her pheromone gland) from a position on one of the cotton leaves (Figure 1), the other females were removed from the chamber with an aspirator. This procedure was conducted in such a way that the pheromone-releasing female was not disturbed. The releasing female's location on the leaf was recorded, by appropriate marks made by the investigator on the outside of the glass chamber, and the corresponding location on the unoccupied leaf was designated as the control location. The female was observed continuously and was removed from the chamber with an aspirator when she ceased her releasing behavior. This aspiration also served to destroy any pheromone gradient which otherwise might have been most concentrated in the area which the female had occupied. Four virgin male moths were then introduced into the chamber, and the number of times they moved to within 0.5 cm of the "female" and "control" locations during a 6-min period was recorded.

The second series of experiments was conducted similarly, except that no cotton leaves were present, and the females released pheromone while clinging to the glass walls of the chamber. Male approaches to within 0.5 cm of "female" locations on the glass and to arbitrarily designated "control" locations, at least 10 cm away, and also on the glass, were recorded as described above.

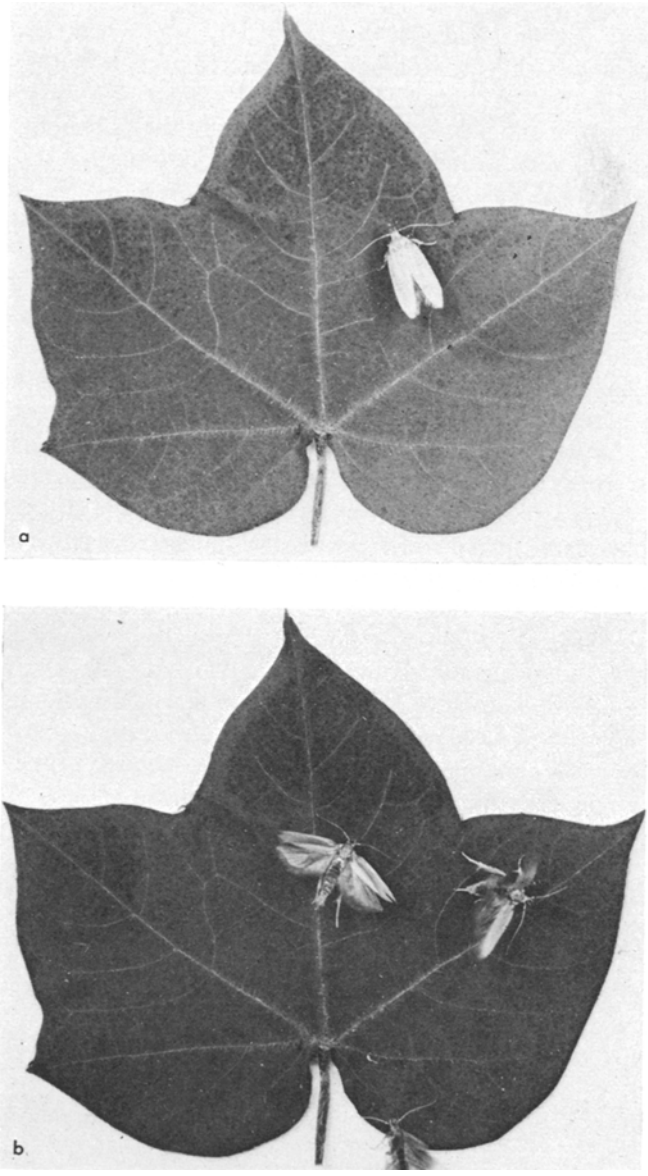

FIG. 1. (a) Female of *P. gossypiella* scent marking on a cotton leaf. (b) Males exhibiting courtship behaviour near the location of the scent mark. Rapid movement of the males caused them to move in and out of a 0.5-cm-radius circle around the mark.

RESULTS AND DISCUSSION

The data (Table 1) indicate that the regions which had been occupied by pheromone-releasing female moths were entered by male moths significantly more frequently than control areas. The males, when near the scent marks, also frequently exhibited characteristic close-range courtship behaviors (Colwell et al., 1978), including wing vibration, upturning of the tip of the abdomen, and extrusion of the hairpencils (Figure 1). In some instances, a male near a scent mark made a copulatory attempt directed towards the scent mark or towards a nearby male. Copulatory attempts by males of *P. gossypiella* typically are elicited only at relatively high concentrations of pheromone (Colwell et al., 1978), so apparently much pheromone was deposited during the scent marking.

Scent marking often is considered to be an advantageous means of communication in that it allows the marking animal to be absent from an area and yet to be advertising its presence in that area by means of the scent-mark odor (Jones and Nowell, 1973). However, based on our present knowledge, the *P. gossypiella* female generally remains at the site of the scent mark with her pheromone gland still exposed. The biological significance of such dual release of pheromone from an exposed gland and a nearby scent mark is not apparent. Perhaps the scent mark ensures the continuity of the pheromone plume. Since the *P. gossypiella* female periodically withdraws her gland during the releasing period (Colwell et al., 1978), a male moving upwind might lose the trail and be in a location where he could no longer perceive the pheromone when the gland was reextended. The scent mark would ensure that the odor was continuously released from the location of the female, so the male might continue to move toward her during periods of gland re-

TABLE 1. NUMBER OF MOVEMENTS BY *P. gossypiella* MALE MOTHS TO WITHIN 0.5 CM OF A FEMALE SCENT MARK OR A CONTROL LOCATION

	Female location		Control location	
Substrate	*N*	Mean[a] ± SE	*N*	Mean[a] ± SE
Cotton leaf	10	18.5[b] ± 5.1	10	3.3[b] ± 0.3
Glass	10	23.4[b] ± 7.0	10	7.1[b] ± 2.0
Grand mean	20	21.0[b] ± 4.3	20	5.2[b] ± 1.1

[a] Each mean represents the number of movements made by 4 males during a 6-min. period. Multiple entries to within 0.5 cm of the designated location by any one male were recorded each time as separate movements.

[b] Difference between mean number of movements to female vs. control location is significant ($P < 0.05$) as determined by Student's t test.

traction. The same continuity of the pheromone message would exist if the female were caused to move a short distance or to retract her gland due to some mechanical disturbance on the leaf near her location.

Another possible function of the scent mark might be to increase the surface area from which pheromone evaporates, thereby increasing both the rate of volatilization and the possible communication distance.

The scent mark might also be involved in close-range orientation by the male to the "correct" part of the female's body. Male copulatory attempts are typically oriented toward the end of the female having the highest pheromone concentration, although visual stimuli probably also guide this orientation (Colwell et al., 1978). The female normally withdraws her pheromone gland when the male first contacts her, and a scent mark beneath her abdomen tip might ensure that the greater pheromone concentration, and thus the zone toward which the male copulatory attempt is oriented, is at her posterior end.

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LETTER TO THE EDITORS

To the Editors:

Since our paper "Oviposition-Deterring Pheromone of *Rhagoletis pomonella*: A Kairomone for Its Parasitoid *Opius lectus*" [*J. Chem. Ecol.* 4:481–494, 1978] was submitted for publication, the work of Corbet (*Nature* 232:481–484, 1971; *Nature* 243:537–538, 1973) has come to our attention. Corbet's studies demonstrated that larvae of the flour moth, *Anagasta kuehniella*, secrete a pheromone which regulates dispersion of other larvae, deters oviposition of the adults, and acts as a kairomone eliciting ovipositional movements of the larval parasite, *Venturia canescens*. Although as far as is known, oviposition-deterring pheromone is not secreted by *A. kuehniella* adults (as is the case in *R. pomonella*), Corbet's findings do in fact constitute the first demonstration of such a pheromone in a phytophagous insect functioning as a kairomone to a parasite. We regret our omission of Corbet's excellent work.

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